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abstracts

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Poster Sessions

Presenters are requested to stand with their posters according to the timetable below:

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Participants wishing to be considered for a poster prize need to put their poster up in the morning coffee break on the day of their poster session.

Poster Prizes

The EMBO Journal, EMBO reports, Molecular Systems Biology and ***EMBO Molecular Medicine*** will each award a poster prize. The journal editors and EMBO Young Investigators will determine winners based on high-quality and exciting unpublished research presented in a clear and appealing manner.

The awards will be presented during the closing ceremony.

A

A 001–211

Poster Abstracts Session A

Sunday 11 September 16:15–17:45

A 001 The nucleolar protein NML regulates the glucose metabolism in vivo

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Background: Intracellular energy balance is important for cell survival. In mammalian cells, the most energy-consuming process is ribosome biosynthesis. We previously identified a novel protein complex, eNoSC, which senses intracellular energy status and epigenetically regulates the rDNA locus by changing the ratio between the numbers of active and silent gene clusters. eNoSC contains a novel nucleolar protein NML, the NAD+-dependent deacetylase SIRT1 and the histone methyltransferase SUV39H.
Observations: In this study, to investigate the physiological role of NML in vivo, we generated mice with a targeted deletion of the gene coding NML (NML-KO mice). The most of NML-KO mice (90%) were embryonic lethal. We generated mouse embryonic fibroblasts (MEF) from NML-KO embryos. We found that metabolites level of glycolysis and TCA cycle in NML-KO MEF have been different from those in WT MEF using the metabolome analysis by CE-MS. Theses result suggested that NML is related to the regulation of glucose metabolism. Next, we evaluated the glucose metabolism of the surviving NML-KO mice. The surviving NML-KO mice showed the increase of serum glucose levels and the decrease of serum insulin levels under the chow diet compared with WT mice. These results showed the suppression of insulin secretion from pancreatic beta cells in NML-KO mice. Now, we are under investigation for the molecular mechanism of glucose metabolism regulation by NML in vitro and in vivo.
Conclusions: Here we showed that NML regulates glucose metabolism under normal glucose condition. On the other hand, NML represses ribosome biogenesis under glucose deprivation. NML may be an important factor to regulate the ribosome biogenesis and the glucose metabolism in response to energy status.

A 002 Analysis of the arginine metabolism during Arabidopsis-nematode interactions

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Background: The plant-parasitic cyst nematode Heterodera schachtii infects next to a variety of plant species also Arabidopsis. It induces specialized feeding structures in roots called syncytia and triggers specific defense reactions. It is known that some of similar adopted responses to biotic and abiotic stress involve several arginine-derived metabolites such as polyamines. Arginine is an essential and proteinogenic amino acid. It is an important medium for transport and storage of nitrogen in plants.
Observations: In order to study the role of arginine metabolism in nematode-induced syncytia, gene expression analysis was performed. We selected genes coding for A.synthase and A. lyase playing a key role in arginine biosynthesis. Further, the expression of ARGAH1 and ARGAH2 coding for arginase was studied. They hydrolyse arginine into ornithine and urea, what results in the two-step oxidation of arginine to NO and citrulline. Relative transcript levels of all selected genes were quantified using qRT-PCR in 5, 10 and 15 day-old H. schachtii-induced syncytia. Promoter::gus lines, when infected with H. schachtii, showed high promoter activity in syncytia, 5 and 10 day post-inoculation. Gene silencing lines of A.synthase and A.lyase both under the control of CamV and PDF2.1 promoters were produced. Lines with PDF2.1 promoter showed a higher susceptibility towards Heterodera schachtii compared to the wild type, whereas infection tests with the T-DNA mutants argah1-1 and argah2-1 did not show any significant difference from wild type plants. Additionally, we measure the amino acid content of syncytia and roots at 5 and 10 dpi, specifically looking at arginine and related amino acids.
Conclusions: Summarizing, all the analyzed genes were significantly up-regulated in syncytia as compared to the non-infected control roots. Our results suggest that arginine cycling plays an important role during nematode feeding site development.

A 003 Diauxic shift in single cells

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Background: Most of the insights into the growth response of cells to environmental changes have been obtained using bulk measurement techniques, in which the average growth rate of a population is followed over time. How the growth rate of individual cells develops during diauxie, a model system for understanding the ability of organisms to regulate their phenotype, is unknown.
Observations: To address this issue, we have developed a single-cell approach. We employ microfluidics to deplete glucose from a mixed glucose-lactose environment, apply novel image analysis algorithms to accurately determine cellular lengths at high time resolution, and use GFP labeling to monitor expression of the lac operon. We find that the majority of the cells first exhibit an abrupt decrease in growth rate to a low but not negligible level, and then exit from that low growth state equally abruptly to adopt the steady-state lactose growth rate. However, a small fraction of the population does not display any detectable decrease in growth rate. We developed a simple stochastic model that explains the observed heterogeneity in growth phenotypes. The data is consistent with the idea that stochasticity in the low lac operon expression during growth with glucose affords a small fraction of the population with sufficient lac proteins to establish a continuous and arrest-free growth during diauxie. This notion is supported by observed correlations in switching times between sister cells.
Conclusions: Given the costs involved in spuriously expressing lac proteins during growth on glucose, these results may indicate that E. coli is hedging its bets for potential environmental changes.

A 004 Cytosolic 5'-Nucleotidase II, an intriguing enzyme

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Background: CN-II is a bifunctional enzyme ubiquitously expressed in human tissues. Taking part in the oxypurine cycle, cN-II may be responsible for regulation of intracellular concentrations of IMP, AMP, GMP and also PRPP/Rib1-P. In vitro studies demonstrated the involvement of cN-II in prodrug metabolism, besides its mRNA level seems to be a prognostic factor in adult AML. Notwithstanding its potential clinical implications, the physiological role exerted by cN-II remains to be unravelled.
Observations: To shed light on the contribution of cN-II to cellular metabolism, we built two eucariotic systems with different cN-II levels: a galactose-inducible expressing model in S.cerevisiae and a lentiviral vector-based, tetracycline-controllable cN-II silencing system in mammalian cells.Thanks to expression of cN-II in gal-induced S.cerevisiae (RS112 strain), we demonstrated that yeasts expressing cN-II and exposed to increasing concentration of MMS have a different trend of Ade2 interchromosomal recombination in comparison with the control, without changes in yeast viability.This finding could be explained by an alteration of intracellular nucleotide pools caused by cN-II. Besides, results obtained with the dox-inducible cN-II knockdown in ADF led to the same hypothesis: cN-II is responsible for the control of intracellular nucleotide levels. In fact, percentages of decrease of PHT activity greater than 40% caused apoptosis of the cultered cells; moreover, cell death was partially overcome by addition of inosine to the medium. To evaluate if these two evidences are due to an imbalance of nucleoside/nucleotide concentrations HPLC analysis of extra/intracellular contents is in progress.
Conclusions: This work not only demonstrates, indirectly, the importance of cN-II in the maintainance of purine intracellular pools, but will be very useful to study the role of cN-II in prodrug metabolism. In this way, we may give important information for the design of personalized chemotherapies.

A 005 Reduction of nuclear encoded enzymes of the mitochondrial energy metabolism in cells devoid of mitochondrial DNA

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Background: Mitochondrial DNA (mtDNA) depletion syndromes are normally associated with reduced activities of oxidative phosphorylation (OXPHOS) enzymes that contain subunits encoded by the mtDNA. Nuclear encoded mitochondrial enzymes, as the citric acid cycle enzyme citrate synthase (CS) and the solely nuclear encoded OXPHOS complex II, usually exhibit normal or enhanced activities. The aim of this study was to elucidate the effect of complete loss of mtDNA on the enzyme activities of OXPHOS enzymes and CS.
Observations: The enzymatic activity of OXPHOS complexes and CS was measured in HEK293 wild-type and rho 0 cells (HEK293 cells devoid of mtDNA). As expected, activities of partially mtDNA encoded OXPHOS complexes I, III, IV and V were significantly reduced in rho 0 cells compared to wild-type cells. Interestingly, the activities of the solely nuclear encoded OXPHOS complex II and of CS were not increased or equal, but also significantly decreased in rho 0 cells. To confirm the results obtained by enzymatic measurements, isolated mitochondria of the respective cell-lines were separated by Blue Native gel electrophoresis. The amount of the mitochondrial membrane protein porin in wild-type did not differ from rho 0 cells. However, complex II was again reduced in rho 0 cells compared to wild-type cells. The general downregulation of all OXPHOS complexes and of CS was not due to a feedback regulation involving the transcriptional regulators nuclear respiratory factor 1 and 2 (Nrf1 and Nrf2) or specificity factor 1 (Sp1), since we did not observe a significant difference in mRNA expression of Nrf1, Nrf2 and Sp1 between wild-type and rho 0 HEK293 cells.
Conclusions: Cells with reduced mtDNA levels tend to increase the expression of nuclear encoded mitochondrial proteins. In contrast, cells devoid of mtDNA show a reduction. This has to be considered in interpretation of enzyme measurements of tissue samples, especially if a depletion of mtDNA is suspected.

A 006 Mitochondrial dysfunction and increased autophagy in cardiomyogenic HL-1 cells with STAT3 knock down

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Background: The transcription factor STAT3 is a key responder to IL-6 type cytokines that play a major role for cardiac survival. The importance of STAT3 itself for cardiac protection was demonstrated in various models of STAT3 overexpression and deletion. The recent identification of STAT3 as a mitochondrial protein with an activating effect on respiratory complex I defined a novel function of STAT3. The aim of this study was to characterise the consequences of STAT3 deficiency for mitochondrial function.
Observations: Cardiomyogenic HL-1 cells with an shRNA induced STAT3 knock down (KD) were used as a model system. Complex I activity measured from mitochondrial suspensions was significantly reduced in STAT3 KD cells. Analysis of mitochondrial morphology with transmission electron microscopy showed ultrastructural damage and swelling of STAT3 KD mitochondria. Mitochondrial damage after treatment with the superoxide producer menadione, however, was less severe in STAT3 KD cells compared to control cells. As complex I is involved in superoxide production through menadione, this finding is consistent with an activating effect of STAT3 on complex I. In addition to the mitochondrial damage observed, STAT3 KD cells displayed a remarkable increase in autophagosome and lysosome formation. As a central proteolytic pathway, autophagy

is involved in maintaining functional homeostasis by removing dysfunctional organelles as well as metabolic homeostasis by providing energy substrates for re-entry into respiration. We are currently investigating, which of these two aspects is the driving force for the autophagy observed here.
Conclusions: Our results demonstrate an important role of STAT3 for maintaining mitochondrial function. Considering that cardiac tissue critically depends on mitochondrial respiration, the data add a novel aspect to explain the protective function of STAT3 for cardiac tissue integrity.

A 007 Time-elapsed effects of extracellular NAADP on cellular calcium reservoirs in undifferentiated skeletal muscle cells and influences of V-type ATPase inhibitors

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Background: Nicotinic acid adenine dinucleotide phosphate (NAADP) is the most potent nucleotide to release calcium. In nanomolar concentrations NAADP targets selectively ryanodine type 1 receptor on the sacroplasmatic reticulum and the two pore channel receptors localized in lysosomes and dense-core secretory vesicles. Beside intracellular targets the extracellular application of NAADP has been found to cause calcium transients in neuronal cells.
Observations: In this study we investigated the role of extracellular NAADP in undifferentiated skeletal muscle cells to evoke calcium transients from intracellular stores. In time series of confocal fluorescence-microscopy images extracellular NAADP triggers calcium transients in a concentration dependent manner. The V-type ATPase inhibitors, bafilomycin A1 and concanamycin A, prevented NAADP induced calcium release. We have therefore investigated the role of lysosomes in the skeletal muscle cells and found efficient destruction of these organelles by bafilomycin A1 or concanamycin A. The latter effects increased the cytosolic calcium concentrations and triggered acidification, observed at low nanomolar concentrations. Finally, we observed an overlap of apoptosis and autophagy triggered in the skeletal muscle cells.
Conclusions: Lysosomal V-type ATPase inhibitors trigger multiple effects in skeletal muscle cells including autophagy, apoptosis and acidification, which in summary may have a profound impact on cellular signalling. Hence, such have to be carefully used to block NAADP triggered calcium signals.

A 008 Characterization of Recombinant Sucrose Synthase Isoform 4 from Potato (Solanum tuberosum L.) in the Sucrose Synthesis Direction

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Background: Sucrose synthase (SuSy) is a retaining glycosyltransferase that catalyses the reversible conversion of fructose and a nucleoside diphosphate glucose into the corresponding nucleoside diphosphate and sucrose. The aim of this work was the characterization of SuSy isoform 4 from potato (Solanum tuberosum L.) in the sucrose synthesis direction.
Observations: Recombinant SuSy was expressed at high levels in Escherichia coli. Chromatography on Niquel affinity column and a Superdex gel filtration column gave a homogeneous protein of specific activity 3.98 ± 0.01 mmol/min·mg protein. SDS PAGE showed a single band which had the expected molecular weight of 96,000 Da. The observed Km for UDPG and ADPG were 0.069 ± 0.009 mM and 0.24 ± 0.05 mM, respectively. The Vmax value for UDPG was 2.5-fold higher than that for ADPG. On the other hand, the apparent Km for fructose was 17.68 ± 0.45 mM when UDPG was fixed and 19.83 ± 0.61 mM when ADPG was fixed; however, the Vmax was 7-fold higher in the first case. The presence of UDP and ADP produced a considerable decrease of SuSy specific activity. Nonetheless, we did not observe inhibition by product up to 10 mM of sucrose. SuSy 4 is a key enzyme in the starch biosynthetic pathway so some metabolites of this pathway were tested. Glucose and fructose 6-phosphate did not have a significant effect on sucrose synthase activity, but glucose 6-phosphate (G6P) and fructose 1,6-biphosphate (F16BP) were inhibitory of the SuSy activity.
Conclusions: Typical Michaelis-Menten kinetics were observed for SuSy 4. UDPG was more effective glucosil donor than ADPG. The enzyme presents inhibition by UDP and ADP. Inhibition of SuSy in the sucrose synthesis direction by G6P and F16BP has probably metabolic significance.

A 009 Gene expression profiles of Entamoeba histolytica in response to different iron environments

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Background: Iron is an essential element for almost all living organisms since it is used at the active site of important redox enzymes dealing with cellular respiration and oxidation. Entamoeba histolytica is the parasite causing amoebiasis, an infectious disease targeting the intestine and liver of humans. The molecules involved in amoeba adaptation to different iron conditions during the invasive process (low levels of iron during intestine colonization and higher levels in blood and liver) are unknown.
Observations: To investigate the effects of iron availability on gene expression in E. histolytica, we determined by microarray experiments the gene expression profile of parasites grown in the presence of different iron concentrations. Conditions included low amounts of iron, iron starvation and iron starvation supplemented with hemoglobin. Genes encoding important proteins involved in iron metabolism, reported in other organism such as bacteria, were identified for the first time in E. histolytica. The influence of iron on the amount of these transcripts was confirmed by quantitative real-time PCR and their protein products were analyzed by Western blots. A heme biosynthesis pathway has not been reported in amoeba, since a ferrochelatase has not been found. With our approach we identified two genes encoding proteins presenting homologies with bacterial

ferrochelatase. In addition, adaptation of E. histolytica to iron depletion was accompanied by increased transcript amounts for a subset of cell signaling molecules including members of the immune-associated nucleotide GTPase gene family for which a role in parasite adaptation to the intestinal environment and virulence could be suggested.

Conclusions: Characterization of the activity of these novel enzymes in heme biosynthesis and iron metabolism as well as the role of newly identified proteins implicated in iron-induced signaling represents an important contribution to the understanding of Entamoeba histolytica metabolism during pathogenicity.

A 010 Retinoids ameliorate insulin resistance in a leptin-dependent manner

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Background: We previously demonstrated that impaired hepatic retinoic acid receptor (RAR) signaling is a possible cause of non-alcoholic fatty liver disease (NAFLD) in clinical and experimental settings. Accumulating evidence suggests a crucial role of insulin resistance in the development and progression of NAFLD. In this study, mouse models of NAFLD with insulin resistance were used to gain more insights into the therapeutic potential of retinoids in NAFLD.

Observations: Dietary administration of all-trans retinoic acid (ATRA) resulted in significant reductions in whole body and visceral fat weights, liver to body ratio, and serum ALT and AST levels, and significant improvement in liver histology of C57BL/6 mice fed a high-fat, high-fructose (HFHFr) diet. In addition, the ATRA treatment markedly ameliorated hepatic insulin resistance in parallel with the upregulation of leptin receptor (Lepr) expression and the activation of leptin signaling pathway including Stat3 and Jak2 in the livers of HFHFr diet-fed C57BL/6 and KK-Ay mice, but not in the leptin-deficient ob/ob mice. These results strongly suggested that a leptin-dependent mechanism is involved in the therapeutic effect of ATRA on insulin resistance. Moreover, in vitro experiments demonstrated that Lepr gene is a direct target of ATRA, and that ATRA significantly enhanced insulin-induced IRS1 tyrosine phosphorylation solely in the presence of leptin. Finally, it was demonstrated that a selective RARa/b agonist, Am80 also ameliorated insulin resistance in KK-Ay mice, suggesting that the RAR signaling is involved in the mechanism of action in the ATRA-induced improvement of insulin sensitivity.

Conclusions: The present study demonstrated for the first time that ATRA ameliorated insulin resistance in a leptin-dependent manner. These data suggest that pharmacological RAR activation has therapeutic potential for the treatment of NAFLD associated with insulin resistance.

A 011 Characterization of the distal promoter of the human pyruvate carboxylase gene in pancreatic beta cells

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Background: Pyruvate carboxylase (PC) is a crucial enzyme that plays a role in many biosynthesis pathways in various tissues including glucose-stimulated insulin secretion. Dysregulation of the PC gene in the pancreatic islet has been shown to be involved in the development of diabetes in humans with type 2 diabetes. In humans, very limited information of transcriptional regulation of the human PC gene is available. Here we determined the transcriptional regulation of the human PC gene in beta cells.

Observations: In this study, RT-PCR analysis performed with cDNA prepared from human liver and pancreatic islets showed that the distal (P2) but not the proximal (P1) promoter of the PC gene was active in pancreatic islets. The 1108 bp upstream fragment of the human P2 promoter was cloned and its 5'-truncated were generated by PCR and ligated to the luciferase reporter gene. These constructs were transiently transfected into the rat insulinoma cell line, INS-1 832/13. This showed that the nucleotides located between -365 and -240, and -114 and -41 contain positive regulatory elements while the nucleotides located between -489 and -365 contain negative regulatory elements. To localize the positive regulatory region in the human P2 promoter, the 25-bp and 15-bp internal deletion constructs of the human P2 promoter were generated and transiently transfected into INS-1 832/13 and non-beta cells (HEK293T). The results showed that four regions of the P2 promoter including -340 to -315, -114 to -99, -69 to -54, and -54 to -39 act as positive regulatory sequences. However, only the -340 to -315, -114 to -99, and -54 to -39 regions are important elements that mediate transcription in beta cells.

Conclusions: In humans, two alternative promoters are involved in the production of multiple mRNA isoforms in different cells. The 1108 bp 5' upstream of the human P2 promoter contains regulatory regions that are responsible for transcriptional regulation of the human PC gene in pancreatic beta cells.

A 012 Biochemical and structural characterization of B23-mediated transcription activation of PCNA gene upon UV irradiation

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Background: B23, also known as NPM1, NO38, is a highly phosphorylated acidic protein that is abundantly expressed in nucleolus of mammalian organisms. B23 has pleiotropic functions in ribosomal biogenesis, centrosome duplication, transcription regulation.The PCNA gene plays a critical role in DNA repair, its transcription is rapidly elevated when cells are exposed to UV irradiation. Our work aims to investigate the mechanism

of B23-mediated transcription activation of PCNA gene upon UV irradiation.

Observations: Our previous data have shown that increased NPM expression results in elevated PCNA transcription level in UV-irradiated cells through recruitment of YY1 and the p300 onto the PCNA promoter. ChIP assay showed that YY1 and NPM bind PCNA promoter after UV irradiation, but B23-YY1-PCNA promoter assembly still needs further characterization. The B23 and YY1 DNA-binding region have been expressed and purified from E.Coli, the promoter region of PCNA has also been amplified. EMSA results show that B23 physically interacts with the PCNA promoter, and further studies are needed to clarify how B23-PCNA promoter interaction might affect the binding of YY1 to PCNA promoter. NMR-based structural studies of B23-PCNA promoter complex will be performed to explore the importance of acetylation on B23-mediated transcription regulation. B23 C-terminal domain has been isotope N15-labelled and purified, and the DNA oligo corresponding to the PCNA promoter region will be titrated to the protein, then the HSQC spectrum will be monitored for significant chemical shifts in order to identify the key residues for B23-PCNA promoter interaction. Our preliminary results in this aspect will also be reported.

Conclusions: Our results show that B23 and YY1 both bind to PCNA promoter afer UV treatment, and the question of B23-YY1-PCNA promoter assembly and B23 acetylation are important to understand the regulatory pathway that enables cells to respond to induction by damaged DNA.

A 013 Chromosome stability: linking cohesin and transcription

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Background: The cohesin complex links sister chromatids until their timely segregation during cell division. Yet despite its crucial role in chromosome stability, the nature of cohesin's interaction with chromosomes remains incompletely understood. We have previously reported that cohesin relocates from its initial loading sites and accumulates at sites of convergent transcriptional termination along S. cerevisiae chromosomes, and that changes in transcriptional status influence cohesin localization.

Observations: To characterize the mechanism of cohesin relocation in vivo, we examined cohesin repositioning along model loci upon transcriptional induction. To identify cohesin binding sites, we utilized chromatin immunoprecipitation in combination with hybridization to whole-genome oligonucleotide microarrays (ChIP-chip). We found that downstream relocation of cohesin still occurred after inactivation of the Scc2/4 cohesin loading complex. In addition, the downstream-relocated pool of cohesin consisted of previously chromosome-bound rather than newly-loaded cohesin. Moreover, 'loading-defective' cohesin repositioned downstream normally upon transcriptional induction, and the relocation of cohesin did not require Wapl, which is thought to promote cohesin turnover. Furthermore, we demonstrated that aberrant transcriptional termination affected cohesin localization, highlighting the correlation between transcription and cohesin positioning.

Conclusions: Thus, a number of factors implicated in cohesin binding are not required for cohesin relocation, while transcription had a significant impact. We are now defining the role of transcription in cohesin movement and determining the importance of cohesin relocation in mediating chromosome stability.

A 014 Kinetics of transcription visualized at the single gene level during the cell cycle

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Background: Is it possible to follow the production of mRNAs from a single gene in a single living cell? Current experimental designs for studying the kinetics of protein interactions with DNA or RNA in vivo mostly rely on over-expressed genes and the subsequent following of recruitment of fluorescent proteins to these gene-arrays. Multiple-copy gene-arrays form non-physiological levels of the gene that can be different from the endogenous state of a single-copy gene.

Observations: We generated a human cell system that enables the detection of transcriptional gene activity at the level of a single allele. Using this system we analyzed and quantified the kinetics of the cyclin D1 gene transcription during the cell cycle at high spatial and temporal resolution. Transcription kinetics of the cyclin D1 allele under either viral (CMV) or endogenous promoter control were examined using time-lapse imaging, fluorescence recovery after photobleaching (FRAP), and quantitative fluorescence in situ hybridization (RNA FISH). Measurements of transcription of a cyclin D1 allele under either viral or endogenous promoter control during the cell cycle allowed the quantification of promoter firing, the number of mRNAs present on active genes, and the in vivo rates of transcription, compared between the two different promoters. This analysis also demonstrated the change in kinetics of active RNA transcription as it proceeded spatially and temporally in parallel to DNA replication during S phase.

Conclusions: This new approach allows for high resolution spatio-temporal analysis of real-time transcription of single genes during the cell cycle, and provides read-outs of mRNA production at the single-molecule level.

A 015 PARP-1, a novel partner of Ets-1 in cancer cells: Study of this interaction on the transcriptional activity of Ets-1

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Background: Ets-1 is a transcription factor of the Ets family. When overexpressed, it is involved in the development of invasive pathologies such as rheumatoid arthritis and cancers. Ets-1 regulates gene expression with partners by binding to specific DNA elements, called Ets binding sites (EBS), found in the promoters of its target genes. Therefore, the understanding of the specificity of its functions is linked to the characterization of its partners.

Observations: For this purpose, we used an affinity purification strategy of Ets-1 partners using biotinylated Ets-1 for streptavidin pull-down. Several potential interaction partners were identified by MALDI-TOF mass spectrometry. Among those, we were able to identified, interacting with Ets-1, the Poly (ADP-Ribose) Polymerase-1 (PARP-1). The interaction between PARP-1 and endogenous Ets-1 was confirmed by co-immunoprecipitation in the cell. PARP-1 is an abundant nuclear protein which catalyzes poly-ADP-ribosylation (PARylation) and plays diverse roles in many molecular and cellular processes, including DNA damage detection and repair and chromatin modification. Previous studies have revealed that besides its role in DNA repair pathway, PARP-1 is involved in the regulation of different transcription factors. In this study, we show that Ets-1 interacts directly with PARP-1 and is parylated in return. Using PARP-1 catalytic inhibitors, our results show that parylation of Ets-1 has direct consequences on its transcriptional activity.
Conclusions: Taken together, these findings strengthen the idea of a functional link between transcription factors and DNA repair proteins. Furthermore, inhibition of PARP-1 could be a new strategy to target Ets-1 activity in tumours and lead cancer cells to death.

A 016 The lack of HMGB1 in mammalian cells (or of NPH6 proteins in yeast) substantially reduces nucleosome number

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Background: It is currently assumed that the number of nucleosomes is fixed, and proportional to the quantity of DNA to be packaged. The location of some nucleosomes may depend on the sequence being packaged (or its propenity to being bent). Strongly positioned nucleosomes may determine the ‘statistical positioning’ of the remaining nucleosomes, like some well-parked cars guide the parking of other cars in a parking lot. Nucleome position determines the accessibility of DNA, and controls gene expression.
Observations: We show that High Mobility Group Box 1 protein (HMGB1) facilitates nucleosome assembly. Mammalian cells lacking HMGB1 contain a reduced amount of core, linker and variant histones, and a correspondingly reduced number of nucleosomes. Yeast nhp6 mutants lacking Nhp6a and –b proteins, which are related to HMGB1, also have a reduced amount of histones and fewer nucleosomes. Nucleosome limitation in both mammalian and yeast cells increases the sensitivity of DNA to damage, increases transcription globally, and affects the relative expression of about 10% of genes. In yeast nhp6 cells the loss of more than one nucleosome in four does not affect the location of nucleosomes and their spacing, but nucleosomal occupancy. The decrease in nucleosomal occupancy is non-uniform, and can be modelled assuming that different nucleosomal sites compete for available histones. Sites with a high propensity to occupation are almost always packaged into nucleosomes both in wild type and nucleosome-depleted cells; nucleosomes on sites with low propensity to occupation are disproportionately lost in nucleosome-depleted cells.
Conclusions: We disprove the notion that the number of nucleosomes is fixed and set by default. At least in yeast, variation in nucleosome number affects nucleosomal occupancy (but not location), both genomewide and gene-specifically, and constitutes a novel layer of epigenetic regulation.

A 017 Transcriptional regulation of the mouse Hspa1a gene: crucial roles for NFkappaB and NF-Y

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Background: Expression of the heat shock protein Hspa1a, a well-recognized molecular chaperone involved in heat-shock and other stress responses, is regulated at the transcriptional and translational levels. However, the mechanism of transcriptional regulation of Hspa1a gene in mouse remains unknown. We undertook a systematic analysis of the mouse Hspa1a gene to identify the cis- elements (promoter domains) and transcription factors that are crucial for its regulation.
Observations: Computational analysis of ~ 1kb promoter region of the mouse Hspa1a gene using CONSITE predicted involvement of several transcription factors (viz. NF-Y, NFkappaB apart from HSF-1, the well-studied transcription factor for heat shock proteins). Generation and transfection of mouse Hspa1a promoter–firefly luciferase reporter plasmids (5’-deletion constructs) into kidney HEK-293 and neuroblastoma Neuro-2a cells showed crucial roles for the promoter domains harbouring NF-Y and NFkappaB binding sites in Hspa1a expression. Co-transfection of NF-Y and NFkappaB expression plasmids with the Hspa1a promoter-luciferase constructs showed enhancement of the luciferase activity. Furthermore, expression of a dominant negative mutant NF-Y protein and a specific siRNA for NFkappaB (p65) in HEK-293 cells resulted in a substantial reduction of the Hspa1a promoter-reporter activity. Elecrophoretic mobility shift assays using NF-Y and NFkappaB oligos displayed formation of specific complexes with HEK-293 nuclear proteins. Consistent with these in vitro results, chromatin immunoprecipitation experiments using Neuro-2a cells showed in vivo interactions of these transcription factors with the mouse Hspa1a promoter.
Conclusions: This study provides evidence for a previously unrecognized mechanism of transcriptional regulation of the mouse Hspa1a gene by the transcription factors NFkappaB and NF-Y.

A 018 Inheritance and establishment of heterochromatin in human pre-implantation embryos

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Background: In mouse early embryos, euchromatic and heterochromatic chromatin states differ between maternal and paternal genomes. While the maternal genome is marked by methylation of histones, the paternal genome is initially devoid of such modifications. To study a possible role of histones in intergenerational inheritance of chromatin states in humans, we studied the dynamics of histone modifications and various chromatin associated proteins in human spermatozoa and pre-implantation embryos.

Observations: In late human zygotes, maternal chromatin is abundantly marked by trimethylation of histone H3 on lysine 9 and 27 (H3K9me3 and H3K27me3). In contrast, we fail to detect H3K27me3 on paternal chromatin, while H3K9me3 is present at a few DAPI-intense regions corresponding to pericentromeric heterochromatin in somatic cells. Over three subsequent cleavage divisions, H3K27me3 staining reduces at the maternal genome suggesting non-maintenance of the maternally inherited mark. In morula embryos, H3K27me3 reappears, concomitant with increased expression of EED and EZH2. For H3K9me3, however, broad maternal and heterochromatic paternal localization is maintained until the 8-cell stage, after which it adopts a somatic pattern. The paternal enrichment of H3K9me3 in human embryos is in sharp contrast with the lack of this modification in mouse early embryos and relates to the presence of H3K9me3 in human spermatozoa. In absence of H3K9me3, pericentromeric heterochromatin in mouse embryos is enriched in H3K27me3 and proteins of Polycomb Repressive Complexes 1 and 2. At human paternal chromatin, we failed to detect PRC1 components that are possibly rendered obsolete by the presence of H3K9me3.

Conclusions: Different from mice, paternal heterochromatin in human embryos is not transiently formatted by PRC1. Instead, it appears to be transmitted in the canonical configuration from spermatozoa to the embryo, suggesting a paternal epigenetic contribution to constitutive heterochromatin formation.

A 019 Transcriptional profiling of shelterin genes uncovers their potential as novel biomarkers in DNA damage response

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Background: Telomeres are primarily regulated by TERT (telomerase reverse transcriptase) and TERC (telomerase RNA component). In addition, protein complex called shelterin is responsible for maintenance and protection of telomeres. So far a range of studies on transcriptional regulation of TERT and TERC has been reported; however, that of shelterin genes are incomplete. In contrast to TERT, there are a limited number of reports regarding the upregulation of shelterin genes during carcinogenesis.

Observations: To investigate the transcriptional regulation of shelterin genes, we performed a TaqMan real-time RT-PCR in HeLa and U2OS cells, and a promoter-luciferase reporter assay in U2OS cells. We prepared pGL3 reporter constructs as follows; TERT -3359/+53 and -523/+50, TERC -798/+69, TNKS1 (TRF1-interacting ankyrin-related ADP-ribose polymerase 1) -391/+7, TNKS2 -560/+65, TPP1 (POT1- and TIN2-organizing protein) -927/+23, PINX1 (PIN2-interacting protein 1) -1008/+32, TRF1 (Telomeric repeat-binding factor 1) -789/+12, TRF2 -221/+71, POT1 (Protection of telomeres) -173/+147, TIN2 (TRF1-interacting nuclear factor 2) -330/+40 and RAP1 (TRF2-interacting telomeric protein) -428/+143. Numbers in the parenthesis is based on the transcriptional start site as +1 nucleotide. We demonstrated that TNKS1, TRF1 and POT1 mRNA were downregulated by topoisomerase II inhibitors etoposide and doxorubicin. In contrast, RAP1 mRNA was upregulated in U2OS cells. Downregulation of POT1 and upregulation of RAP1 were also confirmed by promoter reporter assay, and RAP1 could be regulated by transcription factor SP1. We also demonstrated that E2F1 apoptosis inducer upregulated TRF1 and TIN2 reporter activities.

Conclusions: We first demonstrated that specific shelterin genes are uniquely regulated by DNA damage. Particularly, mRNA and promoter activity for POT1 and RAP1 were similarly regulated by etoposide and doxorubicin. Taken together, shelterin genes could be unique biomarkers for prognosis of certain cancers.

A 020 The tumor suppressor L(3) mbt inhibits neuroepithelial proliferation and acts on insulator elements

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Background: Stem cells need to control the balance between self-renewal and differentiation. In Drosophila neuroblasts tumor suppressor genes regulate this balance. When their function is compromised, neuroblasts create only self-renewing daughter cells – leading to the tumor formation. In addition to known regulators like lgl and brat, screens identified the chromatin binding factor lethal (3) malignant brain tumor. We analyzed the role of l(3)mbt to identify the cause of the tumorous overgrowth.

Observations: Our data show that tumor formation in l(3)mbt mutants is very different from the known Drosophila tumor models. Neuroepithelial cells in the optic lobes overproliferate in an uncontrolled manner in l(3)mbt mutants and generate excess numbers of neuroblasts in late l(3)mbt tumors. Using fly genetics we demonstrate that proliferation control genes that are normally repressed by the SWH signaling pathway are upregulated in l(3)mbt mutants and that the SWH pathway is necessary

for l(3)mbt tumor formation. By Chromatin-Immunoprecipitation and subsequent deep sequencing we show that L(3)mbt is located at DNA sequences bound by the chromatin insulators CTCF, CP190 and BEAF-32. Like in CTCF mutants, the homeotic gene Abdominal-B (Abd-B) is downregulated in the absence of L(3)mbt. Inhibition of the chromatin insulator mod(mdg4) results in upregulation of the SWH target gene bantam.

Conclusions: We show that L(3)mbt controls proliferation of the optic-lobe neuro-epithelium in larval brains by ensuring the correct expression of Hippo pathway target genes. Further we find that L(3)mbt binds to insulator DNA elements, which suggests a role for L(3)mbt as an insulator.

A 021 Chromatin Binding, Nucleosome Spacing and ncRNA-mediated Regulation of the Remodeling ATPase ISWI

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Background: Chromatin modifications, occurring without changes in the DNA sequence, set different chromatin functional states and constitute the epigenetic marks of our genome. Despite the wealth of data concerning the mechanisms of action of chromatin remodeling factors and histone modifying enzymes, relatively little is known about how their activities are coordinated and inherited to regulate chromatin structure, gene expression and other nuclear functions.

Observations: We got interested in dissecting the functional network of regulation existing between ATP-dependent remodelers and chromatin factors. ISWI is an evolutionarily conserved nucleosome sliding factor playing essential roles in transcription, DNA replication, and chromosome organization. Using the fruit fly as a model system and a combination of genome wide and bioinformatic approaches we found that ISWI binds genes near their promoters affecting nucleosome spacing at their transcription start site. Our work shows that higher eukaryote transcription and chromosome organization is regulated genome-wide by the activity of the chromatin remodeling factor ISWI. Further, Using an in vivo assay to identify factors regulating ISWI activity, we recovered a genetic interaction between ISWI and hsromega. The hsromega gene encodes a non-coding RNA that is essential for the assembly and organization of hnRNP-containing nucleoplasmic omega speckles.

Conclusions: Our study shows how in higher eukaryotes the activity of nucleosome remodelling factors regulates gene expression genome-wide. Our work highlights also a novel role for chromatin remodelers in organization of nucleoplasmic compartments through a functional interaction with a large ncRNA.

A 022 Molecular mechanisms of alternative transcription initiation during Xenopus development

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Background: During amphibian development the event of mid blastula transition (MBT) is characterized by the activation of silent embryonic chromatin when transcription becomes clearly detectable. Binding of TATA-box binding protein(TBP) containing TFIID-type complexes to promoters is a rate-limiting step of transcription initiation. Furthermore, other multi-subunit coactivators,such as GCN5-containing complexes are also known to activate gene expression even though they lack any of the TBP-related factors.

Observations: We investigate mechanisms underlying TBP-independent transcription initiation by triple depletion of TBP and its related factors (TRFs):TBP2 and TLF. We use Xenopus embryos, which allow us to study the ´first rounds´ of transcription and the onset of cellular differentiation early after MBT. To determine the relative contributions and roles of distinct TFIID-type and GCN5-containing SAGA complexes we also deplete TFIID-specific and SAGA-specific subunits, such as TAF5 and TAF5L, respectively.Thus, we microinject 1-cell stage embryos with chemically modified antisense oligos for RNA degradation and/or morpholino oligos for translation inhibition. Since it is well established that ySAGA functions as a co-activator for GAL4 by recruiting TBP to promoters we combine our loss-of-function experiments with GAL4-VP16 transcription activation assays. We co-inject in vitro reverse transcribed mRNAs for exogenous GAL4-VP16 activator expression together with a CAT reporter vector construct and determine CAT reporter gene expression levels by Real-Time RT qPCR. Our results show efficient transcription activation by GAL4-VP16 in embryos after MBT upon triple knockdown of TBP, TBP2 and TLF.

Conclusions: This work highlights the intriguing finding that transcription activation does not universally require TBP and TRFs. Our results are unexpected concerning that combined knockdown of these factors did not lead to lethality but rather productive transcription activation has been detected in vivo.

A 023 KLF5-ERb pathway is responsible for the contradictory effects of estrogen on prostate tumor growth

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Background: Estrogen (E2) is known to regulate androgen-insensitive prostate tumor formation. Previously, we reported that E2 enhance the development of androgen-insensitive prostate cancer. On the other hand, E2 is effective against androgen-insensitive prostate cancer. These reports suggest that E2 possesses biphasic functions on prostate cancer progression. However, the

molecular mechanisms underlying the biphasic effects of E2 on androgen-insensitive prostate cancer are not well worked out.
Observations: Our results showed that a low dose of E2 promotes prostate tumor growth. In contrast, a higher dose of E2 significantly inhibits tumor growth. E2 modulates prostate tumor growth by suppressing transcription of the Krüppel-like zinc finger transcription factor 5 (KLF5)-target genes FOXO1 and PDGFA through a non-classical pathway involving estrogen receptor b (ERb) and KLF5. Reduction of FOXO1 levels by E2 inhibits anoikis in prostate cancer cells and promotes prostate tumor growth. In contrast, down-regulation of PDGFA expression inhibits angiogenesis and suppresses tumor growth. Estrogens are effective in the treatment of advanced prostate cancer. However, it is known that estrogen causes side effects through transactivation of ER. We identified novel estrogen receptor (ER) modulator, GS-1405, which exerts anti-estrogenic effects on ER transcriptional activity and its target genes. We showed that GS-1405 inhibits PDGFA expression through ER_-KLF5 pathway, and suppresses angiogenesis in androgen-insensitive tumors. Consequently, GS-1405 abrogates androgen-insensitive prostate tumor growth.
Conclusions: Our findings suggest that the effects of E2 on prostate tumor growth are determined by the balance between FOXO1 and PDGFA expression. And compounds like GS-1405 can inhibit androgen-insensitive prostate cancer progression with minimal side effects which are caused by transactivation of ERs.

A 024 Transcriptional overlap by the imprinted Airn ncRNA is sufficient to repress the Igf2r gene

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Background: The imprinted non protein coding RNA (ncRNA) Airn silences the protein coding gene Igf2r on the paternal chr17 in the mouse. We established an in vitro model in differentiating ES cells that emulates the developmental onset of imprinted expression of Igf2r and Airn. This model was validated by showing that, similar to mice, shortening of the ncRNA Airn to 3kb results in biallelic expression of Igf2r. This makes Airn a paradigm for studying the mechanisms of transcriptional regulation by ncRNAs.
Observations: We used the ES cell model to determine the functional length of Airn. ES lines carrying different Airn truncations were generated and tested for their ability to silence Igf2r upon ES cell differentiation. We demonstrated that truncation of Airn to approximately half of its length (51kb overlapping the Igf2r promoter) does not abolish its silencing function but shortening of Airn to 16kb (not overlapping the Igf2r promoter) results in loss of silencing of Igf2r on the paternal allele. To directly test if the Airn RNA represses Igf2r we used by RNA-FISH to analyze features previously connected with repressive ncRNAs including structure, position within the nucleus and distance to the nucleolus. Surprisingly none of these features differed between the repressive 51kb Airn and a non-repressive 16kb Airn. This indicated that the Airn ncRNA sequence and structure do not play a role in Igf2r repression. Since the Airn transcript overlaps the Igf2r promoter we next tested if transcriptional overlap was sufficient to repress Igf2r. To do this we moved the Airn promoter directly in front of the Igf2r promoter and found that Igf2r is only repressed when Airn transcript overlaps its promoter.
Conclusions: We used gene targeting in ES cells to test parts of the Airn ncRNA for a function in repressing Igf2r. This approach and an RNA-FISH analysis did not support a functional role for the Airn ncRNA. Our data indicates that the Igf2r gene is repressed due to transcriptional overlap by the Airn ncRNA.

A 025 Onset and maintenance of Airn non-coding RNA mediated imprinted expression in an in vitro embryonic stem cell model

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Background: At the mouse imprinted Igf2r locus, the paternally expressed Airn macro non-coding RNA (ncRNA) silences in cis the overlapped protein-coding Igf2r gene, which is thus maternally expressed. Airn has been shown to be necessary to initiate imprinted Igf2r expression, but it is unknown if it is necessary to maintain paternal Igf2r repression or if its repressive effects are confined to a 'window of opportunity' during mouse development.
Observations: I have used an in vitro embryonic stem (ES) cell differentiation system that mimics the developmental onset of Igf2r and Airn imprinted expression and gene targeting technology to establish two inducible systems to turn Airn on or off during ES cell differentiation. To turn Airn on, I created an ES cell line expressing a truncated, non-functional form of Airn in which the truncation stop signal is flanked by loxP sites. A CreER activated by addition of tamoxifen is used to remove the loxP-flanked stop signal, rescuing the truncation and restoring full-length Airn transcription during ES cell differentation. Thus I have established a tool to test if the repressive effects of Airn are confined to a developmental window of opportunity. To turn Airn off, I am in the process of establishing an ES cell line in which the Airn promoter itself is flanked by loxP sites. In this case, activating CreER will knock out the Airn promoter, allowing transcription of the ncRNA to be turned off at will during ES cell differentiation. With this tool I can test if Airn is necessary to maintain paternal Igf2r repression during development.
Conclusions: I established an inducible system to convert Airn from a non-functional to a functional state during differentiation. The reverse system is being developed. Both will shed light on the mechanism of Airn mediated repression, aiding in the design of strategies to block repression by macro ncRNAs.

A 026 Downregulation of the chromatin-remodelling factor Brahma in Sod1 G93A cells modifies transcription and alternative splicing of genes involved in axon growth and guidance

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Background: The human protein Brahma (Brm, SMARCA2 gene) is one of the two ATP-ases subunits of the mammalian SWI/SNF chromatin remodeling complex. Brm-containing SWI/SNF complexes are enriched in neurons, where they play crucial roles in the regulation of genes involved in neuronal differentiation. Moreover, it has been recently demonstrated that Brm associates with components of the spliceosome and with RNA-Polymerase II to regulate the inclusion of alternative exons.
Observations: While investigating with a splicing-sensitive microarray the gene expression changes triggered by mitochondrial stress, we found that Brm is downregulated in the SH-SY5Y neuroblastoma cells overexpressing the SOD1 G93A protein, which is one of the genetic causes of Amyotrophic Lateral Sclerosis (ALS). SOD1 G93A expression also induces changes in transcription and alternative splicing of genes involved in axon growth and guidance. Starting from these observations, we are investigating the link between Brm and the regulation of these genes. We found that only the transcripts encoding the full-length, catalitically active Brm isoforms are downregulated by SOD1 G93A overexpression. In the SMARCA2 promoter, we mapped a region whose activity is directly impaired by mitochondrial stress. Reintroduction of Brm expression in the SOD1 G93A overexpressing cells, and silencing of the same gene in the SOD1 WT overexpressing cells, allowed us to identify two Brm targets: p15RS (which encodes a regulator of Wnt pathway), which is regulated by Brm at the alternative splicing level, and Nrg1 (which encodes a neuron-restricted growth factor), which is regulated by Brm at the gene expression level.
Conclusions: Our results suggest a novel role for Brm in the regulation of the expression of subset of genes involved in axon growth and guidance, two processes which are impaired in neurodegenerative disorders such as ALS. We are currently characterizing additional targets of Brm.

A 027 Hepatitis B virus X protein alters p53 regulation of p53AIP1 gene

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Background: Hepatitis B virus X protein (HBx) has been strongly implicated in the carcinogenesis process of hepatitis B virus-associated hepatocellular carcinoma (HCC). One of the mechanisms by which HBx modulates host processes is through interfering with the activation/repression function of host transcription factors. In this study, we use a genome-wide approach to investigate HBx modulation of p53 transcriptional activity and consequent target gene deregulation, and its association with carcinogenesis.
Observations: We employed genome-wide chromatin immunoprecipitation (ChIP) of HBx and control HepG2 and THLE-3 cells to obtain a global view of HBx-modulated p53 binding to its response elements (RE), and expression microarray to profile gene expression deregulation by HBx. Integration of both genome-wide screens revealed a novel HBx-mediated modulation of p53 binding at 2 p53 RE of p53-regulated apoptosis-inducing protein 1 (p53AIP1), with concomitant increase in gene expression. Both p53 RE were confirmed to be essential for p53-mediated gene regulation using mutation studies and promoter assay. HBx increased gene expression in a p53-dependent manner in promoter assay and RNA interference studies. Gene expression profiling of 100 HCC patients showed general up-regulation of p53AIP1 gene expression in patients with high HBx protein levels, highlighting its clinical relevance. Computational and ChIP studies indicate that HBx-induced alteration of p53 binding depends on the proximal transcription co-regulators at the RE. HBx disrupts DNA binding of p53 in the context of a complex of co-repressors including YY1, GATA1 and HDAC1, but augments p53 binding in a context of co-activators such as Sp1.
Conclusions: This study identified a novel modulation of p53 binding at 2 p53REs of p53AIP1 by HBx, associated with an increase in gene expression. Our findings suggest that HBx-induced alterations of p53 binding depend on the proximal transcription co-regulators that are likely associated with p53 at the RE.

A 028 Role of a Novel Deubiquitinating Complex in Transcription Regulation

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Background: Deubiquitinases (DUBs) form a class of specialized proteases that remove ubiquitin from substrates and play fundamental roles in many cellular processes. BAP1 (BRCA1-Associated Protein1) is a nuclear DUB and homozygously mutated of BAP1 in lung, breast and eye cancers suggesting that this enzyme is a tumor suppressor. Nonetheless, the biological function and mechanism of action of this DUB remain poorly defined.
Observations: To shed new light on BAP1 function, we conducted immunostaining studies and found that BAP1 is mainly associated with euchromatin suggesting a role in transcription regulation. Indeed, using a transcription reporter assay, we found that BAP1 activates transcription in a DUB-activity dependent manner. To provide further evidence for the transcriptional role of BAP1, we conducted a tandem affinity immunopurification of BAP1-associated proteins and found that most of the interacting partners are transcription factors and cofactors. Notably, BAP1 forms a complex with HCF-1, a recently described BAP1-interacting protein, and Yin Yang 1 (YY1) transcription factor. Both proteins are known to be important regulators of cell cycle progression and in particular the G1/S transition by controlling

the expression of E2F target genes. Consistently, global gene expression analysis revealed that BAP1 regulates the expression of critical cell cycle genes including E2F targets.
Conclusions: Further studies are required to delineate the exact role of BAP1 in regulating E2F target genes and to define how deregulation of this pathway contributes to tumorogenesis.

A 029 Impact of genetic variation on chromatin state and genome-wide gene expression phenotypes

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Background: During the last few years, genome-wide chromatin state and histone modification profiling has generated information about specific chromatin signatures related to different functional elements of the genome in multiple cell types. However, the degree of stability and the genetic basis of such signatures across individuals remain largely unknown.
Observations: We have produced genome-wide enrichment profiles of three transcription factors (TF), RNA Pol II, MYC, and SPI1, as well as histone modifications H3K4me1 and H4K20me1, in lymphoblastoid cell lines of two trios sequenced as part of the 1000 Genomes project. In the second phase of the study these marks will be profiled in 100 individuals from the 1000 Genomes CEU population, together with five additional histone modifications. For all individuals, low coverage genome sequence and genotype data is available. We will additionally produce genome-wide mRNA and miRNA sequencing data from the same individuals, which allows us to overlay multiple layers of genomic information for a comprehensive picture of the transcriptional state of the cells. With this dataset we will (i) estimate the degree of heritability of chromatin and TF signatures (ii) compare their enrichment patterns across individuals and around key sequence elements such as eQTLs, shown to affect transcript levels in these individuals (iii) analyze the allele-specific effects of DNA sequence variation on chromatin structure and correlate this information with allele-specific gene expression levels from the same individuals.
Conclusions: This study will improve our understanding of the biological landscape around regulatory and other functional elements of the genome and provide better means to address the cellular basis of phenotypic diversity, such as disease susceptibility, in humans.

A 030 Regulation of Transcriptomes by Stochastic Change in Rho-family GTPases Activity

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Background: Although cancer cell populations are typically derived from a single cell, they show extreme heterogeneity in their replicative abilities, metastatic potential, and anti-tumour drug resistance. In this study, we addressed the cause of this heterogeneity. Epigenetic or post-transcriptional regulation have previously been proposed as explanations for such variability. However, other mechanisms are also likely involved in developing monoclonal heterogeneity.
Observations: Using glioma cells, we tested the hypothesis that the stochastic alteration of intracellular signalling underlies the heterogeneity of cancer cells. In C6 glioma cell lines expressing FRET-based biosensors for Rac1, Cdc42, or RhoA, we found that cells penetrating into the brain parenchyma showed higher Rac1 and Cdc42 activities and lower RhoA activity than those advancing in perivascular regions. We also found that Rac1 activity changes stochastically under normal culture conditions. These observations suggested that stochastic variance in Rac1 activity may determine the migration mode of glioma cells. To characterize genes that affect the activity of Rac1, C6 glioma cells were sorted depending on their Rac1, Cdc42 and RhoA activities, and subsequently analysed for their expression profiles by RNA-Seq method. Significant differences in gene expression were detected between glioma cells with high and low activities of signalling molecules. We named this technique FRET-guided transcriptomics (FRET-GT).
Conclusions: FRET-GT represents a versatile tool to examine the contribution of signalling molecules to transcriptomes, and hence the stochastic behavioural changes of cancer cells.

A 031 swsn-2.1, a genetic interactor of lin-35 Retinoblastoma in Caenorhabditis elegans, encodes a SNF/SWI component involved in the regulation of the intestinal cell cycle

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Background: The functions of the Retinoblastoma (Rb) pathway are altered in the majority of human tumors. Thus, the study of cellular mechanisms in which Rb is involved, might be a prerequisite for the development of novel anti-cancer drugs. Since its C. elegans homolog lin-35 exhibits highly similar modes of operation, this nematode represents an excellent model for the investigation of the implications of the Rb pathway in tumorigenesis.
Observations: A genome wide RNAi-screen in C. elegans enabled the identification of genetic interaction partners of lin-35. One of the identified genes was swsn-2.1. This gene presents homology with the human proteins SMARCD1, SMARCD2 and SMARCD3, which are components of the SWI/SNF chromatin remodeling complex. We showed that the SMARCD genes are differentially expressed in colon carcinomas. We also observed that the inactivation of swsn-2.1 causes enhanced proliferation of intestinal cells in C. elegans. Two different mutant C. elegans strains for swsn-2.1 are at our disposal: one of the mutant alleles, he159, was found by our lab in the course of a screen in a deletion mutant library. Amongst others, this mutation produces phenotypes like sterility and vulva defects, whereby the penetrance is variable. The second mutant allele, tm3309, was generated by a C. elegans knock-out consortium under our request and causes arrest in early developmental stages. We are employing these mutant strains, RNAi and transgenes for the functional characterization of swsn-2.1 and the identification of functional partners. Beyond, we are also investigating swsn-2.2, a paralog of swsn-2.1.
Conclusions: Complementary to our research in C. elegans, we will examine the human homologs SMARCD1, SMARCD2 and SMARCD3 in normal tissues, tumors and cell lines. The achieved data will be integrated to generate a hypothesis of a functional relationship between the Rb pathway, the SMARCD genes and human cancer.

A 032 The functional c-Myc dependent polymorphisms of telomerase promoter control hTERT expression and are associated with telomere length

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Background: Telomere shorting occurs after repeated cell division and has a key role in cell senescence, differentiation, and immortalization. Telomere length is mainly regulated by telomerase activity associated with transcriptional activity of human telomerase reverse transcriptase (hTERT), a subunit of telomerase. The hTERT promoter region was well identified and is rich in transcription factor binding sites including c-myc, SP1, and esterogen.
Observations: In our previous study, hTERT polymorphisms, rs2753940 (-1327C > T), rs2853669 (-244 T > C), rs2736100 and MNS16A, were examined for lung cancer association study. To identity effect of the hTERT polymorphisms on telomere length and transcriptional activity, we determined telomere length according to hTERT polymorphisms. The two hTERT polymorphisms, rs2753940 (-1327C > T), rs2853669 (-244 T > C), have a significant association with telomere length (P = 0.04, P = 0.013, respectively). This telomere length may be determined by hTERT expression which was regulated promoter polymorphism (rs2853669) by changing of c-MYC binding.
Conclusions: Two promoter SNPs have a role in expression of hTERT, which may affect telomere length. These two polymorphisms may be used for risk marker of lung cancer.

A 033 Regulation of Globin Gene Switching by KLF1: New Insights

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Background: Two novel mutations in the human KLF1 gene (p.M39L and p.K288X) were identified in a large family from Malta and linked to high and persistent levels of foetal haemoglobin (Hb F). Studies from other laboratories, in families with different KLF1 mutations, indicate that KLF1 plays a diverse role in Hb F control. These HPFH families provide an excellent opportunity to identify these modifier genes, and a multi center study has been setup to coordinate this effort and support this research.
Observations: DNA from 27 family members that consisted of 10 members with hereditary persistence of foetal haemoglobin (HPFH) was analyzed by whole genome linkage analysis and identified a putative locus on chromosome 19p13.13. DNA sequencing of candidate genes under the LOD score revealed two novel mutations in KLF1 with one resulting in a pre-mature stop codon mutation (p.K288X) ablating completely the three Zinc fingers of KLF1. A list of differentially expressed genes was obtained by RNA expression profiling of human erythroid progenitor cells (HEPs) obtained from normal and HPFH family members. BCL11A was amongst this list of differentially expressed genes, and appeared dependent on KLF1 expression patterns. Knockdown of KLF1 in HEPs revealed a concomitant decrease in BCL11A and an increase in human HBG1/2. A number of CACCC boxes were identified in the promoter of BCL11A, and Chromatin Immunoprecipitation revealed binding of the KLF1 to this sequence thus concluding that BCL11A was regulated by KLF1. A number of KLF1 mutations have recently been reported elsewhere with different phenotypes being strongly dependent on what underlying KLF1 mutation is present.
Conclusions: Mutations in KLF1 affecting these biochemical properties may provide important clues to the functional roles of protein-protein interactions and post-translational modifications. Targeting KLF1 or genes in the same pathway may be a fruitful approach to augment Hb F and treat haemoglobinopathies.

A 034 The cis-regulatory code underlying stochastic and mutually exclusive expression of Drosophila rhodopsins

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Background: The Drosophila retina is composed of single unit eyes (ommatidia) that contain eight photoreceptors (PRs) R1-R8. The six 'outer' PRs R1-R6 form a trapezoid with the two 'inner' R7/R8 PRs positioned in the center. Depending on which rhodopsin (rh) is expressed in R7/R8, two main types of ommatidia can be distinguished that are stochastically distributed: 70% (y subtype) express Rh4 in yR7s coupled with Rh6 in yR8. The remaining 30% (p subtype) express Rh3 in pR7s associated with Rh5 in pR8s.
Observations: To gain a better understanding of the mechanisms that underlie this subtype specific rh expression, we are performing a detailed structure-function analysis of the rh promoters. The proximal promoter region of all rhs contains an 11bp motif, the rhodopsin core sequence I (RCSI, consensus TAATYNRATTN). Mutation of the RCSI causes a loss of reporter expression, suggesting that it is necessary for general activation of all rhs. However, its sequence exhibits subtle variation among rh promoters, which is highly conserved over more than 60 million years of evolution. To address the functional relevance of these subtle differences, we changed the RCSIs to the very similar P3 activator motif TAATYNRATTA that drives expression in all PRs when multimerized. Swapping the RCSI with the P3 motif left rh activation intact, but caused specific reporter derepression into other PR subtypes. Remarkably, promoters of the same ommatidial subtype showed similar derepression phenotypes: The 'yellow' rh4 and rh6 were both derepressed into R1-6 and in the ocelli (three small dorsal eyes that express rh2), whereas the 'pale' rh3 and rh5 were derepressed into their yR7 and yR8 counterparts, respectively.
Conclusions: The RCSI, an 11bp motif found in all Drosophila rhs plays a dual role in general activation and stochastic, subtype-specific rh expression. Subtle, but highly conserved single base pair differences in this motif prevent coexpression of rhs in other PR types, which would compromise color vision.

A 035 ER ligands ameliorate fatty liver via a non-classical ER/LXR pathway

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Background: Fatty liver has emerged as a key feature in the pathogenesis of the metabolic syndrome. The feature of fatty liver is the accumulation of triglyceride (TG) levels in the liver. Several lines of evidence indicate that estrogen (E2) reduces TG levels in liver; however, the molecular mechanism underlying these observations remains unclear.
Observations: Here, we show that in the absence of E2, estrogen receptor beta (ERbeta) binds to Liver X receptor (LXR)-target gene promoters through LXR. At these locations, ERbeta coactivates transcription of genes involved in TG synthesis by recruiting p300. In contrast, in the presence of E2, the recruitment of p300 was abrogated and LXR-dependent transcription was downregulated. Consistent with these results, ERbeta increased LXR-dependent TG accumulation in liver, which was eliminated by E2 treatment. To confirm that this mechanism was responsible for the E2-induced reduction in TG levels, we used the phytoestrogen, Phloretin, which repressed LXR transcriptional activity through ERbeta, but did not enhance ERbeta transcriptional activity in the mouse liver. Unlike E2, Phloretin did not increase uterus size; however, it did reduce TG levels in the liver to a similar extent as E2.
Conclusions: Our results demonstrate that ER ligands reduce TG levels in the mouse liver by inhibiting LXR transcriptional activity via a non-classical pathway.

A 036 Evolution of transcription factor binding in mammals on the million-year scale

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Background: While form and function of organs is well preserved through evolution, the binding of transcription factors (TFs) is not well conserved across species. For example, only 10-20% of TF binding is conserved between human and mouse. In this study we interrogate TF binding evolution in closely related mouse species separated by less than 6 million years of evolution.
Observations: Using chromatin immunoprecipitation combined with sequencing (ChIP-seq) we have determined the binding of three liver TFs in two laboratory strains of Mus musculus (C57BL6 and A/J), one sub-species (Mus musculus castaneus) and two additional species (Mus spretus and Mus caroli, specifically sequenced for this project). By comparing the TF binding in these inbred species we obtained more precise estimates on the rate of TF binding evolution and the underlying mechanisms driving it. We observe a rapid exponential decay of TF binding resulting in less than 45% overlap for three liver-expressed TFs in the livers of Mus musculus and Mus caroli species. This implies that small changes in sequence result in large changes in TF binding.
Conclusions: By taking advantage of the high degree of DNA sequence conservation in their genomes, we are tracking the decay and appearance of shared and unique events and investigating the nature of the cis (the sequence) and trans (the cellular environment) evolutionary changes responsible for this fast decay.

A 037 Induced transcription of transposons in a site of intercalary heterochromatin results in local changes in chromatin structure and DNA replication timing

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Background: Intercalary heterochromatin in salivary gland polytene chromosomes of Drosophila melanogaster is formed by clusters of epigenetically repressed unique genes. Unlike surrounding euchromatin they are characterized by late replication in cell cycle, strong underreplication and genetic silencing. We investigated the changes in chromatin structure, replication timing and DNA polytenization caused by induced transcription of the constructs inserted in the regions of intercalary heterochromatin 11A6-9.
Observations: The inserts EY00353 and EY01976 in the center and at the left edge of the region, respectively, are normally silent. The region (app. 460 kb) has typical morphology of the intercalary heterochromatin and replicates late. GAL4-UAS-induced transcription of the inserts results in formation of a puff, recruiting of RNA-polymerase II and H3K4 methylation. The sites of the activated constructs become early replicating. However changes in chromatin are restricted only to the region around the insert. The rest part of the region remains inactive and late replicating. Artificial transcription induces also local loss of DNA underreplication spanning the inserts and neighboring sequences. Recovery of polytenization is bidirectional and does not depend on the direction of transcription. The size of polytenized area depends on the level of driver activity. Strong activation of the insert EY00353 by means of the da-GAL4 driver results in extended polytenization while leakage of the HSP-GAL4 driver has weaker effect.
Conclusions: We can conclude that the site of intercalary heterochromatin does not represent a single late replicating and underreplicated unit. Every part of the site can be activated and polytenized independently on other parts.

A 038 Resistin regulates adipocyte functions through maintaining the level of transcription factor Srebp-1

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Background: Dramatic changes in adipocytokines (ACs) expression during adipocyte differentiation suggest critical roles of ACs in regulating adipocyte functions. In this study, we examined the expression levels of AC genes at various differentiation stages of primary and 3T3-L1 preadipocytes to gain insight into the biological function of ACs. Especially, focusing on resistin, whose expression was characteristically upregulated during the adipocyte differentiation, we investigated its biological function.
Observations: Although the AC expression levels significantly differed between visceral fat tissues obtained from Zucker obese and lean rats, primary preadipocytes derived from each tissue apparently showed the same expression profile during adipocyte differentiation. Among the ACs, resistin showed characteristic expression profile, which was quite similar to that observed in 3T3-L1 cell line. A siRNA specific to resistin was transfected into undifferentiated 3T3-L1 cells to explore the biological function of resistin. We found that the suppression of resistin expression resulted in a significant decrease in intracellular lipid content in differentiated 3T3-L1 cells. In addition, the expression levels of genes encoding Pai-1 and lipogenic enzymes, such as Fas and Scd-1 were significantly downregulated in the cells, compared to cells transfected with a control siRNA. Since previous reports implicated a transcription factor Srebp-1 as commonly involved in the transcriptional regulation of those genes, its expression was examined. In the cells transfected with the resistin siRNA, Srebp-1 protein was apparently decreased although no change was observed in its mRNA level.
Conclusions: Our study demonstrated that resistin regulates adipocyte functions including lipogenesis and AC production by maintaining the level of functional Srebp-1 protein, possibly in a posttranslational manner. Further studies addressing its underlying mechanism are currently being undertaken.

A 039 Synergistic cis-element based transcriptional cooperation between p53 and Estrogen Receptors in a breast cancer model

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Background: Previous reports have revealed a complex crosstalk between p53 and estrogen receptors (ERs) including transcriptional cooperation mediated by non-canonical cis-elements and not related to their physical association. The tumor suppressor p53 is a sequence-specific transcription factor activated by many stress signals that modulates genes involved mainly in apoptosis or cell cycle control. ERs are steroid hormone receptors and their primary response to estrogens is proliferation.
Observations: Our attention was focused on synergistic transcriptional cooperation between p53 and the ERs mediated through binding to canonical and/or non-canonical p53/estrogen promoter response elements in a human cancer model. The breast cancer-derived MCF7 cells (p53 wild type; ERalpha and ERbeta –weakly- positive but not dependent) were used and subjected to single or combination treatments with specific chemotherapeutic agents and ER ligands to examine the impact of the activation of p53 and ERs. A genome-wide transcriptome analysis was performed in order to identify genes that would be nonresponsive or poorly responsive to p53 or ERs when acting alone but strongly regulated by the combined activation of them. A further validation was done using quantitative real-time PCR. Non-genotoxic p53 activation, p53 silencing and chromatin IP experiments were also performed to investigate direct p53 and

ERs regulation of the selected target genes. Some of them can play an important role in immune response, metabolism, development, or as components of the extracellular matrix. The biological consequences of the identified p53- ER-dependent gene expression co-regulation are under investigation.
Conclusions: p53 and ERs modulate distinct cellular responses but they can cooperate in the transcription of target genes. Given that non-canonical REs are poorly or not responsive to p53 alone, the cooperation with ERs expands the p53 transcriptional network and could have implications for cancer biology.

A 040 The transcriptome of a human embryonic stem cell line

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Background: Gene expression research has advanced considerably during the last few years because of next-generation sequencing (NGS). In addtion, the possibility of differentiation of embryonic stem (ES) cells for personalized medicine has become feasable. However, there are still challenges before clinical treatment of disease using ES cells is made possible. With the appearance of NGS we now have the tools for characterizing cells on whole transcriptome level, a prerequisite to ensure clinical safety.
Observations: In this study we investigated the transcriptome of a human embryonic stem cell line (HS401). The SOLiD sequencing system was used to sequence the transcriptome in order to determine the gene expression. More than 20 000 genes were found to be expressed, leaving approximately 6000 gene transcripts undetected. The expression profile of several of the genes detected in the RNA-Seq were further validated using TaqMan low density arrays and semi-quantitative RT-PCR. First we investigated the expression of stem cell markers in HS401 cells, and found relatively high expression of such markers as GABRB3, DNMT3B, GDF3 and TDGF1. Interestingly, relatively high expression levels of differentiation markers such as COL1A1, FN1 and LAMC1 could be observed. When investigating the gene expression of other specific genes we chose to study members of the HOX gene family, due to the crucial role of these gene products in development and their involvement in cancer.
Conclusions: There is a close relationship between pluripotency and malignancy, and in this study we find expression of genes implicated in cancer. Thus it is important to thoroughly investigate the gene expression patterns of ES cells and cells differentiated from them to ensure the safety of stem cell usage.

A 041 Treatment of lymphoid cells with the topoisomerase II poison etoposide leads to an increased juxtaposition of AML1 and ETO genes on the surface of nucleoli

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Background: The human genes AML1 and ETO are known partners in a t(8,21) translocation that is associated with the development of so-called treatment-related leukaemias in patients receiving chemotherapy with DNA – topoisomerase II poisons.
Observations: To better understand the mechanisms underlying the t(8,21) translocation, we assessed the degree of mutual localization of the AML1 and ETO genes in cultured human lymphoid cells (line Jurkat). Using 3D FISH, we found that in at least 5% of untreated Jurkat cells, alleles of AML1 and ETO are in close proximity. The degree of AML1 and ETO juxtaposition increased two-fold in cells treated with the DNA – topoisomerase II poison etoposide. Surprisingly, in more than 50% of observed cases, colocalization of the AML1 and ETO genes occurred at the surface of nucleoli. The particular role of nucleoli in juxtaposing broken DNA ends is far from clear, although the involvement of this multifunctional nuclear compartment in DNA repair has already been suggested by previous studies. In another set of experiments, we found that treatment of Jurkat cells with etoposide triggers preferential loading of RAD 51 onto breakpoint cluster regions of the AML1 and ETO genes.
Conclusions: Taken together with the results of previous studies, the results presented here strongly suggest that the repair of DNA lesions introduced by topoisomerase II poisons may be mediated simultaneously by multiple mechanisms, which may be the cause of mistakes resulting in translocations.

A 042 Differences in DNA methylation of individual promoter alleles of HLA class II DQA1 gene

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Background: The large polymorphism of HLA class II genes is not restricted to coding region only, but it also applies to the linked promoter region of gene, where it projects to different strength of individual promoter alleles. It is possible that genetic variation between promoter alleles contributes to observed diverse transcription by creating epigenetic variation between them. Here, we aimed to assess the DNA methylation state at promoter region of individual alleles of HLA class II DQA1 gene.
Observations: All analyses were performed with peripheral blood DNA from 89 healthy donors included into this pilot study. The genotyping of HLA-DRB1, HLA-DQB1 and HLA-DQA1 was performed using PCR with sequence specific primers. The genomic DNA was converted by bisulfite treatment and the target

segment in the region -643 – -155 of promoter of HLA-DQA1 gene was amplified using nested PCR. The PCR product was cloned into Escherichia coli (XL-1 Blue). Successful transformants were selected on medium with ampicillin, IPTG and X-Gal. Successful transformation was confirmed by colony PCR and sequencing of individual clones was performed. We found significant differences in methylation of some CpG sites in the region studied, as well as differences in total count of methylated CpG sites between certain alleles. We were not able to identify the relationship between pattern of promoter methylation and its strength.
Conclusions: We found correlation between genotype and epigenotype of promoter alleles of HLA-DQA1 gene. This could be of importance to understanding regulation of normal immune response as well as autoimmunity which is often positively or negatively associated with certain HLA alleles.

A 043 Chromatin marks and spliceosome assembly

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Background: In higher eukaryotes most of the RNA polymerase II transcripts contain intronic sequences that are removed by the spliceosome in a process called pre-mRNA splicing. This process occurs largely co-transcriptionally when pre-mRNA is still attached to the RNA polymerase II complex as it moves along the gene. The close relationship between transcription and splicing was recently extended to chromatin structure and modifications, which affect splicing factor recruitment and splicing outcome.
Observations: Here, we focused on how chromatin modifications affect the dynamics and assembly of the basal splicing machinery in the nucleus. We induced changes in chromatin modifications (inhibition of histone deacetylases, SUV39H1 overexpression, SetD2 knockdown) and assayed U1, U2, U4/U6 and U5 snRNP dynamics using fluorescence recovery afer photobleaching and raster image correlation spectroscopy. Inhibition of histone deacetylases results in overacetylation of H3 and H4 and thus in more open chromatin. No effect was observed on snRNP dynamics implying that snRNP interaction with pre-mRNA is not dependent on histone acetylation. SUV39H1 is an H3K9 methyltransferase and its overexpression causes heterochromatin formation. By 5-FU incorporation assay we showed that a major portion of transcription is sustained after SUV39H1 expression. No change was observed in dynamics of snRNPs forming the catalytic core of the spliceosome. However, in the case of U1 snRNP we detected faster dynamics and shorter residence time on second as well as on milisecond time scale. On the contrary, no change was observed upon downregulation of SetD2, an H3K36 methyltransferase.
Conclusions: Our results demonstrate that the assembly of the active spliceosome follows the same kinetics independently on chromatin state. On the contrary, H3 modifications play a role in U1 snRNP association with pre-mRNA and suggest also additional role for U1 snRNP, likely related to transcription.

A 044 Interconversion between bound and free conformations of LexA orchestrates the bacterial SOS response

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Background: The bacterial SOS response is essential for the maintenance of genomes, but also modulates antibiotic resistance and controls multidrug tolerance in subpopulations of cells known as persisters. In Escherichia coli, the SOS system is controlled by the interplay of the dimeric LexA transcriptional repressor with an inducer, the active RecA filament (RecA*), which forms at sites of DNA damage and activates LexA for self cleavage.
Observations: Our aim was to understand how RecA* formation at any location in a bacterial chromosome can induce the whole SOS system. Here we show that self cleavage of LexA repressor is prevented by binding to specific DNA operator targets and depends on LexA dissociation from the targets. Dissociation rates depend on the operator sequence and this controls the SOS response. Cleavage of unbound LexA proceeds via one subunit first and the uncleaved monomer may be important for resetting of the system. Distance measurements using pulse electron paramagnetic resonance spectroscopy reveal that the DNA binding domains in unbound LexA are mobile, but adopt a defined conformation when bound to operator targets, precluding RecA interaction. Hence, the conformational flexibility of unbound LexA is the key element in establishing a co-ordinated SOS response. We show that, while LexA exhibits diverse dissociation rates from operators, it interacts extremely rapidly with DNA target sites. Modulation of LexA activity changes the occurrence of persister cells in bacterial populations.
Conclusions: Our results reveal how RecA* formation at any location in a bacterial chromosome induces co ordinated induction of the SOS genes.

A 045 White Alleles Suppress Position Effect Variegation

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Background: A central question in epigenetics is to understand how a differentiated daughter cell can inherit the pattern of chromatin modifications from her mother cell during mitosis. It still remains unclear how the complex patterns of covalent and ATP-dependent chromatin modifications are transmitted.A possible mechanism is a daughter cells could 'sense' the relative amount of RNA inherited by her mother cell and transfer the information about the relative abundance of its RNA-pool back to its nucleus.
Observations: In order to genetically test this model we decided to assay if the presence of a non functional gene, that does not

produce a main coding transcript, could influence in trans the expression of a functional copy of the same gene silenced by heterochromatin. In fact, if a chromosomal rearrangement place euchromatic genes adjacent to a region of heterochromatin, it causes a variegated phenotype that results from the random inactivation of the gene by heterochromatin spreading from the breakpoint, a phenomenon known as position effect variegation (PEV).We used this system in Drosophila to screen for non functional alleles of the white gene that could modify the eye color variegation caused by an heterochromatin inversion of the white gene called white-mottled 4 (wm4). Our preliminary data show that several white alleles suppress the variegation of the wm4 line. Remarkably, this effect is inheritable and is correlated with an increase in the white transcript as well as a change in the chromatin structure at the wm4 locus.

Conclusions: Our preliminary data show that several white alleles suppress the variegation of the wm4 line. Remarkably, this effect is inheritable and is correlated with an increase in the white transcript as well as a change in the chromatin structure at the wm4 locus.

A 046 Estrogen receptor ligands regulate prostate tumor formation through a nonclassical pathway that includes ERbeta and KLF5

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Background: Prostate cancer is the most common cancer among men. Androgen ablation therapy is the primary treatment approach for prostate cancer, although most cancers ultimately become refractory to this modality (androgen-insensitive). However, standard treatment options for androgen-insensitive prostate cancers are limited. Recent clinical studies suggest that anti-estrogens inhibit the development of androgen-insensitive prostate cancer, although the underlying mechanism has not been elucidated.

Observations: The pure antagonist ICI 182,780 (ICI) inhibited prostate tumour growth, whereas estrogen (E2) enhanced it. In addition, ligand-dependent changes in tumor growth were prevented by ERbeta knockdown (KD). Next, to investigate the associate pathway, we screened for ERbeta-interacting proteins and identified KLF5, a transcription factor that has been shown to suppress the development and progression of prostate cancer. The effects of ER ligands on prostate tumor growth were abrogated by KLF5 KD. We tried to identify KLF5-target genes involved in modulation of prostate tumor formation by ER ligands, and identified FOXO1. We also found that apoptosis increased in the presence of ICI, whereas it was inhibited by E2, an effect that was abolished by FOXO1 KD. Moreover, our results showed that in the presence of ICI, the recruitment of CBP to the promoter of FOXO1 through ERbeta and KLF5 was increased, which enhanced the transcription of FOXO1 and apoptosis. In the presence of E2, ERbeta acts as an adaptor for KLF5 and the E3-type ubiquitin ligase WWP1, which enhanced the ubiquitination and degradation of KLF5. As a result, E2 suppressed the transcriptional activity of KLF5 and apoptosis.

Conclusions: Our results demonstrate that ER ligands affect prostate cancer tumor growth via ERbeta-mediated regulation of KLF5. In addition, our results showed that FOXO1 as a critical ERbeta and KLF5 target gene that induces apoptosis and suppresses prostate tumor growth.

A 047 Methylation-dependent SUMOylation of chromatin factors

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Background: High mobility group A2 (HMGA2) is a chromatin-associated protein involved in the regulation of stem cell function, embryogenesis and cancer development. Although the protein does not contain a consensus SUMOylation site, it has been shown to be SUMOylated, and this modification affected its ability to downregulate the promyelocytic leukemia protein.

Observations: In this study, we demonstrate that a lysine residue next to the suboptimal SUMOylation motif (K66KAE) can be methylated in vitro and in vivo by the Set7/9 methyltransferase. By editing the lysine, the increased hydrophobicity of the resulting 6-N-methyl-lysine transforms the sequence into a consensus SUMO motif and greatly stimulates its SUMOylation in vitro. Furthermore, similar putative methylation-dependent SUMO motifs are found in a number of other chromatin proteins, and we confirm methylation-dependent SUMOylation of a site in one such protein, the Polyhomeotic complex 1 homolog (PHC1).

Conclusions: Together, these results suggest that crosstalk between methylation and SUMOylation is a general mode for regulation of chromatin function.

A 048 Nuclear chaperone NPM1 stabilizes EBNA2 and RBPJ association and is essential for continuous lymphoblastoid cell growth

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Background: We seek to uncover the knowledge concerning whether cellular factors in addition to classical transcription factors and co-activators are involved in EBNA2 mediated transcription.

Observations: Nucleophosmin (NPM1) was identified as one of the cellular proteins bound to EBNA2 and the specific interaction between EBNA2 and NPM1 was further addressed to be interplayed by EBNA2 aa 300-360 and the oligomerization domain (OD) of NPM1. Activation of NPM1 expression was specifically observed in both malignant B cells and lymphoblastoid cells, and EBNA2 and EBNALP were further addressed to play a role in modulation of NPM1 transactivation. Chromatin-immunopre-

cipitation and co-immunoprecipitation assays showed NPM1 readily stabilized the interaction of EBNA2 with RBPJ in order to facilitate EBNA2 recruitment onto target promoters. Of importance, NPM1 knockdown caused abrogation of both EBNA2 mediated transcription and cell maintenance of IB4 lymphoblastoid cells.

Conclusions: These data allow us to propose a new version of picture depicting NPM1-assisted recruitment of EBNA2 onto viral chromatins can lead to initiate the transcription activation of latency-associated genes and consequently the persistence of EBV transformed B cells is maintained.

A 049 Quantitative live imaging of a dynamic epigenetic system in Drosophila melanogaster

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Background: Polycomb (PcG) and Trithorax (TrxG) group proteins provide epigenetic memory of transcriptional patterns during development. PcG proteins assemble two major complexes, PRC1 and PRC2, which are bound to chromatin of target genes. Although PcG proteins ensure stable inheritance of expression states, PRC1 proteins have been shown to be in constant flux. We want to know whether other PcG/TrxG proteins show similar kinetics or whether they statically bind once they are attached to specific sites.

Observations: To understand kinetics of the PcG/TrxG system it is essential to measure concentrations and kinetic properties of involved components. We have generated Drosophila lines expressing functional EGFP fusion proteins of E(z), Ash1, Dsp1 and Pho. Fusion protein concentrations were determined by imaging in blastoderm embryos and calibration by virus like particles containing 120 EGFP molecules. Ratios of transgenic and endogenous proteins were determined using quantitative western blotting. These measurements and an independent approach using EGFP-ELISA allowed us to calculate the number of endogenous PcG/TrxG proteins per nucleus. Dynamics of Pc, E(z), Ash1 and Dsp1 in embryos were measured by FRAP. Diffusion constants as well as association/dissociation rates were extracted by fitting a reaction-diffusion model to the FRAP data. The TrxG protein Ash1 shows a higher bound fraction then the PcG proteins. Moreover, two PcG/TrxG proteins remain attached to mitotic chromatin during mitosis. Since epigenetic memory has to be maintained during cell division, mitotic chromatin attachment could have a role in the maintenance of expression states.

Conclusions: Quantitative measurements of kinetic properties of PRC1, PRC2, TrxG and DNA-binding proteins in embryos allowed us to determine that these proteins have fast but different kinetics. Future work will aim to quantify and understand the attachment of PcG/TrxG proteins to mitotic chromatin.

A 050 Tissue-specific differences in chromatin interactions in the Igf2r imprinted gene cluster

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Background: Imprinted expression in the mouse Igf2r imprinted gene cluster is controlled by the macro non-protein-coding (nc) RNA Airn, which silences Igf2r, Slc22a2 and Slc22a3 on the paternal chromosome. The Igf2r promoter is overlapped by anti-sense transcription of Airn, but Slc22a2 and Slc22a3 are not overlapped and lie 157 and 321kb upstream. Imprinted gene silencing of Slc22a2 and Slc22a3 by Airn presents a model to examine cis-acting gene silencing of non-overlapped genes by macro ncRNAs.

Observations: Previous studies in placenta have indicated that Airn becomes associated with the Slc22a3 promoter and recruits the repressive histone modification complexes G9A and polycomb. The localisation of Airn to the Slc22a3 promoter could be explained by induction of a paternal chromosome specific loop. To test this we are using Chromosome Conformation Capture (3C) to detect interactions between the Slc22a2 or Slc22a3 promoter and the Airn gene. We distinguish interactions on the maternal and paternal allele using tissues or cells derived from mice where one allele of the Igf2r imprinted cluster is deleted. Embryonic cells do not have imprinted expression of Slc22a2 and Slc22a3, and showed a similar interaction pattern between the maternal and paternal alleles. The visceral yolk sac (VYS) is an extra-embryonic tissue that, like placenta, shows imprinted expression of Slc22a2 and Slc22a3. Interaction patterns in VYS were different from the embryo, and currently we are investigating if any of these VYS specific interactions are parental allele specific.

Conclusions: Similar 3C chromatin interaction patterns between parental alleles in embryo indicate no role in overlapped silencing of Igf2r by Airn. The VYS shows a different interaction pattern, but it is yet to be determined if this relates to non-overlapped silencing of Slc22a2 and Slc22a3 by Airn.

A 051 Combinatorial regulation of E2F target genes by the oncogenic ETS transcription factor EWS-FLI1

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Background: Ewing's sarcoma is a highly aggressive pediatric cancer characterized by the chimeric ETS transcription factor EWS-FLI1. By whole genome gene expression and ChIP-seq analyses we obtained evidence that EWS-FLI1 directly binds 50% of E2F target genes and possibly cooperates with E2F transcription factors. Furthermore, several E2F factors are themselves direct targets of EWS-FLI1.

Observations: To unravel the hierarchical structure of this EWS-FLI1/E2F transcriptional network module we performed luciferase reporter gene assays in combination with DNA motif

mutation analyses and ChIP-PCR. The promoter regions of ten candidates for direct target genes of EWS-FLI1 and E2F factors were chosen for in-depth analysis. Corroborating the transcriptomic data, promoter activity of all ten genes showed reduced levels upon knockdown of EWS-FLI1. By chromatin immunoprecipitation, we confirmed that EWS-FLI1 directly binds to the promoter regions. For some of those genes, mutation of only a single ETS binding site leads to a reduction of luciferase activity comparable to knockdown of EWS-FLI1 (for example the E2F promoters). Furthermore, ChIP-PCR revealed that upon EWS-FLI1 repression an EWS-FLI1 induced activating E2F factor is exchanged for a repressing E2F factor on the promoter of this gene. In the absence of an ETS binding site the activating E2F factor is no longer able to bind.
Conclusions: Our results imply that EWS-FLI1 directly activates E2F factors and then actively recruits them to their jointly regulated target genes, thereby potentiating their activity.

A 052 The nucleolus connecting intracellular energy status with p53 activation

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Background: In response to a shortage of intracellular energy, mammalian cells suppress ribosome biogenesis and induce cell cycle arrest, both of which contribute to cell survival. We previously found that a novel nucleolar protein, nucleomethylin (NML), reduces rRNA synthesis in response to glucose limitation. Moreover, our recent work revealed that the nucleolar protein MYBBP1A activates p53 when nucleolar RNA content was reduced by inhibition of rRNA transcription.
Observations: Here, we hypothesize that the reduction in nucleolar RNA content in response to glucose starvation may cause the translocation of MYBBP1A, which eventually leads to the activation of p53. First we found that glucose limitation causes translocation of MYBBP1A from the nucleolus to the nucleoplasm. Subsequently translocated MYBBP1A is implicated in acetylation and accumulation of p53. Next, we examined the effect of NML depletion on the localization of MYBBP1A under the glucose-starved condition. The translocation of MYBBP1A into the nucleoplasm in response to glucose limitation was compromised by the depletion of NML. To assess the physiological role of NML-MYBBP1A-mediated p53 activation, we performed experiments using a human normal fibroblast cell line, WI-38. We tested the effect of the NML-MYBBP1A pathway on cell proliferation under the glucose-starved condition. We found that NML-MYBBP1A pathway contributes to G1 cell cycle arrest by activating p53 in normal WI-38 cells when glucose concentration is reduced, which may contribute to limiting energy consumption under glucose starvation.
Conclusions: Here we showed that a novel nucleolar pathway involving the NML and MYBBP1A is implicated in reduction of energy consumption by inducing cell cycle arrest. Our results indicate that the nucleolus functions as a sensor that transduces the intracellular energy status into the cell cycle machinery.

A 053 Analysis of the interaction domain between MYBBP1A and p53

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Background: Recent reports have shown that a number of nucleolar proteins activate p53 in response to cellular stresses. It is also reported that nucleolar structure is disrupted by various stresses. These finding indicate that there is a strong relationship between p53 activation and nucleolar disruption. Recently, we identified nucleolar protein MYBBP1A as a key component of nucleolar stress signaling. However, the molecular mechanism for the interaction between p53 and MYBBP1A is remains unknown.
Observations: Using GST pull-down assay, we found that p53 directly bound to MYBBP1A and the C-terminal 30 amino acids of p53 were required for the binding to MYBBP1A. The C-terminal region of p53 contains six lysine residues which are received posttranslational modifications, such as ubiquitination, acetylation, and methylation. We found that six lysine residues and one arginine residue were essential for p53-MYBBP1A interaction. Next, we determined p53-binding region in MYBBP1A. We found that p53 bound to two distinct regions (central region and C-terminal region) of MYBBP1A. Father analysis revealed that lysine and arginine residues in p53 C-terminal region were necessary for the binding to each of them. To identify new factors which could interact with MYBBP1A in the same way as p53, yeast two-hybrid screening was performed, and Ring1B was identified as a new partner for MYBBP1A. Ring1B is a member of the polycomb repressive complex 1. We found that Ring1B had similar amino-acid sequence to C-terminal region of p53. Now, we analyze the effects of Ring1B to p53-MYBBP1A interaction, posttranscriptional modification of p53, and its transcriptional activity.
Conclusions: We found that MYBBP1A bound to C-terminal region of p53, and this interaction required lysine and arginine residues of p53. We also found that MYBBP1A possessed two p53-binding regions, and Ring1B bound to these regions. These results suggest that Ring1B regulates p53 activation through MYBBP1A.

A 054 MYBBP1A degradation induced by nucleolar stress requires p53 and APC/C

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Background: A number of external and internal insults were shown to induce nucleolar stress by disrupting nucleolar structure. Nucleolar disruption induces acetylation and accumulation of p53. Our previous report showed that nucleolar protein MYBBP1A is a signal transducer of nucleolar stress, which directly binds to and activates p53. Interestingly, after the

activation of p53, MYBBP1A protein levels are down regulated. Here we show the mechanism of MYBBP1A down-regulation after nucleolar stress.

Observations: First, we examined MYBBP1A protein and mRNA levels after nucleolar strss. MYBBP1A protein levels were decreased followed by nucleolar disruption, whereas MYBBP1A mRNA levels were unchanged. Treatment of cells with MG132, a proteasome inhibitor, blocked the reduction of MYBBP1A protein levels. These results suggest that MYBBP1A is degraded by the proteasome pathway after nucleolar stress. Next, to investigate whether p53 is involved in the degradation of MYBBP1A, we knocked-down p53. Knockdown of p53 inhibited MYBBP1A degradation after nucleolar stress. In addition, treatment of cells with alpha-amanitin, an RNA polymerase II inhibitor, did not markedly affect MYBBP1A degradation. These results indicate that MYBBP1A degradation requires p53; however, p53-dependent transcription is not necessary for the degradation. Next, to identify an E3 ubiquitin ligase involved in MYBBP1A degradation, we purified MYBBP1A interacting proteins and identified APC/C subunits, APC5 and APC7. Knockdown of APC5 or APC7 abrogated MYBBP1A degradation after nucleolar stress, suggesting that APC/C is an E3 ubiquitin ligase for MYBBP1A.

Conclusions: Our results demonstrate that MYBBP1A is degraded by the proteasome after nucleolar stress, and degradation of MYBBP1A is p53 and APC/C dependent. In addition, our results suggest that MYBBP1A forms a complex with p53 and APC/C, and this complex formation is important for the degradation of MYBBP1A.

A 055 Regulation of nonconventional initiation complex assembly at the Nos2 promoter

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Background: Infection of macrophages with the intracellular bacterial pathogen Listeria monocytogenes leads to profound alterations of gene expression. Transcription factors NFkB and ISGF3 are activated by the IkB kinase complex and signaling by the type I interferon (IFN-I) receptor, respectively. Both are prominent regulators of infection-induced genes. A subset of such genes including Nos2, encoding the inducible nitric oxide synthase (iNOS), requires cooperative activity of NFkB and ISGF3.

Observations: ISGF3 and NFkB interact to form a clearance/elongation-competent transcription initiation complex at the Nos2 promoter. Whereas ISGF3 alone can recruit the general transcription factor TFIID and RNA polymerase II (pol II), NFkB is essential for the deposition of TFIIH and the associated S/T kinase CDK7 which phosphorylates the amino acid hepta-repeat sequence of the Pol II carboxy-terminal domain (CTD) at serine 5. NFkappaB mediated TFIIH deposition is stable for >24 hrs and primes the Nos2 gene for the subsequent pol II recruitment by ISGF3. Inversely, Pol II deposition by ISGF3 is similarly stable and thus able to prime the Nos2 promoter for subsequent TFIIH activity. Binding of the pTEFb-associated CTD serine 2 kinase CDK9 also requires the presence of NFkB. We further demonstrate the infection-induced appearance of acetyl marks at histone H4 and the concomitant binding of the pTEFb recruitment factor 'bromodomain-containing protein 4' (BRD4).

Conclusions: Cooperative transcription initiation complex formation by NFkB and ISGF3 at the iNOS promoter generates a transcriptional memory effect through stable TFIIH or Pol II deposition. NFkB is required for the binding of pTEFb through a mechanism involving histone-acetylation-dependent binding of Brd4.

A 056 Autocatalytic differentiation of epigenetic modifications within the Arabidopsis genome

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Background: In the plant genomes, genes and transposons are differentially regulated by epigenetic modifications. DNA methylation and histone H3 lysine 9 dimethylation (H3K9me2) are high in transposons, and are important for constitutively silencing transposons. On the other hand, these modifications are excluded from active genes to ensure their expression. So far, the mechanisms that epigenetically discriminate genes and transposons have been largely unexplored.

Observations: By using ChIP-chip approach in several Arabidopsis mutants, we explored this issue. In the mutant for Jumonji-domain histone demethylase IBM1, accumulation of H3K9me2 was induced ectopically in genes. This associated with ectopic accumulation of the non-CG methylation in genes. On the other hand, the level of H3K9me2 was unaffected by ibm1 mutation in transposons. These results suggest that IBM1 specifically target genes and exclude heterochromatin marks from genes. In the mutant for histone methyltransferase KYP/SUVH4, or non-CG DNA methyltrasferese CMT3, the level of H3K9me2 was reduced and the transcription was de-repressed in many transposons. Interestingly, these reduction of H3K9me2 and transcriptional de-repression were largely dependent on IBM1, suggesting that IBM1 also target transposons when they are transcribed. These results indicate that IBM1 stabilizes active state of chromatin by preventing accumulation of H3K9me2 in transcribing sequence. We also found that the ibm1-induced accumulation of H3K9me2 at genes is completely dependent on KYP/SUVH4 and CMT3, suggesting a self-reinforcing loop of H3K9me2 and non-CG methylation stabilizes inactive state of chromatin.

Conclusions: Taken together, we propose that epigenetic modifications in genes and transposons are autocatalytically differentiated by two self-reinforcing loops; one is loop of transcription and H3K9 demethylation by IBM1, and the other is loop of H3K9 dimethylation and non-CG methylation.

A 057 Role of CDYL in establishment and maintenance of heterochromatin

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Background: Histone post translational modifications (PTM) can modulate chromatin structure via the recruitment of modi-

fication dependent proteins. Heterochromatin is associated with particular PTMs like histone H3 lysine9 methylation a PTM that does not alter biophysical properties of the chromatin fiber and may therefore relay its function via the recruitment of binding proteins. CDYL constitutes a novel factor implicated in the formation of heterochromatin via the recognition of histone H3K9 methylation.

Observations: The unusual structural organization of CDYL combines an amino-terminal chromodomain involved in the recognition of methylated lysines and a carboxy-terminal domain with similarity to the enoyl-coenzyme A hydratase family. We are using biochemical and biophysical methods such as analytical ultracentrifugation, atomic force microscopy or fluorescence polarization measurements to study direct effects of CDYL on the chromatin fiber in vitro. By these experimental approaches we intend to decipher how this factor can contribute to heterochromatin formation. Our results point to a role of CDYL as scaffolding protein for both DNA and nucleosomes that can transfer the chromatin fiber into a compacted state, which may hamper transcriptional activation and thereby contribute to heterochromatin formation. By further refining our in vitro measurements we intend to fully characterize the structural properties of the CDYL-chromatin complex. Finally expanding our in vitro studies to cellular and in vivo model systems we seek to understand the biological relevance of CDYL.

Conclusions: Our in vitro studies indicate that CDYL can function as a molecular 'cross-linker' that scaffolds DNA and nucleosomal templates and in this way could contribute to chromatin structural organization.

A 058 RNA content in the nucleolus alters p53 acetylation

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Background: Recently, a number of external and internal insults were shown to induce nucleolar stress by disrupting nucleolar structure. It is reported that nucleolar disruption is sufficient for inducing p53-dependent cell cycle arrest and apoptosis. Thus, the existence of a stress sensor that monitors nucleolar structure and function and regulates p53 levels was proposed but the molecular details remained to be determined.

Observations: Here, we show that nucleolar disruption induces acetylation and accumulation of p53. Using an siRNA library, we screened nucleolar proteins involved in the accumulation and acetylation of p53, and identified three gene products MYBBP1A, RPL5, and RPL11. We found that MYBBP1A was anchored in the nucleolus through nucleolar RNA. When rRNA transcription was suppressed by nucleolar stress, MYBBP1A translocated from the nucleolus to the nucleoplasm, and strengthen the interaction between p53 and p300 acetyltransferase to increase acetylation of p53. We also showed that RPL5 and RPL11 were necessary for rRNA transport. Knockdown of RPL5 or RPL11 abrogated export of rRNA from the nucleolus and counteracted reduction of nucleolar RNA levels caused by inhibition of rRNA transcription. As a result, RPL5 or RPL11 knockdown inhibited MYBBP1A translocation to the nucleoplasm and p53 activation.

Conclusions: Our observations indicated that nucleolar RNA content was maintained through a dynamic equilibrium between RNA generation and export. The loss of this balance due to stress altered the nucleolar RNA content and modulated p53 activity.

A 059 NML plays a key role in NF-kappaB signaling

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Background: Nucleolus is traditionally the site of rRNA synthesis and ribosome assembly. We previously found a novel nucleolar function and identified NML. NML senses intracellular energy status and epigenetically controls the rDNA locus in order to change the ratio between the numbers of active and silent gene clusters. We generated NML KO mice to investigate the role of NML in vivo. The most of NML KO mice were embryonic lethal. Thus we generated mouse embryonic fibroblasts (MEF) from NML KO embryo.

Observations: The result of DNA microarray analysis using NML KO MEF cells showed that NF-kB target gene expression was decreased in NML KO MEF cells. In NML KO MEFs, induction of NF-kB target gene by TNF-alpha was lower than that in WT MEFs. Next, we analysed the molecular mechanism of reduction of NF-kB pathway in NML KO MEF cells. It is well known that phosphorylation of p65 is crucial for regulating its transcriptional activity. We found that the induction of phosphorylation of serine 536 within p65 one of component of NF-kB was reduced in NML KO MEF cells. Furthermore, ChIP analysis revealed that recruitment of p65 to IL-6 promoter region in response to stimulation was attenuated in NML KO MEF cells. Finally, we tested the affects of NML on TNF-alpha induced apoptosis by TUNEL assay. TUNEL-positive cells markedly increased in TNF-alpha-treated NML KO cells compared with that in TNF-alpha treated WT cells compared with that in TNF-alpha treated WT cells (WT:KO = 7.56%:47.3%). These results indicate that NF-kB signaling is suppressed in NML KO cells.

Conclusions: In this study, we found that NML regulate NF-kB activity via modification of phosphorylation of p65 and is essential for suppression of TNF-alpha induced apoptosis by NF-kB. We currently examine this mechanism and analysis the phenotype of NML KO mice.

A 060 Systematic Analysis of Epigenetic Reprogramming Process Accompanied by Generation of Mouse Induced Pluripotent Stem Cell

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Background: Recently we reported that a single transfer of protein extract from mouse embryonic stem cells (ESCs) could reprogram adult somatic cells into iPSCs. This protein-based method is regarded as a relatively easy and safe technique when

compared with previous gene transfer methods using repeated transfer or prolonged exposure to potentially harmful gene carriers. However, the epigenetic characterization of protein derived-iPSCs was not undertaken at genome-wide level.

Observations: Here, we globally analyzed chromatin signatures such as histone H3 K4me3 and K27me3 as well as gene expression profiles in two different mouse iPSCs derived from cardiac and skin fibroblasts and ESCs. Our data indicate that chromatin states of somatic cells were dramatically changed during dedifferentiation and thus gene expression profiles of two types of iPSCs were almost identical to that of ESC even though iPSCs were generated from two different somatic cells. Histone modification patterns of all 20,550 genes are almost similar at the promoters. The H3K4me3, an active marker shows higher correlation of iPS with ES cells ($r = 0.9885$). In addition, the alterations in chromatin structure during reprogramming process were tightly correlated with the changes in gene expression.

Conclusions: The epigenetic reprogramming should be faithfully accompanied to change the cell fate from somatic cells to ESC-like iPSCs.

A 061 DNMT1 depletion activates a pathway p14ARF/TP53 controlled that induces G1 arrest preventing DNA demethylation and aneuploidy

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Background: Aneuploidy is considered the result of chromosome segregation errors caused by defects in the mitotic spindle assembly, centrosome duplication, cell-cycle checkpoints and epigenetic changes. Usually, aneuploidy affects negatively proliferation of normal cells. However, it is frequently associated with cancer that is characterized by a uncontrolled proliferation. Thus, understanding the pathway(s) that block proliferation of aneuploid cells might open new avenue to exploit new cancer therapies.

Observations: We found that in primary human fibroblasts (IMR90) knocking down of DNMT1, a member of epigenetic machinery is perceived by the cell as a stress signal that induces p14ARF activation followed by TP53 stabilization that in turn transactivates p21waf1 triggering the G1 arrest. DNMT1 depleted cells bypassed the arrest and became aneuploid when TP53 or p14ARF were simultaneously silenced by RNAi. In addition by using stable near-diploid human tumor cells (HCT116), which are p14ARF-null and TP53-wt, we found that DNMT1 depleted HCT116 cells did not arrest in G1, underwent overall DNA demethylation and become aneuploid.

Conclusions: Our results suggest that Dnmt1 depletion triggered G1 arrest in human fibroblasts by activating a pathway p14ARF/TP53 dependent thus avoiding aneuploidy caused by DNA demethylation coupled with incorrect cycle progression.

A 062 Are multinucleated cells and multipolar spindles associated with chromosomal instability in tumor cells?

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Background: Aneuploidy is a feature associated to the aggressiveness of most solid tumors and it can result from multipolar mitosis. Disruptions in centrosome cycle and cytokinesis can lead to multipolar spindles. We have previously shown that chrysotile, a type of asbestos fibers, and vincristine, a chemotherapeutic agent, are able to induce multipolar mitosis. The present work aimed to go further in studying the effects of chrysotile and vincristine treatments on the ploidy of cultured human cancer cells.

Observations: Lung cancer cells were submitted to 2 different treatments: chrysotile for 48h or vincristine for 24h, followed by 24-48h of recovery in normal culture medium. The presence and localization of AuroraA, pericentrin and gamma-tubulin in the spindle poles of multipolar mitosis were similar to those detected in control cells centrosomes. Besides, in interphase multinucleated cells were detected multiple focuses of gamma-tubulin and pericentrin. The number of chromosomes analyzed in metaphase preparations was different when comparing the treatments: chrysotile increased the tetraploid cell population while vincristine increased the number of chromosomes randomly. In both treatments the ploidy deviations remained similar even after a 96h recovery period, as well as the presence of multinucleated cells and multipolar mitosis.Time-lapse imaging of GFP-alpha-tubulin-transfected cells indicated that chrysotile-multipolar mitosis can be arrested in metaphase or finish M phase, thus generating 2 or 3 daughter cells and contributing to aneuploid cell formation. Time-lapse experiments with GFP-histone cells are contributing to the understanding of the relation between multinucleation and multipolar spindles.

Conclusions: All together, these data indicate that both agents interfere in mitosis, leading to multinucleation, aneuploid cells and multipolar mitosis with multiple functional centrosomes, and help us to understand how multipolar mitosis affect chromosome instability after chrysotile or vincristine treatments.

A 063 Coexpression of mammalian retrotransposons with the MAST2 gene in testicles

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Background: Retrotransposable elements (REs) are mobile genetic elements that are able to self-replicate in the host genome by reverse transcription of their RNA intermediates. In

mammals, they constitute ~40% of the genomic DNA. Transcription of the actively proliferating REs is especially high in the germ tissues like testicles and ovaries. However, RE mRNA levels in germ line tissues vary greatly among the individuals.
Observations: Gene MAST2 encodes microtubule associated serine/threonine kinase that functions in a multi-protein complex in spermatid maturation. We have recently shown that a fragment of MAST2 was fused with the 3'-terminal part of an SVA retrotransposon, thus forming a novel hybrid MAST2-SVA RE family presented in the human DNA by ≥76 copies. In qRT-PCR tests, we explored transcriptional activities of MAST2 and of the active human REs in various tissue samples. We provide evidence that in testicles, MAST2 gene demonstrates a strong cotranscriptional pattern with the active human RE families L1, Alu and SVA. We further show that such a correlation also exists for the L1 and B2Y RE family members in rat and for the L1 and B2 elements in mouse, with the exception of the mouse B1 RE family.
Conclusions: Such a remarkable correlation may shed light on the extraordinarily successful propagation of the MAST2-SVA family in the human genome and suggest to consider REs as the universal biomarkers for some types of mammalian germ cells.

A 064 Investigating the Interplay of Double Strand Break Repair and Chromosome Dynamics in Escherichia coli

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Background: Homologous recombination repair is a ubiquitous and crucial process that safeguards the integrity of the inherited genetic information. Pathways and factors involved in DSB repair have been extensively studies in E. coli, however the way these events are organised within the cell compartment remain elusive. Our study characterises the intracellular dynamics of DSB repair and its coordination with chromosome segregation over the course of the cell cycle.
Observations: The experimental approach is to use the yeast I-SceI endonuclease to introduce double strand breaks (DSB) at specific chromosome loci, at chosen points in the cell cycle. The system we designed allows monitoring of the intracellular dynamics of both DNA double-strand break ends in living cells. It was combined with fluorescent derivates of protein factors such as RecA (the main effector of homologous recombination), DnaN (replisome component), PriA (involved in replication restart) in several relevant genetic backgrounds. This experimental setup enables us to describe the effects of DSB in vivo, and to observe their fate in HR-proficient or HR-deficient strains (recA-). For the first time, we observe DNA repair at work in real time, as witnessed by modified chromosome dynamics, DNA degradation, cell division inhibition, replication restarts or SOS induction.
Conclusions: The intracellular dynamics of the chromosome locus damaged is dramatically modified when compare to non-damaged. The analysis of the damages-induced segregation pattern provides important breakthroughs regarding the way HR between sisters' loci is organised in the cell.

A 065 ATMIN is required for maintenance of genomic stability and suppression of B cell lymphoma

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Background: ATM is the protein kinase mutated in ataxia telangiectasia patients that display immune deficiencies, cancer predisposition, neuronal degeneration and radiosensitivity. ATM responds to DNA double-strand breaks (DSBs) and alterations in chromatin structure to promote repair of damage or arrest the cell cycle. ATM is activated by two known co-factors in a stimulus-dependent manner: after DSBs NBS1 is required for activation of ATM while changes in chromatin structure also activate ATM via ATMIN.
Observations: Using conditional inactivation of ATMIN in B cells, we demonstrate that ablation of ATMIN-dependent non-canonical ATM activation results in oncogenic chromosomal translocations and subsequent tumor development. These translocations occur because programmed breaks generated during somatic recombination are not repaired effectively, leading to a defect in class switch recombination and genomic instability. Furthermore, B cell maturation is affected, and there is severe defect in ATM signaling (Loizou et al, in press Cancer Cell).
Conclusions: These findings indicate that noncanonical mode of ATM activation is absolutely required for ATM function in cancer.

A 066 ARM1 is a novel regulator of ATM function

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Background: The kinase ATM activates cell cycle checkpoints and orchestrates the DNA damage response. In response to different types of damage, ATM activation is triggered by molecularly distinct modes. Firstly, DNA double strand breaks (DSBs) result in ATM autophosphorylation, monomerision and subsequent recruitment of activated ATM to DSBs through the Mre11-Rad50-NBS1 complex. Secondly, in response to oxidative stress cysteine residues on ATM are oxidised, resulting in an active ATM disulfide-linked dimer.
Observations: We performed a screen for novel Ataxia-telangiectasia mutated (ATM) regulatory proteins, leading to the identification of ARM1 (ATM Regulatory Molecule 1). ARM1 deficient cells were radiosensitive and had a defective G2/M checkpoint, both of which are hallmarks of ATM deficiency. We then tested ARM1 function in both modes of ATM activation. We observed markedly reduced phosphorylation of ATM substrates, including Smc1, KAP-1 and Chk2, after ionising radiation (IR) in siARM1 cells. Furthermore, immunofluorescence staining for DNA damage foci (pH2AX, pATM, pSMC1) demonstrated a delay

in foci formation after IR in ARM1 deficient cells. Moreover, treatment of siARM1 cells with H202 showed that ARM1 is also required for ATM activation by oxidative stress. In contrast, ARM1 is not required for activation and substrate phosphorylation of the related kinase ATR (ataxia telangiectasia and Rad 3 Related) after UV induced DNA damage. Thus ARM1 is required for the activation of the ATM, but not ATR, signalling pathway.
Conclusions: We have identified and characterised ARM1 as a key regulator of ATM function. ARM1 is required for ATM activation in response to both DNA DSBs and oxidative stress. Therefore ARM1 is a novel key component of the ATM signalling pathway that plays a fundamental role in ATM biology and function.

A 067 The use of artificial 'zinc-finger' protein for directed DNA integration into safe position in the human genome

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Background: Retroviruses are naturally positioned as a base of choice for constructing gene therapy vectors due to their ability to integrate viral DNA into the host cell genome. Unfortunately, insertional mutagenesis caused by random integration catalyzed by retroviral integrases remains a considerable obstacle for successful use of such vectors. The reason for random integration lies in the properties of retroviral integrases that show a substantial nonspecific DNA-binding activity.
Observations: This obstacle can be potentially overcome by a direct modification of retroviral integrase by the addition of a protein domain with specific DNA-binding activity. Such attempts were made earlier but none of them produced hybrids that would target integration into a specific and safe site in the human genome. Herein, we present hybrid proteins consisting of an artificial 'zinc-finger' domain (tRL18) attached to either HIV-1 integrase or the integrase of the prototype foamy virus. The tRL18 protein was designed and constructed in such a way that it recognizes an 18 bp site within human leucine tRNA gene, which we consider a safe location taking into account that tRNA genes are presented in tandem copies in the human genome and that several integration events should not undermine the entire cellular protein synthesis. Our hybrids were tested for their catalytic activity, which appeared to be unaffected by the addition of tRL18. Their ability for directed integration in the vicinity of the target site was assayed in an in vitro system. All hybrid proteins were capable of changing the integration pattern of the wild type enzyme and showed a preferred integration around the target site.
Conclusions: Although the ability of our constructs to direct integration in vivo remains to be tested, we consider our system promising because it is designed for direct integration into a potentially safe region in the human genome and because it has proved worthy for two different retroviral integrases.

A 068 Identification and characterization of genomic lesions that suppress lethality in the absence of spindle assembly checkpoint component, MDF-1/Mad1, in Caenorhabditis elegans

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Background: To ensure genome integrity, the spindle assembly checkpoint (SAC) delays anaphase onset until all of the kinetochores have achieved proper attachment to the spindle. To identify genes that are crucial for genome integrity, we used the dog-1(gk10) mutator strain to create genomic lesions that can rescue the lethality in the absence of MDF-1/Mad1, a conserved component of the SAC in C. elegans.
Observations: From an initial screen of mdf-1(gk2); dog-1(gk10) homozygotes, we isolated the first suppressor candidate, such-4(h2168). One clone from this strain was outcrossed from the dog-1 background to avoid further accumulation of mutations, while the second clone, which was nicknamed 'MAD_DOG', was cultivated in the lab for 470 generations, with periodic freezing, to allow further clonal accumulation of non-lethal dog-1-induced mutations. We cloned such-4, the first suppressor in the MAD_DOG strain, which increased the percent of fertile progeny from 2%, observed in the absence of MDF-1, to 10%. We showed that such-4 is a tandem duplication containing 62 protein-coding genes. Increased dosage of a duplicated gene, CYB-3 (Cyclin B3), rescues the mdf-1 lethality. To determine whether additional suppressors have accumulated in the MAD_DOG clones in the period of 470 generations, we phenotypically characterized three of them and discovered further striking improvement in fitness (beyond cyb-3 dosage suppression) over time. Namely, mdf-1 mutant worms cultivated in the absence of DOG-1 for 70, 270 or 470 generations produce ~20%, ~50%, and ~80% of fertile adult progeny, respectively.
Conclusions: Our analysis suggests that multiple suppressors have accumulated in MAD_DOG clones. We showed that the initial suppressor is due to increased dosage of CYB-3. To identify other suppressors, we have sequenced the 470-generation MAD_DOG genome using complementary next-generation sequencing methods.

A 069 Saccharomycodes ludwigii: Chromosome segregation in the absence of crossovers in meiosis

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Background: Meiotic recombination ensures both faithful chromosome segregation during meiosis I and genetic diversity. The lack of crossover (CO) leads to chromosome missegregation and aneuploidy. However previous segregation analysis suggested the absence of CO in the yeast species Saccharomycodes ludwigii. In

order to understand the mechanisms involved in chromosome segregation and the impact of the lack of recombination on the genome structure, meiotic recombination has been investigated in Sd. ludwigii.

Observations: To conduct this study, the establishment of Sd. ludwigii as a model organism was a prerequisite and was initiated by the sequencing of the genome and the establishment of genomic engineering protocols. Orthologs of all main genes involved in meiosis were identified in the Sd. ludwigii genome. Segregation analysis confirmed the absence of any detectable meiotic exchange between homologs, in agreement with the previous genetic study. Meiotic double strand breaks (DSBs) were observed and turned out to be required for proper chromosome segregation. The initiation and processing of these DSBs appeared to be performed as in S. cerevisiae. However, their resolution seemed to require a single pathway, which lead to the formation of interfering COs in S. cerevisiae. The presence and requirement of this pathway was confirmed by observing the synaptonemal complex. Importantly, few meiotic DSBs were detected compared to S. cerevisiae, in the range of one per chromosome. Preliminary analysis of the DSB localization showed that they do not occur at telomeres, which constitutes regions of the genome that are not covered by genetics and the SNP segregation analysis.

Conclusions: Taken together, the results of both genetic and cytological studies strongly support the existence of meiosis specific DSBs in Sd. ludwigii and indicate their major role in meiotic chromosome segregation.

A 070 Antigen receptor regulation of B lymphocyte development by calmodulin inhibition of E-proteins

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Background: B lymphocyte development to antibody producing cells is characterized by the ordered rearrangement of gene segments of first an immunoglobulin (Ig) heavy (H) chain gene and then an Ig light (L) chain gene to create antibody diversity. Early in the development, signaling from the pre-BCR with surrogate light chain (SLC) proteins instead of IgL is a critical checkpoint for functional IgH rearrangement. Later in the development, the BCR is used to judge antigen-binding strengths of B cells.

Observations: During somatic hypermutation (SH) of the antibody genes, BCR signaling has to be able to detect successive improvements in antigen affinity, even if relatively small, over an extremely broad range of affinities. Here we show that both the pre-BCR and the BCR are subject to broad negative feedback regulation. For both receptors, activation of signaling leads to down-regulation of the receptor proteins as well as many co-receptors and proteins participating in signal pathways from the receptor. We show how interactions change within the signaling scaffold of the BCR during the feedback inhibition process. We also show that the feedback inhibition of the BCR signalosome and most of its proteins, and several other key regulations controlled by the pre-BCR or the BCR at different B cell development checkpoints, are through inhibition of the transcription factor E2A by Ca2+/calmodulin.

Conclusions: Down-regulation of the pre-BCR is much broader than the previously established SLC proteins, and down-regulation of the BCR enables sensitive detection of improvements in antigen affinity over a very large span of affinities during SH. Calmodulin inhibition of E2A is important in B cell development.

A 071 Galanin is a vital activation agent for human polymorphonuclear neutrophils (PMNs)

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Background: PMNs comprise a fundamental component of the innate immune response, being recruited rapidly to sites of infections. Galanin is a bioactive neuropeptide that is involved at different levels during inflammatory processes. Recently, we were able to show that accumulation of PMNs upon induction of neurogenic inflammation is disturbed in galanin knockout mice. Consequently, we aimed to elucidate if galanin is directly influencing human PMN function in vitro.

Observations: In PMNs, isolated from healthy individuals, galanin exposure increases the release of myeloperoxidase, which is a marker of degranulation of azurophil/ primary granules that occurs during PMN activation. In addition, we found that galanin boosts lactoferrin and metalloproteinase 9 (MMP 9) activity, indicating that it can mobilize specific secondary (lactoferrin) and gelatinase granules, which are secreted during cell activation. Galanin also enhances the expression of the beta-2-integrin CD11b together with expression of CD66b and CD63, markers of secondary and azurophil granules, respectively, that are activated during cellular activation and adhesion. In accordance with these findings, we were able to demonstrate that treatment of galanin at concentration of 10-7 Molar results in significant changes of neutrophil adherence. These effects are mediated possibly through the galanin receptor subtypes GalR2 and/or GalR3, which are expressed in human PMNs.

Conclusions: Here, we have identified and characterized galanin as another crucial regulator for PMN activation in human neutrophils, making this peptide another possible agent for the development of novel therapies.

A 072 Conditional Stat1 ablation reveals the importance of interferon signalling for innate and adaptive immunity to Listeria monocytogenes infection

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Background: Listeria monocytogenes is facultative intracellular bacterium able to cause life threatening disease in immunocompromised hosts. Type I and type II interferons (IFN) are major

cytokines produced during infection. Responses to both show an absolute need for signal transducer and activator of transcription 1 (Stat1). Nonetheless, IFN types have opposing effects on systemic infections with L. monocytogenes, as type I IFN are detrimental and type II IFN strictly required for host defense.

Observations: We used the loxP system for conditional gene targeting and Cre-mediated deletion to generate tissue-specific Stat1 ablation. Specifically, mouse lines devoid of Stat1 in myeloid cells (macrophages/neutrophils), dendritic cells (DC), or T-cells were obtained. These animals were used to investigate both the innate and adaptive immune responses to infection with L. monocytogenes. Whereas IFN signalling was required in the myeloid cell compartment during the innate phase of immunity, it was of minor importance for recall responses of immunized mice. Conversely the IFN response of DC was not important for innate resistance, but contributed to the generation and/or mobilization of immunological memory. Consistent with this, the IFN response of T-cells contributed to protection against L. monocytogenes in immunized animals.

Conclusions: Taken together, our findings highlight the differential importance and deployment of interferon signalling in different compartments of immune cells as well as in distinct phases of the immune response to L. monocytogenes infections.

A 073 Expression of miRNAs miR-133b and miR-206 in the Il17a/f locus is co-regulated with IL-17 production in alphabeta and gammadelta T cells

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Background: The genes for two miRNAs miR-133b and miR-206 are located directly upstream of the Il17a/f gene locus. So far, miR-206/133b have been reported to be specifically important for muscle regeneration and development and their expression has been suggested to be largely restricted to skeletal muscle and osteoblasts. However, based on their close proximity to the Il17a/f gene locus we hypothesized that expression of these two miRNAs and secretion of the main Th17 cytokine IL-17A might correlate.

Observations: Differentiation of T helper 17 cells (Th17) is a multistep process that involves the cytokines IL-6, TGF-beta, and IL-23 as well as IL-1beta, IL-21, and TNF-alpha. Thereby, robust induction of the capacity to produce IL-17 involves epigenetic modifications of the syntenic Il17a/f locus. Using inbred mouse strains, we identified co-regulation of gene transcription at the Il17a/f locus with the nearby microRNAs miR-133b and miR-206 that are clustered approximately 45 kb upstream of Il17a/f. Expression of these microRNAs was specific for Th17 as compared to other CD4+ T cell subsets and this was equally valid for in vitro polarized and ex vivo derived cells. From all factors analyzed, IL-23 was the most important cytokine for the in vitro induction of miR-133b and miR-206 in naive CD4+ T cells of wild type mice. However, analysis of IL-23R deficient mice revealed that IL-23R signaling was not essential for the induction of miR-133b and miR-206. Importantly, we found a similar co-regulation in CCR6+ and other gammadelta T cell subsets that are predisposed to production of IL-17.

Conclusions: Taken together, we discovered a novel feature of T cell differentiation towards an IL-17-producing phenotype that is shared between alphabeta and gammadelta T cells. Notably, the specific co-regulation of miR-133b and miR-206 with the Il17a/f locus also extended to human Th17 cells.

A 074 HIV unveils: Synapse vesicle fusion keeps T cells signaling

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Background: Immune synapse is the hallmark of the adaptive response. The molecular mechanism that generates immune synapses can be subverted by HIV to downregulate T cell responses and improve virus propagation. The goal is to elucidate the molecular mechanisms by which HIV-1 detours the polarized endosomal traffic of key signaling molecules that traffic through endosomes to the immune synapse. HIV molecular specificity, compartments involved and viral proteins responsible are to be investigated.

Observations: Several key immune signaling molecules are transported through recycling endosomes to the immune synapse where they are activated: TCRzeta, Lck, and LAT. Our results shown: HIV-1 differentially affects the distribution of Lck, TCRzeta and LAT signaling molecules: It extensively confines Lck to its intracellular compartment, partially retains TCRzeta and does not affect LAT. Lck, TCRzeta and LAT reside in distinct intracellular compartments: Different signaling molecules are carried in distinct compartments which are differentially affected by HIV. Lck, TCRzeta and LAT are sequentially released from intracellular compartments to the plasma membrane following TCR triggering: Lck, TCRzeta and LAT distribution into distinct compartments enables their sequential recruitment to the plasma membrane following TCR engagement. TCRzeta and LAT distribution into distinct compartments enables their sequential single TCR recruitment: Lck precedes and is pivotal for TCRzeta and LAT clustering. By targeting Lck traffic, HIV-1 controls the extent of synapse formation. Methods: confocal microscopy and live TIRF imaging. Experimental systems: non-infected and HIV-infected primary CD4 T cells.

Conclusions: Using HIV as synapse modulator, we disclosed a novel mechanism for TCR signal amplification controlled by hierarchic vesicle recruitment. At its inception lays Lck delivery to induce TCRzeta and LAT recruitment. Such mechanism may explain how T cell outcomes are integrated at the immunological synapse.

A 075 Regulation of macrophage function by Inhibitor of Apoptosis Proteins

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Background: Inhibitor of apoptosis proteins are a family of conserved proteins including cIAP1, cIAP2 and XIAP. cIAP's are known to regulate signalling from TNF family receptors. More

recently IAP's have been shown to regulate innate immune signalling pathways such as TLR and NOD signalling. Given the potential role of IAP's in regulating immune function and inflammatory signalling we examined the effect of IAP loss or antagonism on the function of macrophages.

Observations: Using an HOXB8 immortalised macrophage system, the effect of the IAP antagonist LBW242 on macrophages was tested. At normal doses (10uM) that are able to degrade cIAP1, wild type, cIAP1-/- and cIAP2-/- macrophages survive for extended periods, whereas XIAP-/- macrophages die rapidly. XIAP -/- macrophages show hypersensitivity to LBW242 treatment dying at doses down to 2.5uM. Wild type macrophages were killed by LBW at higher doses of LBW. Interestingly HOXB8 immortalised macrophage progenitors also show significant sensitivity to LBW induced death in a similar fashion to XIAP-/- macrophages. When examined, myeloid progenitor cells immortalised by HOXB8 express no detectable XIAP. During differentiation XIAP levels increase, thus protecting the cells from LBW. To test wether LBW treatment would affect macrophages in an infection model, macrophages derived from HoxB8 immortalised progenitors were infected with LCMV to examine the effect of IAP antagonists on viral replication. Significantly, macrophages treated with LBW showed significantly lower viral titres after 48-72 hours.

Conclusions: XIAP plays a key role in regulating the sensitivity of macrophages to IAP antagonists. Sensitivity of myeloid progenitors to IAP antagonists may be an important consideration when treating patients with IAP antagonists. IAP antagonism results in reduced viral replication in LCMV infected macrophages.

A 076 A role for the RNA pol II-associated PAF complex in AID-induced immune diversification

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Background: Antibody diversification requires the DNA deaminase AID to induce DNA instability at immunoglobulin (Ig) loci upon B cell stimulation, leading to increased antibody affinity for antigen and alterations in effector function. Catalytically, AID deaminates cytosine to uracil requiring single-stranded DNA. For efficient cytosine deamination, AID needs to gain access to ssDNA and the Ig loci, with transcription by RNA polymerase II (RNA pol II) possibly providing both aspects.

Observations: To understand these mechanisms, we isolated endogenous AID-containing protein complexes from the chromatin fraction of diversifying B cells by FLAG affinity purification, and identified their components by mass spectrometry. The majority of proteins associated with AID belonged to RNA processing, RNA pol II elongation and chromatin modification complexes. Besides the two core polymerase subunits, members of the PAF complex, SUPT5H, SUPT6H, and FACT complex associated with AID. We demonstrate that PAF1 is a novel, direct AID interacting partner, and that depletion of PAF complex members in B-cells inhibits AID-induced immune diversification (Class Switch Recombination).

Conclusions: We show the importance of the Paf complex, which controls transcription elongation and transcriptional chromatin changes, for AID function. Our findings, together with current findings in the field allow for a model to emerge of how RNA pol II elongation and pausing induce and resolve AID lesions.

A 077 The DNA binding mode of human DAI, a cytosolic DNA sensor and an activator of innate immune response

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Background: The DNA-dependent activator of IFN-regulatory factors (DAI), also known as DLM1/ZBP1, initiates an innate immune response by binding to foreign DNAs in the cytosol. For full activation of the immune response, three DNA binding domains at the N-terminus are required: two Z-DNA binding domains Zalpha and Zbeta and an adjacent putative B-DNA binding domain. However, the mechanisms of the DNA binding and of the following immune activation are not clearly understood.

Observations: To gain structural insights into the DNA-binding mechanism of hZbetaDAI, the solution structure of the DNA-free hZbetaDAI was solved and its bindings to B- and Z-DNAs were analyzed by NMR spectroscopy. Compared to the Z-DNA-bound structure, the conformation of DNA-free hZbetaDAI has notable alterations in the alpha3 recognition helix, the 'wing' and Y145, which are critical in Z-DNA recognition. Unlike some other Zalpha domains, hZbetaDAI appears to have conformational flexibility and structural adaptation is required for Z-DNA binding. Chemical shift perturbation experiments revealed that hZbetaDAI also binds weakly to B-DNA via a different binding mode. The C-terminal domain of DAI is reported to undergo a conformational change on B-DNA binding, thus, it is possible that these changes are correlated.

Conclusions: Current results provide clues for understanding how hZbetaDAI contributes to the recognition of foreign DNA in both B and Z conformations. Furthermore, it is also proposed that the dimerization of DAI accompanied by its DNA binding might be correlated with the signaling in the innate immune response.

A 078 Infection with a virulent strain of Mycobacterium avium induces the development of thymus atrophy and bone marrow dysfunction

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Background: HIV-infected individuals are the main targets of infection by Mycobacterium avium. We showed that infection of mice with a highly virulent strain of M. avium (ATCC 25291 SmT) induces T and B cell depletion in the spleen, dependent on IFN-gamma production, and premature thymic atrophy (TA). In this study, we determined the mechanisms of TA, the implications to the pathogenesis of peripheral lymphopenia (PL) and a putative relation to bone marrow (BM) dysfunction during infection.
Observations: We established that TA found during infection is accompanied by a depletion of the four main populations (CD4 + CD8 + , CD4-CD8-, CD4 + CD8-, CD4-CD8 +) dependant on IFN-gamma production. Thymectomy and infection showed additive effects in the development of PL. We determined the role of glucocorticoids (GCs) on TA and PL by determining the levels of corticosterone present in the blood by radioimmunoassay, the effect of blocking the GC receptors by RU486 treatment in the thymus and spleen cell populations and thymocyte sensitivity to dexamethasone (Dex)-induced cell death in vivo by flow cytometry analysis. We determined if TA and PL were related to a BM dysfunction by analysing whether adoptive transfer of BM-cells from non-infected animals or infected mice might reconstitute the thymus of Rag-deficient mice. Serum level of GCs was increased after day 60 post infection, treatment with steroid-GC receptor antagonist RU486 seem not able to revert TA or PL but Dex sensitivity of thymocytes was increased during infection. We found that BM is compromised during infection, since we were not able to reconstitute Rag-deficient animals adoptively transferred with BM from infected animals.
Conclusions: M. avium-induced PL is not exclusively a consequence of TA and factors such as GCs and IFN-gamma influence the TA found during infection. Dysfunction of BM precursors as a consequence of infection with M. avium might be responsible for the absence of thymocytes and peripheral lymphocytes.

A 079 The Role of Histone Methyltransferase, Ezh2, in Dendritic Cells

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Background: Polycomb group protein, Ezh2, serves as a crucial regulator of gene expression pattern during development, through its histone methyltransferase activity. Ezh2 also has an unexpected role in cytosolic signaling pathway (Su et al., 2005). However, Ezh2's exact function and mechanism of action in the cytosolic signaling is not yet known. Our aim is study the function of Ezh2 in dendritic cells (DC), a key player in the immune system.
Observations: We studied the function of Ezh2 in DCs using a conditional Ezh2 KO mouse, from which we can culture bone marrow-derived DC. By immunostaining of DC markers, and flow cytometry, we observed that Ezh2 is not required for the development, as well as maturation of DCs. We also looked at Ezh2-deficient DC populations in several organs, and again, we found that Ezh2 deficiency do not significantly change DC populations in vivo. This, then allows us to look at the function of Ezh2 in the physiological functions of DC. Endocytosis assay, such as FITC dextran and opsonized latex beads uptake assay, revealed that Ezh2 is crucial for the antigen capture activity of DC. By time lapse imaging, we also found that Ezh2 deficient DCs are much less motile. Furthermore, we looked at the formation of adhesion complex formation, such as podosomes and focal adhesion complexes, by allowing the DCs to attach to extracellular matrix-mimicking coating. Immunofluorescence of several members of the adhesion complex reveals that Ezh2 is involved in the dynamic of adhesion structure formation.
Conclusions: Our studies with Ezh2 deficient DC showed that Ezh2 is crucial for the basic cytoskeletal functions of DC, such as antigen capture and motility. Our studies have further revealed that Ezh2 regulates DC interaction with the cellular matrix, through the intricate dynamic of adhesion structures.

A 080 Expression of molecular mediators in the lung of mice with abdominal polymicrobial sepsis

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Background: Sepsis is still a major cause of postoperative morbidity and mortality in human patients. Therefore, it is important to identify biochemical mediators for their potential in predicting prognosis of sepsis and their suitability as drug targets. The aim of the present study was to elucidate the changes of the expression of potential markers of disease severity in an animal model of polymicrobial sepsis.
Observations: The expression of over eighty potential inflammatory mediators was investigated in murine lungs in an animal model of abdominal polymicrobial sepsis, the colon ascendens stent peritonitis (CASP). In agreement with the literature we found significant upregulation of TNF-alpha (47-fold), IL-1 beta (37-fold), IL-10 (64-fold) and the chemokines CCL2 (47-fold), CCL3 (207-fold), CCL4 (90-fold), CXCL1 (230-fold), and CXCL10 (37-fold). Furthermore, we detected an increase of CCL7 (29-fold), CCL19 (6-fold), CXCL5 (7-fold), CXCL13 (11-fold) and chemokine receptor CCR8 (7-fold) mRNA expression in murine lungs 12 hours after CASP surgery. The mRNA expression of the neuropeptides substance P (27-fold) and neuropeptide Y (NPY)

(5-fold) were significantly increased in lungs of septic mice, whereas the expression of galanin (-3-fold) was significantly down-regulated.

Conclusions: In the present study new potential markers for sepsis were identified. In future studies it had to be determined whether altered concentrations of these markers are found in the serum of septic animals and if they are changed under septic conditions in humans.

A 081 Induction and function of type I interferons during infection with Streptococcus pyogenes

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Background: The Gram-positive human pathogen S. pyogenes causes a variety of diseases that range from mild pharyngitis to rare but severe invasive infections. The innate immune system was shown in animal models to be essential for host defense yet it is not known how the innate immune cells recognize this pathogen.

Observations: We have recently demonstrated that mouse macrophages and dendritic cells produce type I interferons (IFN) in response to S. pyogenes and that type I IFN signaling is required for successful immune response of mice. The mechanism of type I IFN induction and how IFNs protect the host are not well understood. In studies employing cells from knockout animals and RNAi-mediated gene silencing we show that type I IFNs are induced independently of known pattern recognition receptors (PRRs) including TLR3, TLR7, TLR9 and the RIGI pathway. The type I IFN-inducing ligands are S. pyogenes-derived DNA (in macrophages) and RNA (in conventional dendritic cells, cDCs). In macrophages the IFN induction depends on TBK1, STING and IRF3 whereas cDCs require MyD88 and IRF5. Phagocytosis of S. pyogenes is required for IFN production in both cell types. The lack of type I IFN signaling causes increased recruitment of neutrophils to the site of infection in a subcutaneous infection model. Consistently, macrophages exposed to IFNs produce less CXCL1 and CXCL2, the neutrophil-attracting chemokines, in response to S. pyogenes.

Conclusions: We speculate that type I IFNs prevent excessive neutrophil-elicited tissue damage thereby reducing dissemination of bacteria. Currently we investigate whether macrophages or dendritic cells are the relevant cells types for the protective IFN production in vivo.

A 082 Friend or foe? Insect-directed strategies for differentiating between microbial pathogens and beneficial mutualists

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Background: The invertebrate immune system comprises an innate set of immune-reactive molecules of extreme high specificity and efficiency, allowing many invertebrates to prosper even in microbe-rich environments. Key players are innate effector proteins named antimicrobial peptides (AMPs). Intimate associations of many insects with ubiquitous alpha-proteobacteria Wolbachia challenge the host's immune system, but the molecular mechanisms by which Wolbachia and host immunity interact remain to be elucidated.

Observations: Drosophila paulistorum consists of six semispecies currently under incipient speciation. All semispecies harbor different but conspecific Wolbachia, each serving vital mutualistic functions to their natural hosts; but in hybrids they trigger reproductive incompatibilities. Wolbachia titer levels are controlled strictly by the host, since any deviation from their natural levels transform the mutualist to a pathogen. We hence propose that Wolbachia coevolves rapidly with the innate immune system. Thus we have determined the evolutionary dynamics and potential functional roles of six candidate AMPs in regulating Wolbachia densities, by isolating and sequencing their homologues in all six semispecies. Some of them show clear signals for diversifying selection, i.e. extremely high rates of replacement substitutions. Attacin C has emerged as our prime candidate for triggering evolutionary adaptation to a consistent immunological challenge by the rapidly evolving symbiont, since AttaC shows highest rates of diversifying selection among the six semispecies. In addition some AMPs have furthermore diversified rapidly by recent gene duplications and the evolution of new paralogous genes.

Conclusions: Analyzing fast evolving AMP genes in this Drosophila system provides an excellent opportunity to shed light upon the ongoing coevolutionary arms race, titer regulation and functional dynamics between intracellular symbionts and immune system of the host.

A 083 Runx1 controls B cell development by positively regulating Ebf1 expression

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Background: Runx complexes are critical transcriptional factors in development of numerous tissues and cells. Even though their roles in differentiation of T cell precursors to CD4+ or CD8+ lineage have been well characterized, their function in B cell development remains largely unknown. Since a germline deletion of Runx1 results in a complete lack of B cells as well as general hematopoiesis, it is necessary to use conditional gene deletion to study the function of Runx1 in B cell development.

Observations: We generated several mouse lines in which genes of Runx1, Runx3 or CBFbeta (a common binding partner for all Runx proteins) are flanked by two loxP sequences. These transgenic mouse strains were crossed with another transgenic mouse line (Mb1-Cre) expressing Cre recombinase from the very early B cell development, thus conditionally deleting loxP-flanked target genes only from early B cell precursors. Conditional deletion of Runx1 resulted in serious defects in B cell development from the early pro-B cell stage with virtually no peripheral B cells. Interestingly, this phenotype was accompanied by an impaired V(D)J recombination and a significantly reduced mRNA expression of Ebf1, a critical transcriptional factor for B cell lineage specification, suggesting that the presence of Runx1 influences Ebf1 expression. A critical role of Runx1 in Ebf1-mediated B cell

differentiation was further confirmed by rescue of defective B cell development in Runx1-deficient cells with overexpression of Ebf1. Consistent with these findings, we further discovered that Runx1 specifically binds to Ebf1 proximal promoter region and positively regulates Ebf1 transcription.

Conclusions: Our data identify Ebf1 as a novel target for Runx1-mediated transcriptional regulation. We propose that Runx1 transcriptionally regulates B cell development by binding to Ebf1 promoter region and maintaining its transcription.

A 084 Host response to pulmonary coinfection: Tradeoffs and vulnerabilities

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Background: Secondary bacterial pneumonia is a common complication following influenza virus infection. Much of the morbidity and mortality associated with influenza virus infection is due to pulmonary bacterial infections. Influenza virus infection can often alter the lung microenvironment and suppress the immune response, leading to an increase in bacterial adherence and/or dissemination. It is difficult to separate the pathology caused by the pathogen from the changes caused by the host response.

Observations: We used the pulmonary pathogen Legionella pneumophila to examine the effect of influenza virus on the innate immune response in the lung. The viral/bacterial coinfection led to a rapid lethal pneumonitis that was not seen with either infection alone. However, unlike secondary infections with other lung-tropic bacterial pathogens the bacterial clearance was not impaired by viral infection. This enabled the clear separation of host responses from the effects of increased bacterial load. We examined alterations of the lung microenvironment including the immune response, the location of the pathogens, and changes in the lung epithelium throughout the course of single infections and coinfections. Influenza infection in the context of coinfection dramatically altered the lung microenvironment in a variety of ways. Coinfection led to an increase in many aspects of the immune response. In addition, genes involved in tissue repair and stress response were decreased after influenza virus infection, leading to decreased resistance to the effects of a bacterial infection.

Conclusions: Using a model system of viral/bacterial pulmonary infection we were able to examine changes in the lung microenvironment that occurred independent of increased bacterial burden. Infection with two pathogens led to increased inflammation and decreased ability to tolerate the host response.

A 085 Dissecting the expression patterns of genes involved in autophagy after interactions between macrophages and bacterial pathogens

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Background: Autophagy is a defense mechanism that involves formation of double-membrane vesicles, called autophagosomes, which sequester cytoplasm, damaged organelles or protein aggregates for degradation inside cells. In addition, autophagy has been linked to innate and adaptive immune responses to numerous infectious microorganisms. Here we examine how autophagy, a potentially vital facet of the innate immune response to pathogens, is differentially modulated by intracellular and extracellular signals.

Observations: Detection and quantification of autophagy gene expression in macrophages were performed using q-RT-PCR arrays. The arrays include diverse genes that encode components of the molecular machinery and key regulators modulating autophagy in response to both extracellular and intracellular bacterial pathogens, i.e. genes involved in autophagic vacuole formation, genes responsible for protein targeting to membrane-vacuole, genes responsible for protein transport, genes linking autophagosome to lysosome, and genes involved in protein ubiquitination. As pathogens, strains of Listeria monocytogenes, Rhodococcus equi, Streptococcus pneumoniae, Corynebacterium pseudotuberculosis, and Mycobacterium smegmatis were used.

Conclusions: Despite some similarities in the intracellular trafficking between intracellular pathogens, the gene expression is diverse and differentially expressed genes are reported. The specific transcriptome profiling reveals novel molecules and signatures associated with autophagy.

A 086 Tracking a Transcriptional Host Immune Response of Experimental Listeria monocytogenes Infection Using an ex-vivo Nervous System Model

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Background: Listeria monocytogenes is a Gram-positive, facultative intracellular bacterium that causes invasive diseases in humans and animals. L. monocytogenes has been extensively studied as a model pathogen largely from the perspective of innate and adaptive immune responses. The aim of this work is to describe the transcriptomic changes underlying the complex mechanisms of the brain immune host response to L. monocytogenes using an ex-vivo model.
Observations: Listeria monocytogenes strain HUMV-4251 was isolated from human cerebrospinal fluid (CSF). Rat tridimensional organotypic slice cultures (3D-OSC) were infected with L. monocytogenes and after different time points and total RNA was extracted using TriZol. Detection and quantification of gene expression in the 3D-OSC were performed using q-RT-PCR to study diverse genes important in the innate and adaptive immune response, including genes encoding chemokines, interleukin cytokines, genes involved in NF-kB signaling, and genes of apoptosis, and acute-phase response. Infections were also tracked by means of Scanning Electron Microscopy (SEM). The Ccl2 chemokine, a monocyte chemoattractant was a leading immunomodulatory signal expressed from the beginning of the infection. We detect also genes related to the negative regulation of apoptosis that may promote neuronal survival. Increased expression of proinflammatory cytokines such as TNF-alpha, and members of the IL-1 family was characteristic of the early immune response in our model. In SEM studies, we detect phagocytic activity as early as 15-30 min p.i., and cells with typical apoptotic phenotype after 1h p.i.
Conclusions: The present model, which allows the direct contact between pathogen and brain provides a better tool than isolated cell cultures for further host-pathogen interaction studies with L. monocytogenes mutants and also with other pathogens.

A 087 Interaction of Streptococcus iniae with rat and mouse macrophages

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Background: Streptococcus iniae has become one of the most serious aquatic pathogens in the last decade, causing large losses in wild and farmed fish worldwide. There is clear evidence that this pathogen is capable not only of causing serious disease in animals but also of being transferred to and infecting humans. To gain more insight as to the ability of S. iniae to initiate infection, we studied the interactions between S. iniae strains and rodent macrophages.
Observations: Three reference strains, and a outbreak strain isolated from a natural infection in farmed fishes in the Canary Islands (Spain) were used to study the interaction with the macrophage cell lines J774 (mouse) and R2 (rat). We study the interaction by immunofluorescence, scanning electron microscopy, and antibiotic protection assays. Internalization of S. iniae occurred in J774 macrophages by a zipper-like mechanism and some strains survive and-or replicate for at least 24 h. A different pattern of interaction with the rat macrophage cell line was observed.
Conclusions: The present study demonstrates for the first time that S. iniae is largely bound and internalized by rodent macrophages and thus remains intracellular. Moreover, a possible role of the bacterial capsule in attachment to macrophages is discussed.

A 088 Escherichia coli K1 invasion of human brain microvascular endothelial cells via a dynamin-independent pathway

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Background: Escherichia coli (E. coli) K1 is one of the most common Gram negative bacteria that cause neonatal bacterial meningitis. Previous studies have extensively investigated bacterial entry pathways into human brain microvascular endothelial cells (HBMEC) to gain insight into how the bacteria cross the blood brain barrier in vivo. Some evidence suggests that the bacterium invades, and possibly transcytoses, HBMEC by exploiting caveolin-mediated uptake, a clathrin-independent endocytic pathway.
Observations: To explore caveolin-mediated uptake, we looked at the role of dynamin, a large GTPase that has been implicated in the membrane fission of caveolae buds, in bacterial entry into HBMEC. We found that overexpression of green fluorescent protein (GFP) tagged dominant negative dynamin 2 [Dyn2(aa) K44A] did not affect the bacterial invasion based on quantitative microscopy scoring. Instead, our immunofluorescence staining found that flotillin, a lipid raft marker also associated with clathrin-independent endocytosis, accumulated around invading bacteria as well as intracellular bacteria. The role of flotillin in transcytosis of the bacteria has been further explored with mutant flotillin constructs.
Conclusions: Our observations suggest that E. coli K1 is able to invade HBMEC via a dynamin-independent endocytic route, which requires flotillin, and that flotillin appears further involved in bacterial transcytosis.

A 089 Coronavirus S and M protein interplay: Mapping S protein domains relevant for S-M protein interaction

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Background: Coronaviruses (CoV) are RNA viruses that infect avian and mammalian species, including humans. The CoV spike protein (S) plays a crucial role in viral infection. It promotes virus entry by binding to cellular receptors and inducing membrane fusion. Incorporation of CoV S is achieved by its interaction with the viral membrane protein (M). We investigate the S-M inter-

action of transmissible gastroenteritis virus (TGEV) to get new insights in the formation of infectious virus particles.

Observations: In this study the region of the cytoplasmic tail of TGEV S, which is important for interaction with M was mapped. Therefore, different S deletion mutants were co-transfected with M, C-terminal fused to an HA-tag. Immunoflurescence analysis was performed and co-localisation studies of S and M were investigated by laser scanning confocal microscopy. Furthermore, the cell surface expression of S proteins has been dissected in M co-expressing cells. The results show that the S wildtype protein is intracellularly retained due to a tyrosine-based retention signal and it co-localises with M. In contrast, the mutant S(Y/A), in which the retention signal is destroyed is located at the cell surface. However, in co-expression with M it is again intracellularly retained and co-localises with M. The S deletion mutants, in which different parts of the cytoplasmic tail were deleted (including the retention signal) show a cell surface localisation, but in co-expression with M these mutants display a reduced intracellular retention. Compared to S(Y/A), we observed a strengthened cell surface expression of S in M co-transfected cells concomitant with increased truncations of S cytoplasmic tail.

Conclusions: Our results raise the suggestion that a truncation of the S cytoplasmic tail leads to a disruption of its interaction with M by eliminating crucial domains. Likely, the membrane-proximal cysteine-rich part of the S protein cytoplasmic tail plays an important role in CoV S-M protein interaction.

A 090 The role of the effector NleA in bacterial virulence correlates with its interaction with the mammalian protein Sec24

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Background: Enteropathogenic E. coli (EPEC) and enterohemorrhagic E. coli (EHEC) are food-borne pathogens that cause severe diarrheal illnesses in humans. Citrobacter rodentium is a pathogen of mice that causes similar illnesses in mice. EPEC, EHEC, and C. rodentium cause disease by injecting virulence proteins directly into host cells via a syringe-like apparatus known as the Type III Secretion System. These virulence proteins work synergistically to by-pass, inhibit, or hijack host cell functions.

Observations: NleA (non-LEE-encoded effector A), also known as EspI, is a Type III secreted effector that is common to EPEC, EHEC and C. rodentium. We have shown that the deletion of the gene encoding NleA from C. rodentium leads to a striking attenuation of bacterial virulence. NleA inhibits vesicle trafficking in mammalian cells by interacting with mammalian COPII (coatamer protein II) complex. COPII is involved in the transport of newly synthesized proteins from the endoplasmic reticulum to the Golgi apparatus. COPII is made up of five subunits and we have shown that NleA interacts specifically with the Sec24 subunit. Mammalian cells express four separate paralogues of Sec24: A-D. These four paralogues of Sec24 mediate selection of cargo proteins for transport and possess distinct, but overlapping cargo specificities. By studying the interaction of NleA with Sec24 proteins, we have created a mutant NleA protein that no longer interacts with Sec24. In vivo C. rodentium experiments demonstrate that bacterial strains expressing this non-binding protein are fully attenuated in mice.

Conclusions: When studying the biology of host-pathogen interactions it is very rare to find a single protein interaction to have drastic in vivo effects. This work provides strong evidence that NleA's interaction and inhibition of COPII is key to C. rodentium-related disease.

A 091 Genetic characterization of antibiotic resistant and virulence determinants in Escherichia coli strains recovered from Iberian lynx and Iberian wolf

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Background: The intensive use of antibiotics in human and veterinary medicine, associated with mechanisms of bacterial genetic transfer, caused a selective pressure that contributed to the dissemination of antimicrobial resistance in different bacteria groups. We intended to analyse the prevalence of antimicrobial resistance and the mechanisms implicated in faecal E. coli of Iberian lynx and Iberian wolf. The identification of phylogenetic groups and virulence determinants was also performed.

Observations: A total of 303 E. coli isolates obtained from 365 faecal samples from Iberian lynx (Lynx pardinus) and Iberian wolf (Canis lupus signatus) were studied. The Iberian lynx faecal samples were obtained from 30 wild animals and 98 captive animals in Spain and the Iberian wolf faecal samples were collected in the northeast of Portugal. Moderate levels of resistance (from 8.4% to 29.8%) were observed to ampicillin, tetracycline, streptomycin, sulfamethoxazole/trimethoprim, chloramphenicol, and nalidixic acid. Resistance genes detected by specific PCR included blaTEM, blaSHV, tet(A), tet(B), aadA, strA-strB, aac(3)-II, aac(3)-IV, different combinations of sul1, sul2, and sul3, and cmlA. In the Iberian lynx isolates 14.8% were ascribed to the phylogenetic group A, 37% to B1, 38% to B2, and 10.2% to D. In the Iberian wolf isolates 28.7% were ascribed to group A, 46.7% to B1, 6.7% to B2, and 17.9% to D. In respect to the presence of virulence determinants, in the Iberian lynx isolates was detected the presence of aer (13%), papC (11.1%), cnf1 (13%), papG3 (10.2%), and fimA (94.4%) genes, whereas in the Iberian wolf was identified aer (15.4%), papC (5.1%), cnf1 (0.5%), and fimA (91.3%) genes.

Conclusions: The virulence determinants and resistance genes detected in our study represents a serious public health problem. Iberian lynx and Iberian wolf can act as reservoirs of resistance genes and as they travel large distances they could spread resistant bacteria throughout the environment.

A 092 Nanoscale organization of the prokaryotic cell division machinery imaged by super-resolution microscopy

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Background: Cell division is highly spatially and temporally regulated, to ensure viability of each daughter cell. In bacteria, cytokinesis is mediated by the ubiquitous protein FtsZ, which forms a ring structure at midcell (the Z-ring) prior to septum formation. The Z-ring recruits proteins essential for cytokinesis, and later constricts the cell membrane. FtsZ has been characterized biochemically and structurally, however, it remains unclear how the in vivo structure of the Z-ring leads to cytokinesis.
Observations: To investigate the in vivo structure of the Z-ring and a binding partner, we combine multiple recently developed super-resolution imaging techniques, including photoactivated activated localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM), structured illumination (SIM) and stimulated emission depletion microscopy (STED). With spatial resolutions ranging from 25 – 125 nm, we show that FtsZ does not form a ring of uniform thickness around the circumference of the bacterium. Consistent with this observation, STORM and SIM images of the bacterial membrane reveal that midcell curvature is also not uniform around the cell circumference. Analysis of live-cell 3D-SIM images and fixed-cell STORM images, demonstrates that Z-ring constriction leads to compaction of the Z-ring. The number of FtsZ subunits is constant throughout the cell cycle, thus the density of FtsZ within the ring increases during division. STORM images of the cell membrane in E. coli expressing FtsZ-GFP reveal how FtsZ intensity is related to cell curvature.
Conclusions: We demonstrate how various super-resolution imaging techniques can be used in concert to study the organization of large protein complexes inside cells. Additionally, we present a model for Z-ring constriction and force generation throughout the cell cycle.

A 093 Hierarchy in transcriptional responses to carbon sources in Neisseria meningitidis

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Background: Bacteria developed carbon catabolite repression (CCR) to optimize metabolic versatility while improving their competitiveness in their natural habitats. Neisseria meningitidis (Nm), an obligate human pathogen, colonizes the nasopharynx but can cause septicemia and meningitis. Nm uses a restricted range of carbon sources including glucose and lactate, the availability of which varies in diverse host niches. Nm lacks components of known CCR mechanisms, suggesting a novel mechanism may exist.
Observations: To investigate Nm regulatory responses to sugar availability we performed transcriptome analysis of bacterium grown in vitro in defined media in presence or absence of glucose and lactate. Approximately 3% of the global gene expression responded to glucose availability. Genes of the Entner-Doudoroff and pentose phosphate pathways both involved in the glucose catabolism are upregulated in the presence of glucose. On the other hand genes related to the lactate catabolism are found repressed in presence of glucose, indicating a hierarchy on carbon sources utilization, and evidence for a mechanism of CCR. Lactate has been reported to play a critical role for Nm colonization and serum resistance, however surprisingly there was little evidence of lactate-specific transcriptional responses. We found the lactate permease gene, important for lactate uptake, significantly repressed by lactate probably through a feedback loop that we are currently investigating. Genomic analysis allowed us to identify several putative C-source responsive regulators that we have mutagenized. We are currently investigating their importance in CCR and the regulation in response to glucose.
Conclusions: CCR is one of the main regulatory phenomena in bacteria, and is often used by pathogens to regulate the expression of virulence factors. Here we provide the first evidence of CCR in Nm and are currently investigating the regulators that could be responsible and its implication in Nm physiology.

A 094 The genome of Chlamydophila psittaci: a comparative genome analysis

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Background: Chlamydophila psittaci is a Gram-negative obligate intracellular bacterium and the pathogenic agent of psittacosis. Although this pathogen's primary hosts are birds, human infections can occur with clinical symptoms ranging from mild indisposition to septic multi-organ failure and, ultimately, death. As part of an ongoing effort to investigate the genetic basis of virulence and host specificity in this species we sequenced the full length genome of the C. psittaci type strain 6BC.
Observations: The comparison of the genome of C. psittaci to other members of the Chlamydiaceae family showed a high similarity in gene content and a high level of synteny. The contentious division of the Chlamydiaceae into the genera Chlamydia and Chlamydophila is corroborated by the genomic data. Genome-wide patterns of non-synonymous variation suggest strong purifying selection on large parts of the genome. Only the G family of the polymorphic outer membrane proteins (PMPs), which are suspected to play a central role in host–pathogen interactions, display a high level of evolutionary change. Another group of proteins essential for chlamydial development within the host cell are type III secretion proteins. In silico prediction of

type III secretion proteins suggested several putative proteins of so far unknown function for further analysis.
Conclusions: The genome of C. psittaci compared to other members of the Chlamydiaceae gives insights into the genome organization, as well as the evolutionary dynamics of the PMP family proteins.

A 095 Interaction of antigenic phytoplasma membrane proteins with leafhopper vector proteins: a role in transmission specificity?

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Background: Phytoplasmas are wall-less plant bacteria associated with crop diseases worldwide. They live in the plant phloem and are transmitted from plant to plant by insect vectors in a persistent and propagative manner. Since thousands of insect species feed in the phloem, but less than 100 are known phytoplasma vectors, there must be a recognition between pathogen and vectors. We identified vector proteins specifically interacting with the most abundant antigenic membrane protein (Amp) of phytoplasmas.
Observations: Non-denaturing and denaturing far Western blotting and affinity chromatography showed interactions of 'Candidatus Phytoplasma asteris, CY strain Amp with several membrane proteins of the vector, among which the alpha and beta subunits of ATP synthase, and actin. No interactions with membrane proteins of non-vector insects were observed. The presence of ATP synthase, generally known as a mitochondrial protein, on the plasma membranes of the midgut and salivary gland cells of the insect vector was demonstrated by serology, immunolabelling and confocal microscopy. Membrane localization and in vitro interaction with phytoplasma membrane proteins indicated that, besides being involved in energy metabolism, ATP synthase (a multi-function protein) may act as a receptor for Amp. Amp also interacted with actin of vector, but not non-vector, insect species, consistent with previous findings for the closely related OY phytoplasma strain of 'Ca. P. asteris' and several of its vector species. Other vector proteins also interacted with Amp, but peptide mass fingerprinting could not identify them.
Conclusions: Amp of CY specifically interacted with plasma membrane proteins from vector species only, among which actin and ATP synthase. Other vector proteins interacted with Amp, suggesting the presence of a complex network of insect proteins interacting with Amp in the determination of vector specificity.

A 096 Host Cellular Mechanisms Regulating Intracellular Survival of Mycobacterium tuberculosis

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Background: Intracellular survival of Mycobacterium tuberculosis depends largely on the ability of the pathogen to manipulate host responses targeted against it. Autophagy is one such physiological process, discovered originally as a key mechanism for cellular survival under stress, and now implicated extensively in clearing intracellular pathogens including viral (dengue) and bacterial (Mycobacterium tuberculosis, Salmonella sp.etc) pathogens.
Observations: This study aims to delineate the central role played by autophagy in intracellular Mtb survival. We used avirulent and virulent strains of Mycobacteria to establish that the difference between killing of avirulent (H37Ra) and survival of virulent (H37Rv) strain mainly relied on the ability of the virulent strain to suppress the anti-microbial autophagy process. Having established the role of autophagy in Mtb survival, we also tried to establish molecular mechanisms regulating it. The two molecular indicators used in the study were conversion of LC3BI to LC3BII and phosphorylation status of central negative regulator of autophagy mToR. We found higher conversion of LC3BI into LC3BII in case of H37Ra infection (indicating higher autophagy rate) and also lower activation of mToR. We also observed greater localization of H37Ra into LC3 positive compartment, as compared to H37Rv through confocal microscopy, clearly indicating differential regulation of autophagy under the two conditions. Subsequent studies with siRNAs against the molecules known to be important for intracellular Mtb survival yielded novel insights into the molecular mechanisms regulating antimicrobial autophagy.
Conclusions: Our results show that several independent pathways may regulate antimicrobial autophagy. A more detailed analysis and integration into global autophagy regulatory network would lead to newer insights into host autophagy machinery and help add novel dimensions to anti-tuberculosis research.

A 097 A role for Staphylococcus aureus EsxA and EsxB proteins at the host-pathogen interface

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Background: The staphylococcal proteins EsxA and EsxB are secreted by the specialized secretion system Ess (ESAT-6 secretion system) and have been implicated in staphylococcal abscess formation in a murine model of staphylococcal infection. However, the precise biological function of these proteins in the context of staphylococcal pathogenesis is not known. This study aims at investigating the role of the Esx proteins A and B in the interactions of S. aureus with the host.
Observations: To understand if the Esx proteins play a role in host cell entry or intracellular persistence, isogenic single and double mutants for esxA and esxB were used for infection of various epithelial cell lines. Invasion assays did not reveal any major differences in internalization and survival efficiency between S. aureus wildtype and mutant strains in the host cells. However, microscopic analysis showed significant differences between host cells infected with wildtype and esx mutants. In addition, infected host cells demonstrated clearly different host immune responses towards wildtype and mutants. Preliminary FACS analysis of infected cells did not demonstrate any significant alterations in numbers of dead or apoptotic cells. This Esx-mediated modulation of the host cellular and immune responses is currently being investigated further.

Conclusions: Our results show that EsxA and EsxB may be implicated in the interaction between S. aureus and the host, possibly by affecting host response upon bacterial infection. This may suggest an important role for these proteins in establishment and maintenance of a staphylococcal infection.

A 098 Vaccinia virus as a dynamic tool to study signalling pathways that lead to cell blebbing

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Background: Vaccinia is a widely studied member of the poxvirus family. Throughout its life cycle, vaccinia manipulates cellular processes of its host to aid its replication and spread. Early in infection host cells contract, bleb and migrate, reminiscent of the amoeboid migration of cancer cells. Amoeboid migration is often dependent on the activity of the GTPase RhoA and its effector ROCK, which phosphorylates myosin light chain resulting in actin-myosin fibre assembly and contraction of the cell cortex.

Observations: Vaccinia induced contraction and blebbing is a transient event, beginning at 2 hours post infection and lasting for a period of 2-3 hours, after which cells respread. Using a panel of inhibitors we found that infection-induced contraction requires Rho and ROCK signalling but not Rac, Pak1-3, Src family kinases nor the activation of the EGF receptor. Infected cell contraction is dependent on the expression of early vaccinia proteins whilst re-spreading is dependent on late proteins. We have found that vaccinia induced cell contraction and blebbing is dependent on the expression of the early vaccinia protein, F11, as a virus that does not express F11 cannot induce the phenotype. Importantly, live cell imaging of F11-GFP revealed that F11 is recruited to the plasma membrane during membrane blebbing, further supporting a role for F11 in regulating these membrane events. To gain further molecular insight into vaccinia induced contraction, we performed a thematic siRNA screen using a motility library. We identified a kinase ordinarily involved in the regulation of innate immune responses. In the absence of F11, siRNA mediated knockdown of the kinase permits infection induced contraction.

Conclusions: Future work will focus on elucidating the role of the kinase in infection-induced contraction; its relationship to F11 and whether this is via its canonical role in innate immunity or by a novel mechanism. These findings may also identify a new signalling pathway in cancer cell migration.

A 099 Phosphorylation of TbCentrin2 by TbPLK is an important regulator of flagellum attachment zone biogenesis in Trypanosoma brucei

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Background: T. brucei is a protist parasite that causes African sleeping sickness. The parasite has a highly specialized cytoskeleton that gives the organism its awl-like shape and facilitates its motility in its hosts. T. brucei has a single flagellum that is adhered to the surface of the cell via a cytoskeletal structure known as the flagellum attachment zone (FAZ). Flagellar adhesion is essential for T. brucei viability, making the proper inheritance of the FAZ an essential feature of the cell cycle.

Observations: The single T. brucei homolog of the mitotic kinase PLK (TbPLK) is essential for the duplication of a cytoskeletal structure known as the bilobe, which may play a role in the inheritance of the Golgi apparatus. The bilobe is continuous with the FAZ, suggesting that they might be substituents of an even larger cytoskeletal structure. TbPLK phosphorylates the bilobe component TbCentrin2 in vitro. To test the in vivo relevance of this phosphorylation, the phospho-sites present on TbCentrin2 were mapped and compared to those generated by TbPLK in vitro. Mutants of the subset of in vivo TbCentrin2 phospho-sites that could be generated by TbPLK were expressed in T. brucei using a conditional knockout strategy. Removal of the TbCentrin2 phospho-sites caused a slow growth phenotype, while replacing them with aspartate phospho-mimetics caused cell cycle arrest and the accumulation of cells with aberrant DNA content and detached flagella. Further analysis showed that cells containing the phospho-mimetics had defects in bilobe duplication and were not able to generate a new FAZ. Some cells were able to undergo cytokinesis, which yielded a daughter cell with a detached flagellum.

Conclusions: Phosphorylation of TbCentrin2 by TbPLK plays a role in the formation of a new bilobe and FAZ. In the absence of a new FAZ, the new flagellum is not adhered to the cell body, which means that cell division will produce a cell that lacks proper motility and will not be able to divide effectively.

A 100 Involvement of the toxin-antitoxin system HigBA in nalidixic acid resistance of Caulobacter crescentus

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Background: Caulobacter crescentus is intrinsically resistant to the antibiotic nalidixic acid (Nal), but is rendered sensitive to it by loss of function of the polarity marker TipN. As the primary role of this protein is to localise at the division plane during cytokinesis to mark the new pole, it is unclear how it might mediate Nal resistance. We carried out genetic screening for mutations which restored Nal resistance to the tipN mutant in order to clarify this pathway.

Observations: Random transposon mutagenesis of the tipN mutant produced two independent insertions into the antitoxin gene higA, which conferred the ability to form single colonies on Nal solid media and improved growth in Nal liquid media relative to the tipN mutant parent strain. Disruption of higA could have implied that the cognate toxin higB was non-functional, however, cloning and overexpression of higB efficiently inhibited growth of the cells. Promoter activity assays confirmed that higA represses the promoter of higBA, consistent with other toxin-antitoxin systems, and that the promoter was also regulated by the SOS repressor LexA. An in-frame deletion

mutant of the higBA operon did not exhibit any change in Nal resistance, either in the wild type or tipN mutant background. The tipN higBA double mutant was subsequently complemented with higA or higBA in trans, and overexpression of higA alone caused an increase in Nal sensitivity, while higBA had no effect. Therefore, the antitoxin HigA is able to affect Nal resistance in the absence of its cognate toxin HigB.

Conclusions: This is the first example of a toxin-antitoxin system in which the antitoxin, rather than the toxin, is the effector of the phenotype. Screening of pathogenic bacteria, which typically carry many toxin-antitoxin systems in their genome, may uncover new antitoxin regulators of antibiotic resistance.

A 101 Human liver sinusoidal endothelial cells respond to Entamoeba histolytica by changes in morphology, integrin signalling and cell death, dependent upon contact and amoebic cysteine proteinase activity

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Background: Invasive infection with the extracellular parasite Entamoeba histolytica causes amoebiasis, a human infectious disease provoking dysentery and liver abscess. Initial amoeba-liver cell interactions are potentially decisive for the outcome of hepatic infection. We focussed on interactions with human hepatic endothelial cells, for these cells form the barrier the parasites cross before penetrating into the parenchyma and importantly, they participate in the modulation of liver immune responses.

Observations: We characterized the early responses of a human liver sinusoidal endothelial cell line to virulent and virulence-attenuated E. histolytica, using cell cultures and combining imaging technologies (scanning electron, two-photon and video microscopy), transcriptome analysis and cell biology methods (fluorescence-activated cell sorting, immunocytochemistry). In particular, we addressed the question of parasite-induced target cell death. Within the first minutes of incubation human cells start to retract, enter into apoptosis and die. Virulent parasites killed faster and more efficiently. In the presence of virulent amoebae, expression of genes related to cell cycle, cell death and integrin-mediated adhesion signalling was modulated rapidly and intracellular localisations of actin fibres and of key components of focal adhesion complexes, focal adhesion kinase and paxillin, changed in the human cells. Effects of galactose, cysteine protease-specific inhibitors and of amoeba strains not expressing virulence factors amoebapore A and cysteine protease A5 indicated that cell death and cytoskeleton disorganization are dependent upon parasite adhesion and amoebic cysteine proteinase activity.

Conclusions: The data suggest that E. histolytica interference with integrin-mediated host cell adhesion signalling accounts for morphological changes and death of the human cells. We propose a major role of this pathway in liver endothelium integrity and function in early steps of the hepatic infection process.

A 102 Host-microbe interactions: Influence of symbiotic Wolbachia on sex pheromones and mating behaviour in Drosophila

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Background: It has been proposed recently that quantitative alterations in invertebrate cuticular hydrocarbon (CHC) profiles contribute significantly to sexual isolation, a key driving mechanism of speciation. Members of neotropical D. paulistorum, a natural speciation complex in statu nascendi, are infected with maternally inherited endosymbiotic bacteria, Wolbachia. Obligate mutualistic Wolbachia of D. paulistorum manipulate sexual behaviour by influencing mate choice.

Observations: In this model system, we have analyzed, via gas chromatography/mass spectrometry, CHC profiles of D. paulistorum semispecies, which differ significantly in compound quantities but not in composition. In a pilot proportion screen, we have evaluated potential influences of Wolbachia on pheromonal signatures. Preliminary results show massive changes in CHC profiles between natural benign infection status and artificially-depleted flies, suggesting strong influences exerted by these bacteria on D. paulistorum CHC signatures. Studies of D. melanogaster and related sibling species have indicated that desaturase genes are essential for sex pheromone production, and that they are the most rapidly evolving genes. Since Wolbachia might manipulate expression of desat genes in D. paulistorum, we have determined (i) the divergence of desat genes in the desat1-desat2-desatF-clade among D. paulistorum semispecies, plus (ii) their expression levels in naturally-infected and artificially-depleted flies.

Conclusions: Obligate mutualistic Wolbachia of the natural speciation system D. paulistorum are capable of modulating pheromone patterns adaptively and so they have the potential to contribute significantly to host speciation by mediating impacts on sexual behaviour and mate recognition.

A 103 Processive movement of MreB-associated cell wall biosynthetic complexes in bacteria

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Background: The rod-shaped model gram positive bacterium Bacillus subtilis expresses three isoforms of the prokaryotic actin: MreB, Mbl and MreBH. All three proteins are thought to polymerize into dynamic filamentous helical structures underneath the cell membrane and together with the cell wall (CW) control cell morphogenesis. The prevailing model postulates that membrane-associated MreB filaments spatially organize

elongation-specific peptidoglycan-synthesizing complexes along sidewalls.
Observations: We have used Total Internal Reflection Fluorescence microscopy (TIRFM) to quantitatively characterize the in vivo distribution and dynamics of fluorescently-labelled MreB proteins and visualize the dynamic relationship between MreB isoforms and CW synthesis proteins in Bacillus subtilis cells. We show that during exponential growth MreB proteins do not form helical structures. Instead, together with other morphogenetic factors (MreC, MreD, PBPH, PBP2a and RodA), they assemble into discrete patches that processively move along peripheral tracks perpendicular to the cell axis. We show with Fluorescence Recovery After Photobleaching (FRAP) experiments that patch motility is not driven by MreB polymerization. Patch motility arrest using CW inhibitors vancomycin and phosphomycin, strongly suggest that the motive force for MreB patches is provided by peptydoglycan (PG) synthesis itself. We also provide evidence that MreB determines rod shape by restricting mobility of elongation complexes.
Conclusions: We propose that 1) CW elongation complexes insert new PG along tracks largely normal to cell long axis, 2) complexes motility is powered by PG polymerization, and 3) MreB acts as a polymeric clamp to restrict the diffusion of CW complexes and allow processive movement in correct orientation.

A 104 Topographical continuity of bacterial populations in the healthy human respiratory tract

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Background: Defining the biogeography of bacterial populations in human body habitats is a high priority for understanding microbial-host relationships in health and disease. The healthy lung was traditionally considered sterile, but this notion has been challenged by emerging molecular approaches that enable comprehensive examination of microbial communities. However, studies of the lung are challenging due to difficulties in working with low biomass samples.
Observations: Our goal was to use molecular methods to define the bacterial microbiota present in the lungs of healthy individuals and assess its relationship to upper airway populations. We sampled respiratory flora intensively at multiple sites in six healthy individuals. The upper tract was sampled by oral wash and oro-/nasopharyngeal swabs. Two bronchoscopes were used to collect samples up to the glottis, followed by serial bronchoalveolar lavage and lower airway protected brush. Bacterial abundance and composition were analyzed by 16S rDNA Q-PCR and deep sequencing. Bacterial communities from the lung displayed composition indistinguishable from the upper airways, but were 2-4 logs lower in biomass. Lung-specific sequences were rare and not shared among individuals. There was no unique lung microbiome. In contrast to other organs systems, the respiratory tract harbored a homogenous microbiota that decreased in biomass from upper to lower tract.
Conclusions: The healthy lung does not contain a consistent distinct microbiome, but instead has low levels of bacterial sequences largely indistinguishable from upper respiratory flora. These findings establish baseline data for healthy subjects and sampling approaches for sequence-based analysis of diseases.

A 105 Morphodynamics of spore differentiation in Clostridium difficile

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Background: Clostridium difficile is a strict anaerobic spore-forming bacterium and the major causative agent of nosocomial diseases associated to antibiotic therapy in adults. Spores produced by C. difficile are highly resistant, infective and are the primary cause of transmission in health care institutions. Despite the central importance of spores in the pathogenesis of C. difficile, a detailed description of the cell differentiation process leading to spore formation in this organism is lacking.
Observations: Evidence suggests that the main morphological stages of sporulation are conserved in the Clostridia. Moreover, the four cell type-specific RNA polymerase sigma factors that govern developmental gene expression in the well-studied model organism Bacillus subtilis are present in the C. difficile genome. In B. subtilis, SigF in the forespore, and SigE in the mother cell, control early stages of development, and are replaced by SigG and SigK, respectively, which control the final stages of development. Using a recently developed genetic system based on the use of type II introns, we have inactivated sigF, sigE, sigG and sigK of C. difficile. Disruption of sigF, sigE and sigG abolishes spore formation and their function largely conforms the B. subtilis paradigm. In contrast we found that SigK is not essential for sporulation. Rather, SigK appears to be specifically required for the release of the spore into the surrounding medium. The sigF mutant is the earliest that is specifically blocked in the sporulation pathway. This mutant is being used to determine if and to what extent is spore formation important for host colonization and infection.
Conclusions: While offering new insights into the genetic orchestration of spore morphogenesis in an important human pathogenesis, our analysis will contribute to clarify the role of sporulation in C. difficile infection.

A 106 Laser trap manipulation reveals specific functions of surface adhesions from motile malaria parasites

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Background: Plasmodium sporozoites are transmitted during the bite of a mosquito. Deposited in the skin, they migrate at high speed through the dermis to find and enter a blood vessel. Sporozoite motility is essential for establishing a malaria infection and depends on initial adhesion to a substrate as well as the continuous turnover of discrete adhesion sites as mediated by a dynamic actin cytoskeleton and possibly different members of the TRAP family of adhesins.

Observations: Using laser tweezers to manipulate individual sporozoites at will, we reveal differences in initial parasite adhesion by sporozoites lacking two members of the TRAP family of sporozoite surface adhesins. trap(-) and s6(-) sporozoites showed a deficit in initial adhesion strengths compared to wild type sporozoites. We probed the cohesive and adhesive strength of a distinct adhesion event. This showed a different functionality of TRAP and S6 in the cohesive strength of the primary adhesion site and revealed a spatially separated function for both proteins along the sporozoite surface. It furthermore showed differences between TRAP and S6 in their functional interaction with actin filaments during the transition between adhesion and gliding.

Conclusions: We were able to show a functional difference between two different TRAP family adhesins. Investigating how these differences arise will be essential for our understanding of gliding motility, which is essential for salivary gland invasion, migration in the skin and infection of the liver.

A 107 Nanotubes facilitate the contact between malaria parasites during sexual reproduction in the mosquito midgut

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Background: Physical contact is important for the interaction between animal cells, but can represent a major challenge for protists like malaria parasites. During recent years, filamentous membrane tubules have been identified in an increasing number of eukaryotic cell types, termed nanotubes, which are considered an ancient feature to establish long-distance connections during intercellular communication.

Observations: We discovered similar tubular structures in the malaria pathogen Plasmodium falciparum, which emerge from the newly formed gametes following parasite transmission from the human to the mosquito during the blood meal. The filaments can exhibit a length of more than 100 micrometer and contain the F-actin isoform actin 2. They actively form within a few minutes after gametocyte activation in the mosquito midgut and persist until the zygote transforms into the ookinete. The filaments represent dynamic surface protrusions, which originate from the parasite plasma membrane after the pathogen has egressed from the enveloping red blood cell. They are close-ended and express adhesion proteins on their surface that are typically found in gametes, like Pfs230, Pfs48/45 or Pfs25, but not the zygote surface protein Pfs28. We show that these tubular structures represent long-distance cell-to-cell connections between sexual stage parasites and demonstrate that they meet the characteristics of nanotubes.

Conclusions: We conclude that malaria parasites utilize nanotubes to establish contact between newly formed gametes in the mosquito midgut. We hypothesize that the filaments are tools to locate, connect and pool sexual stage parasites within the blood meal in order to facilitate fertilization.

A 108 The STE20 kinase MAP4K4 (NIK, HGK) is required for Kaposi's Sarcoma-Associated Herpesvirus (KSHV) lytic reactivation in endothelial cells

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Background: KSHV is a causative agent of Kaposi's sarcoma, primary effusion lymphoma, and the plasma cell variant of multicentric Castleman's disease. Both viral latency and productive reactivation are required for KSHV persistence and tumour establishment. Various cellular signaling rearrangements can trigger virus reactivation from latency. In order to better understand KSHV pathogenesis, we investigate cellular mechanisms underlying the lytic switch of the virus.

Observations: To identify 'druggable' cellular kinases required for KSHV reactivation we screened a library of kinase inhibitors. We then profiled the most promising compounds and verified their targets using siRNA mediated silencing. In this way, we identified a member of STE20 family MAP4K4 (also known as NIK or HGK) as a novel cellular kinase involved in KSHV reactivation from latency. Silencing of MAP4K4 strongly reduces the amount of infectious viral progeny. The expression of viral early and late (structural) lytic genes is significantly downregulated at mRNA and protein levels upon MAP4K4 knockdown. In KSHV infected endothelial cells MAP4K4 does not appear to signal via SAPK/JNK, one of its reported downstream targets, or ERK1/2 and p38, suggesting that other downstream players are important for KSHV reactivation. MAP4K4 was originally described as Nck-interacting kinase (NIK). Interestingly, the KSHV K15 non-structural transmembrane protein, required for viral reactivation, recruits Nck1 and Nck2 via its C-terminal domain. This suggests that MAP4K4 could interact with K15 via Nck adaptor proteins.

Conclusions: Our observations suggest that the cellular kinase MAP4K4 is required for the expression of KSHV lytic genes and the production of new infectious virions. MAP4K4 may, therefore, represent a novel target for the development of inhibitors, which can be used for the treatment of KSHV-associated tumours.

A 109 Multiple signalling pathways are involved in apoptosis and cell cycle arrest induced by periodontal pathogen in human trophoblast

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Background: Epidemiological and interventional studies of humans have revealed close associations between periodontal diseases and preterm delivery of low birth weight. P. gingivalis, a periodontal pathogen, can harbor in gestational tissues following oral-hematogenous spread. The pathogen was shown to induce apoptosis by G1 arrest. P. gingivalis-induced DNA damage response pathway has not been investigated. In this study, we examined these pathways involved in the cellular disorder caused by P. gingivalis.
Observations: Human trophoblast-derived cells (HTR-8) were incubated with P. gingivalis for 6, 12, 24, and 48 at a multiplicity of infection of 200. Cell cycle and apoptosis were analyzed by fluorescence-activated cell sorting. DNA damage triggers several signal transduction pathways that lead either to damage repair coupled with cell cycle arrest, or to apoptosis. Then activation of DNA damage response pathways was determined by western blotting. DNA damage was associated with increased expression of phospho-ATR (Ser428), phospho-Chk2 (Thr68), phospho-p53 (Ser15) by P. gingivalis. Levels of phospho-MDM2 (Ser166) were dephosphorylated and total MDM2 were degraded. In addition, G1 arrest and apoptosis were observed with p53-sustained expression during P. gingivalis infection. Interestingly, Ets1, transcription factor that plays an important role in apoptosis, was overexpressed following P. gingivalis infection. At the same time, expression of Ets1 led to G1 arrest and apoptosis in P. gingivalis-infected HTR-8 cells.
Conclusions: These results suggest that the expression of both Ets1 and DNA damage signaling pathways mediates G1 arrest and apoptosis in P. gingivalis-infected HTR-8 cells.

A 110 Lipocalin 2 modulates inflammation and impairs host defense against Streptococcus pneumoniae

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Background: Lipocalin 2 (Lcn2) is an antibacterial protein, known to interfere with bacterial siderophore-dependent iron acquisition. Thus, Lcn2-/- mice are highly susceptible to infections with siderophore-dependent pathogens. Although pneumococci acquire iron independent of siderophores, they induce tremendously high levels of Lcn2 in lungs of infected patients. The biological implication of this finding is unknown. We therefore attempted to investigate the role of Lcn2 during pneumococcal pneumonia.
Observations: Studying a murine pneumonia model, we discovered an improved bacterial clearance in Lcn2-/- mice 48h after infection. Consistently we found lung inflammation reduced in Lcn2-/- mice at this time-point, indicating a harmful role for Lcn2. As an explanation for this improved host defense we found the early inflammatory response (6h after induction of pneumonia) to be augmented with a significantly increased neutrophil influx in Lcn2-/- mice. In vitro, Lcn2-/- macrophages exhibited enhanced KC, IL-6 and TNF alpha secretion and decreased IL-10 expression in response to TLR ligands as compared to wild-type cells. Finally, recombinant mouse Lcn2 dampened the macrophage response to TLR ligands.
Conclusions: We postulate that Lcn2 modulates the early inflammatory response upon Streptococcus pneumoniae infection and thus impairs clearance of bacteria.

A 111 Probe pattern transitions in a bacterial oscillating system using multi-color in vivo fluorescence imaging and nanofluidic confinement

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Background: Faithful cell division of bacterium E. coli relies on remarkable oscillations of the MinCDE system to perform accurate and symmetric septation. Perturbing this bio-oscillator by disrupting minB locus or altering MinDE expressions is known to cause minicelling and otherwise filamentous phenotypes. Recent reports suggested MinE plays a pivotal role in spatiotemporal pattern formation of MinDE cohorts; yet it remains elusive if their pattern transitions led to morphological changes, and vice versa.
Observations: In the present study, the coupling dynamics of MinDE interplays is investigated by time-lapse microscopy. We used multi-color fluorescence imaging to detail the mode transitions in vivo in distinct MinDE pattern formations. Despite E-ring capping viewed as a drive of pole-to-pole MinD oscillations, we found peculiar expression signatures in MinD and MinE spatiotemporal distributions, corresponding to MinDE patterns other than the E-ring type, correlate to their relative expression levels. Alternatively, bacteria were confined in nanofluidic devices, to mimic curvature changes of cell peripherals. Interestingly, under strong confinement of 400 nm depth, bacteria are able to proliferate, but show irregular pancake-like shapes and intermittent oscillation episodes were observed in MinDE dynamics. The transitions between intermittent episodes display period-doubling signature of bifurcation from spatial time-frequency analysis. Apart from distinct MinDE patterns in aberrant cell shapes, filamentous cells (length > 50um) with traveling/standing waves of MinDE become dominant once they exit from nano region into microenvironment, and resume to unit cell length in 12 hours via septation.

Conclusions: Our results indicate MinDE pattern fluctuations/transitions correspond to aberrancy in septation and morphology. The study synergizes the join merits of in vivo imaging, single cell analysis, and nanofluidics to grasp the insight of noise-driven pattern transition and phenotypic changes in bacteria.

A 112 Targeting to the Trypanosome bilobe

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Background: The aim of our current research is to characterize a novel cytoskeletal structure, termed the bilobe, which we identified in the unicellular parasitic protist Trypanosoma brucei. This parasite is the causative agent of Sleeping Sickness in humans and Nagana in livestock. Organisms such as T. brucei, which is an early-branching lineage on the Eukaryotic tree, can also play a role in studying fundamental biological mechanisms.
Observations: So far, only three proteins have been shown to localize to the bilobe – TbCentrin2/4, TbLRRP and TbMORN1. The TbMORN1 protein is composed of fifteen Membrane Occupation and Recognition Nexus repeats each comprising twenty-three amino acids. The function of these repeats is unknown. Our aim is to determine the specific targeting of TbMORN1 to the bilobe by observing T. brucei cells transiently expressing various TbMORN1 truncation constructs. Transfected cells were analyzed by immunofluorescence and biochemical fractionation. So far, we have investigated more than fifteen different TbMORN1 truncations. We find that the N-terminus is dispensable for targeting. Interestingly, successive C-terminal truncations showed alternating localizations between the bilobe and the cytosol. We hypothesize that the reason for this pattern is linked to the hydrophobicity profile of the TbMORN1 protein.
Conclusions: The investigation of TbMORN1 targeting is one step in the detailed characterization of its function and that of the bilobe as a whole. Future work will concentrate on analyzing further TbMORN1 truncations and identifying the targeting motif responsible for localizing it to the bilobe.

A 113 Identification of novel surface exposed Clostridium difficile proteins potentially involved in the colonization of intestinal mucosa

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Background: Clostridium difficile, a Gram-positive spore forming anaerobic bacterium, is a frequent cause of hospital-associated infections. C. difficile infection is primarily associated with the use of broad-spectrum antibiotics that suppress the normal gut flora allowing the bacterium to colonize the intestine. Pathogenicity of C. difficile is mediated by toxin A and B and extensive studies have defined their precise modality of action and their interferences in cellular pathways of the host.
Observations: Whereas it is assumed that colonization is a prerequisite to toxin pathogenicity, little is known yet about the role of nontoxin proteins in C. difficile virulence, either during colonization or in the pathogenic process of the bacterium. The recent availability of the whole C. difficile genome allows the identification of putative surface proteins of C. difficile that could mediate the adherence to epithelial cells and the colonization of the intestine. Searching for sequence homologies to known virulence factors and protein motifs involved in interaction with the host, we have selected three proteins of C. difficile exposed on the bacterial surface with a potential ability to adhere to extracellular matrix components and to epithelial cells. Preliminary studies aimed to characterize the selected proteins have been carried-out by two approaches: i) ability of the recombinant proteins to bind to human cells, tissue sections and extracellular matrix components and ii) contribution of selected proteins to bacterial adherence to human intestinal cells.
Conclusions: We demonstrated that the recombinant proteins show adhesive properties suggesting their involvement in the interaction of C. difficile to the intestinal epithelium.

A 114 Characterization of the icosahedral, membrane-containing, archaeal virus STIV2

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Background: The members of the Archaea often live in extreme, harsh environments such as acidic, hot springs and hypersaline waters, and they are the least understood from the three domains of life. Known archaeal viruses (~40) constitute less than 1% of the ~8000 species of viruses that have been described to date. These isolated extremophilic viruses exhibit an exceptional diversity of morphotypes most of which are not encountered among viruses of the Bacteria and Eukarya.
Observations: To date, only two icosahedrally-symmetric, membrane-containing archaeal viruses, SH1 and Sulfolobus Turreted Icosahedral Virus (STIV), have been described in detail. We report the sequence and three-dimensional structure of a third such virus – STIV2 – isolated from an Icelandic terrestrial acidic hot spring. Characterization of this new isolate revealed it to be similar to STIV on the level of genome and structural organization. The genome organisation indicates that these two viruses have diverged from a common ancestor. Interestingly, the prominent surface turrets of these two viruses are strikingly different. By mass spectrometry, we mapped several large insertions and deletions in the known structural proteins that could account for these differences, and showed that both viruses can infect the same host. Of the structural proteins that we have purified and characterized, especially the postulated viral genome packaging ATPase is of interest. We will present its specificity, its activity, and a model of its structure.
Conclusions: We have revealed important new insights into the genome, assembly and structure of the pathogenic viruses of the archaea.

A 115 Genome wide expression profiling reveals Burkholderia cenocepacia adaptive strategies to long-term colonization of the lungs of cystic fibrosis patients under antibiotic therapy

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Background: Burkholderia cepacia complex bacteria are serious opportunistic pathogens in cystic fibrosis (CF) patients. To get insights into the adaptive strategies adopted by B. cenocepacia during long-term colonization of the CF lungs, the genome wide expression profiles of two clonal variants, phenotypically characterized in a previous work and obtained from the same CF patient sputa, were obtained using 2D-DIGE-based quantitative proteomics and transcriptomic analysis.
Observations: The two clonal variants compared were: B. cenocepacia IST439 thought to have started the infection and isolate IST4113. This variant exhibit higher resistance to antimicrobials and was obtained after 3 years of chronic colonization and following intravenous antimicrobial therapy. The comparison of the proteomes and transcriptomes of the two variants indicates an increased expression in isolate IST4113, compared to IST439, of proteins/genes, involved in Ribosome structure, Translation, Nucleotide synthesis, and Protein folding and stabilization. This observation suggests increased protein synthesis, DNA repair and stress resistance in IST4113. The up-regulation of several genes encoding drug-efflux pumps in this more antibiotic resistant isolate was also registered. This genome-wide analysis also allowed the identification of other adaptive responses, namely a more efficient utilization of alternative carbon sources, in particular of amino acids, an alteration in the peptidoglycan, membrane lipids and lipopolysaccharide biosynthesis, the up-regulation of genes involved in adhesion and in iron uptake from the environment and the stimulation of a microaerobic-based metabolism.
Conclusions: The comparison of the proteome and transcriptome of B. cenocepacia clonal variants suggests a genetic adaptation to the stress conditions to which they are exposed over time in the CF airways, promoting the sucessful colonizing and long-term survival in the lungs.

A 116 Detection of single molecules of HIV-1 nucleic acid via an oligo fluorescent in situ hybridization (O-FISH)

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Background: Visualizing a single copy of nuclei acid in its native sub-cellular compartments is a major challenge in cell biology, and this is further exacerbated if the nucleic acid molecules are limited in size, such as miRNAs.
Observations: Here, we present a generic approach to detect nucleic acids using 26 nucleotides oligo probes. Using HIV-1 as a model system, we demonstrate our oligo-fluorescent in situ hybridization (O-FISH) can detect nucleic acids down to 1-2 copies per cell. The specificities of our O-FISH HIV probes are validated with both HIV uninfected cells and HIV-1 reverse transcription inhibitors. The transient detectability of viral RNA suggests HIV genomes undergo conformational rearrangement at the early steps of reverse transcription.
Conclusions: In addition of demonstrating the first generally applicable tool to discern functional viruses from non-functional particles based on the synthesis of viral genome, our O-FISH technology highlights the potential and means to visualize single copies of miRNA in cells.

A 117 Ebola virus entry requires the cholesterol transporter Niemann-Pick C1

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Background: Entry of Ebola (EBOV) and Marburg (MARV) filoviruses into cells is mediated by the viral spike glycoprotein, which attaches viral particles to the cell surface, delivers them to endosomes, and catalyzes fusion between viral and endosomal membranes. Additional host factors in the endosomal compartment are likely required for viral membrane fusion. However, despite considerable efforts, these critical host factors have defied molecular identification.
Observations: Here we describe a genome-wide haploid genetic screen in human cells to identify host factors required for EBOV virus entry. Our screen uncovered 67 mutations disrupting all six members of the HOPS multisubunit tethering complex, which is involved in fusion of endosomes to lysosomes, and 39 independent mutations that disrupt the endo/lysosomal cholesterol transporter protein Niemann-Pick C1 (NPC1). Cells defective for the HOPS complex or NPC1 function, including primary fibroblasts derived from human Niemann-Pick type C1 disease patients, are resistant to infection by EBOV and MARV, but remain fully susceptible to a suite of unrelated viruses. We show that membrane fusion mediated by filovirus glycoproteins and viral escape from the vesicular compartment requires the NPC1 protein, independent of its known function in cholesterol transport. In agreement herewith, the small molecule U18666A, which causes a cellular phenotype similar to NPC1 deficiency, specifically inhibits EBOV infection. Finally, we assessed the effect of NPC1 mutation in lethal mouse models of EBOV and MARV infection and show that NPC1 knockout mice are largely protected against filovirus infection.

Conclusions: Our findings uncover unique features of the unusual entry pathway used by filoviruses. The unanticipated role for the hereditary disease gene NPC1 in filovirus entry, infection, and pathogenesis may facilitate the development antiviral strategies to combat these deadly agents.

A 118 In search of cell machinery employed by HIV-1 protein Nef: optimising a whole genome siRNA screen

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Background: Nef is a multifunctional accessory protein of the HIV-1 virus required for progression from HIV infection into full-blown AIDS. Present work sets out to study its two independent functions. Firstly, Nef downregulates MHC I from cell surface which is part of the virus's imunno-evasion strategy. Secondly, Nef causes downregulation and degradation of CD4, the virus's receptor of entry, preventing super-infection. In order to find further host proteins a whole-genome siRNA screen will be performed.

Observations: A HeLa-M cell line was used as a base for creating a model system. A clonal cell line was created, which expresses HLA-A2, CD4 and a modified Nef protein which can be activated using a small molecule. The read out of the assay is the amount of HLA-A2 and CD4 on the cell surface after Nef activation as compared to cells in which Nef has been left inactive. The assay was optimised for a 96-well format in a plate reader. From previous studies it is known that Nef requires certain host proteins to function. Clathrin is required for the downregulation of both HLA-A2 and CD4. Nef also requires Adaptor Protein 1 (AP-1) to downregulate HLA-A2, but not CD4. AP-2 is required for downregulation of CD-4 but its absence enhances Nef's effect on HLA-A2. These phenotypes have been repeated in the present system and they have been selected as controls in the screen.

Conclusions: The results replicate phenotypes known from T-cell studies which suggests that the cell line is a good model in which to study Nef mediated downregulation of cell surface receptors. The screen conditions and exact protocol have been optimised and the screening is ongoing.

A 119 Detecting host targets of bacterial effector proteins: A chemical reporter for protein AMPylation

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Background: Protein AMPylation is an emerging regulatory posttranslational modification (PTM), employed by several bacterial pathogens to interfere with host signaling processes. A plethora of putative AMPylation enzymes (AMPylators), with an unknown set of substrates, are present in many bacterial pathogens, suggesting a more general role for protein AMPylation. Due to the lack of analytical tools for this neglected PTM we developed a chemical reporter for the detection of AMPylated substrates.

Observations: AMPylators transfer adenosine monophosphate (AMP) from the endogenous cofactor adenosine triphosphate (ATP) onto side-chain hydroxyl groups of their corresponding protein substrates. Therefore, we synthesized an ATP analog with an alkyne group, which serves a bioorthogonal reporter moiety. Covalent functionalization of AMPylated substrates with an alkynyl chemical reporter in lieu of AMP allows their subsequent detection by click-chemistry mediated ligation to a fluorophore. We tested this novel ATP analog with three diverse bacterial effector proteins, whose AMPylation activity towards host small GTPases has been previously described. All tested effectors, comprising Fic domain and adenylyl transferase domain AMPylators, modified their corresponding substrates with the chemical reporter. Point mutant analysis revealed that the chemical reporter covalently modifies the same residues as AMP. In addition, the chemical reporter was also transferred selectively in cell lysates, allowing substrate detection at endogenous levels.

Conclusions: We present the first chemical reporter for protein AMPylation and show that it provides a general method to detect host substrates of secreted bacterial AMPylators. This reporter should be a powerful chemical tool for the analysis of novel AMPylated substrates of many bacterial pathogens.

A 120 Legionella pneumophila chronobiology: a tool for its adaptation to natural niches?

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Background: The intracellular bacterium Legionella pneumophila replicates in aquatic protozoa and may cause a severe pneumonia in humans. Its genome codes for homologues of KaiB and KaiC, the circadian clock core proteins of Cyanobacteria. There is little evidence for circadian rhythms in prokaryotes but several metabolic processes are rhythmic like chromosome compaction or cell division. We speculate that protozoa might have circadian rhythms and these proteins could help the bacterium to adapt to these.

Observations: Analyses of L. pneumophila kaiC and kaiB mutants in different infection models indicate that KaiB and KaiC are involved in virulence in amoeba and in a pulmonary mouse model. Furthermore, the Kai proteins seem to be implicated in regulating cell division processes and in increased resistance of L. pneumophila to oxidative stress. Transcriptional analyses of the mutant strains compared to the wild type strain, indicate that KaiB and KaiC indeed regulate genes implicated in oxidative stress response. Phylogenetic analyses of prokaryotic KaiC homologues revealed that the L. pneumophila and Cyanobacteria KaiC protein sequences share ancestry, however it seems that the Kai proteins of L. pneumophila have evolved divergently of those of Cyanobacteria, suggesting that they also may have evolved different functions.

Conclusions: In aquatic environments sunlight leads to the production of reactive oxygen species (ROS). ROS is also a key defense mechanism of human cells in response to intracellu-

lar pathogens. Thus, the KaiBC proteins of L. pneumophila may improve its fitness in the environment and the host cells.

A 121 Free fatty acids – new inducers of dormant mycobacterial cell reactivation

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Background: Family of secreted bacterial Rpf proteins participates in dormant cells (DC) reactivation. However, Rpf concentration at the begginning of reactivation could be very low and not enough to promote cell growth. Probably, there is another inducer that may play a role of the 'first signal' controlling reactivation. The aim of this study is to find and to characterise new inducers of dormant mycobacteria reactivation.
Observations: We found that upon DC reactivation, rpf genes expression begins later than [H3]-uracil incorporation and followed by OD increase of the culture. Obviously, the metabolic reactivation process could be initiated by some other factors than Rpf. Zhang et al has reported that growth stimulation of the aged mycobacterial cells might be under control of exogenous phospholipids [Zhang, 2001]. We found that the reactivation process in DC of Mycobacterium smegmatis is stimulated by different phospholipids (phosphatidylcholine, cardiolipin, and lyso-phosphatidylcholine). Since fatty acid residues are common for phospholipids, we hypothesized that fatty acids (FA) may be considered as true reactivation inducers. Indeed, low concentrations of free FA added to the medium stimulated DC resuscitation. Unsaturated fatty acids (oleic, linoleic) at concentration of 1 microgram / ml added to 10E6 DC cells shows better reactivation effect in comparison with saturated fatty acids and higher alcohols. Resuscitation effect of phospholipids and oleic acid was concentration-dependent. The range of oleic acid concentrations (0.05-3.0 microgram / ml) was significantly lower than phospholipids (50-250 microgram / ml).
Conclusions: This study fir the first time demonstrates that free FA are the substances responsible for reactivation of dormant mycobacteria, while phospholipids probably serve as their sources. In this regard, it is worth noting that free fatty acids are abundant in host cells and tissues.

A 122 Physiological function of mechanosensitive channels in Synechocystis sp. PCC 6803

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Background: Microbial possess adaptation mechanisms to the environmental stress. Mechanosensitive channels are one of the major machinery for the release of cytoplasmic solutes to achieve a rapid reduction of the turgor pressure during the transition from media of high osmolarity to low. However the adaptation mechanism of the Synechocystis to the hypoosmotic stress remains elusive.
Observations: We have isolated the genes encoding mechanosensitive ion channel homologs from Synechocystis sp. PCC 6803 genome and have performed osmotic-downshock assays in Escherichia coli lacking endogenous mechanosensitive -ion-channels. syMscL (slr0875) well rescued the mutation of the osmotic-shock sensitivity of the E. coli. The point mutation at valine 32, which is conserved among MscL family proteins, showed gain of function phenotype. Mechanosensitive channel deletion strains of Synechocystis sp. PCC 6803 showed the hyper sensitive phenotype in the osmotic-downshock assay. Moreover, hypo-osmotic swelling during osmotic-downshock was observed in the cell volume monitoring by using the stopped-flow spectrophotometry. Membrane fractionation followed by immunoblotting revealed that syMscL was localized in the plasma membrane of Synechocystis. A real-time bioluminescence monitoring system revealed that the expression of symscL was controlled by the circadian rhythm and the gene expression peaked at the beginning of subjective night.
Conclusions: These results indicate that syMscL extrudes intracelluar osmolytes to decrease the intracellular osmolarityg. The circadian rhythm of the expression of syMscL may be needed for the adaptation to the daily osmotic changes in the cells.

A 123 Base J is required for proper termination of RNA polymerase II transcription

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Background: The genomic DNA of trypanosomatids contains a hypermodified base, beta-D-hydroxymethyluracil or J, that replaces a fraction of thymines. In Leishmania, base J is found almost exclusively at the telomeres. The approximately 1%, chromosomal internal J has now been mapped by the isolation of DNA fragments containing J using anti-J DNA antibodies or a protein that specifically binds J DNA followed by high throughput sequence analysis of the isolated fragments.
Observations: We find that J is located at the transcription termination region between convergent protein-coding gene clusters in L. major and L. tarentolae. J is also found upstream of all presumed internal transcription initiation sites within protein-coding gene clusters, which are marked by acetylated histone H3 and at some transcription initiation sites between divergent protein-coding gene clusters, especially those with a relative long distance between transcription starts. Finally, J is found flanking the rRNA repeat cluster, and upstream of the mini-exon (SL) locus. Reducing J in the genome of L. tarentolae results in massive read-through at transcription termination sites. The read-through leads to a genome wide alteration of transcript

levels, which provides a plausible explanation why Leishmania parasites die when J is lost.

Conclusions: The specific localization of J and the read-through observed when J is reduced strongly suggests that J is required to terminate RNA polymerase II transcription at specific locations in the genome.

A 124 Analysis of Salmonella typhimurium host cell invasion using micropatterned cells

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Background: Salmonella Typhimurium (S. Tm) is a Gram-negative, flagellated bacterium, which triggers its entry into non-phagocytic cells in the mammalian gut by injecting different effector proteins. These proteins force the cell to form actin enriched membrane protrusions (membrane ruffles), which engulf the docked bacteria. The analysis of the infection process of S. Tm at the single cell level is challenging, as mammalian cells cultured in vitro show a high variability in morphology and physiology.

Observations: Cells grown on microfabricated patterns, enforcing them to adopt a specific shape and preventing them from migrating, allow studying single cells in a more defined environment and with less cell-to-cell variation at the single cell level. In this study, we compared the infection process of S. Tm in classical unconstrained cell culture and micropatterned cells. We found that S. Tm infections follows the same infection steps (docking, ruffling, membrane closure and SCV repositioning near the nuclei) and actin rearrangement kinetics, demonstrating that the invasion mechanisms into this two types of cells were comparable.

Conclusions: Cell micropatterning allowed us to 'standardize' the infection process and quantitatively compare the preferred entry points of different Salmonella strains and the induced actin rearrangements using statistical tools.

A 125 Functional diversification of the GALA type-three effector family contributes to adaptation of Ralstonia solanacearum on different plant hosts

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Background: Plant pathogenic bacteria use large type-3 effectors (T3Es) repertoires in order to manipulate their host's cellular functions. Because most of these effectors act redundantly, selective pressures leading to the generation and maintenance of such repertoires are unclear. This is illustrated by the GALA T3Es family from Ralstonia solanacearum (seven members in our reference strain) which are collectively, but not individually, required for disease on Arabidopsis thaliana and tomato.

Observations: Availability of GALA sequences from several R. solanacearum strains enabled us to reconstruct the GALA phylogeny. We show that the family was probably generated by several duplication events in the ancestral strain, and subsequently conserved during R. solanacearum strains divergence. We then investigated evolution, redundancy and diversification of this family in order to understand which selective forces (beneficial dosage effect of fully redundant proteins or functional diversification) were responsible for the conservation of this T3Es family. Positive selection was detected on some GALA members, suggesting a functional diversification scenario. We then focused on the GALAs from the reference strain GMI1000 to examine their expression levels, subcellular localization, ability to suppress plant defense responses and contribution to pathogenicity on three different host plants: A. thaliana, tomato and eggplant. All these experiments tend to confirm the functional diversification hypothesis of the GALA gene family. Moreover, differential GALA requirement for complete disease on different hosts provides a new example of pathoadaptation for plant pathogen effectors.

Conclusions: Functional diversification and pathoadaptation of the GALA family likely accounts for its remarkable conservation during R. solanacearum evolution. Duplication of important pathogenicity genes may represent a general mechanism enabling pathogens to adapt on new hosts.

A 126 Down-regulation of EBNA2 mediated transactivation of EBV latency specific LMP1 promoter by p53 is implicated in cellular protection against EBV infection

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Background: Epstein-Barr Virus (EBV) nuclear antigen EBNA2 and EBNALP are essential for immortalization of B lymphocytes upon virus infection. The evidence showing that EBNALP is biochemically associated with p53, suggesting that p53 may play a role in EBV infection. The role of p53 in modulation of transcription mediated by EBNA2 and EBNALP has not been documented yet since the first evidence of protein-protein interaction between EBNALP and p53 was described by Szeklely and colleagues in 1993.

Observations: The interactions between p53 and EBNA2, or EBNALP were emphasized in our recent studies. Among the EBV associated malignant cells, the expression levels of p53 were observed to be reversely correlated with the presence of EBV latent infection, suggesting p53 has a negative impact on EBV infection. Accordingly, we found plasmid mediated p53 overexpression completely abrogated EBNA2 and EBNALP dependent luciferase activity of a latency specific promoter reporter, LMP1-Luc, in BJAB B lymphoma cells, or LMP1 expression from the endogenous EBV genome in EBV latency I infected AKATA cells. In particular, both activation and oligmerlization (Olg) domains of p53 were identified as the major determinants participated in down-regulation of EBNA2 mediated transcription, whereas Olg and proline rich domains were shown to have a role in repressing EBNALP co-activation.

Conclusions: We demonstrated PXXP1 motif of p53 can recruit corepressor sin3A to negatively regulate transactivation potency of EBNA2. Our current data lead us to propose p53 mediated down-regulation of transcription triggered by EBNA2 and EBNALP is an innate cellular defense against EBV infection.

A 127 Adaptation to osmotic stress in E. coli: old song, new melody

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Background: Bacteria encounter widely varying environmental conditions and increase in salinity is one of the most frequent ones. Adaptation to this case, which is referred to as hyperosmotic stress, involves a modification of transcription patterns with downstream effects on physiology. Moreover, the nucleoid structure is highly sensitive to these changes (supercoiling has been reported) and to global gene expression through RNA polymerase binding and distribution.
Observations: Here, we probed the impact of hyperosmotic stress (0.5 M NaCl) on the nucleoid structure of Escherichia coli K-12 coupled with a detailed survey of RNA polymerase binding using microscopy and ChIP-on-chip, respectively. Our observation showed surprising dynamics of the E. coli chromosome that appears consistent with the observed RNA polymerase distribution. Interestingly, RNA polymerase binding events appear to be less frequent during the stress period while the nucleoid shows global expansion. Furthermore, we assessed the transcriptional changes underlying the response to hyperosmotic stress and observed the activity of previously reported and unknown-to-date genes with respect to time. As previously reported, we did not observe any significant change in expression of rpoS, that is the gene encoding the osmotic stress sigma factor. Lastly, we reconstructed the regulatory interactions governing this adaptattion and established the preferential involvement of simple transcriptional motifs, namely feed-forward loops and simple-input modules.
Conclusions: Our study thus demonstrates the crucial involvement of the RNA polymerase in the dynamics and topology of the bacterial chromosome and represents the first complete and comprehensive map of the events directing the adaptation of E. coli to increased salinity in the medium.

A 128 Detection and isolation of antimicrobial substance exhibiting activity against MRSA induced from Hermetia illuscens

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Background: Rapidly spreading pathogenic microorganisms, such as Methicillin-resistant Staphylococcus aureus (MRSA), that have developed resistance to common antibiotics have elicited the search for effective, alternative antimicrobial substances. The larvae of Hermetia illucens are scavengers that live in extremely harsh environments inhabited by various microbes, suggesting that they may be rich in generations of antimicrobial peptides (AMPs) possessing activity against drug-resistant 'superbugs'.
Observations: In this study, we induced anti-MRSA product from H. illucens and purified a novel antimicrobial product from the immunized haemolymph. Immunized haemolymph of H. illucens with Escherichia coli did not show anti-MRSA activity, whereas that with Staphylococcus aureus exhibited a potent, broad-spectrum antibacterial activity against Gram negative and Gram positive bacteria including MRSA. The larvae of H. illucens were induced with Gram positive bacteria, and their haemolymph extracts were preparatively purified with C18 SPE against various acetonitrile concentrations and, subsequently, using RPC. Fractions exhibiting antibiotic activity against MRSA, E. coli, and both were separately collected and pooled, and the first pool was further purified by C18 HPLC column, confirming the presence of a singlet exhibiting anti-MRSA activity.
Conclusions: The data provide evidence that this pool possesses a novel AMP that could be developed into therapeutically useful anti-infective agents and thus, is worth further studies on its physical/structural and functional properties and, in particular, investigations on its antimicrobial mechanisms.

A 129 Lipoteichoic acid induced cytokine release is inhibited by apolipoprotein B100

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Background: The initial immune response to pathogens depends on specialized receptors including Toll-like receptors and nucleotide-binding domain proteins. In addition to these cell associated immune receptors, a number of soluble serum proteins have been described to contribute to the recognition of LPS of Gram-negative bacteria. However, little is known about the role of serum proteins during immune recognition of lipoteichoic acid (LTA), an important immunostimulatory component of Gram-positive bacteria.
Observations: Comparative SDS-PAGE profiles of chromatography fractions of human plasma preincubated with staphylococcal LTA revealed an interaction between LTA and apolipoproteins (ApoA1, ApoA2, ApoA4 and ApoB100), which was confirmed by solid-phase binding assays. In the presence of ApoB100 but not ApoA1 or ApoA2 a significant inhibition of LTA-induced cytokine release from human peripheral blood cells (PBMC) was observed. Comparable to the human data, PBMCs and peritoneal macrophages of LDL-R. knockout mice, comprising increased ApoB100 levels, showed reduced cytokine induction compared to WT cells upon stimulation with LTA as well as with heat-inactivated S.aureus. Moreover, mice pretreated with a drug (4APP) that inhibits low-density lipoprotein secretion by the liver were significantly more susceptible to infection with heat-killed S.aureus that WT mice, indicating that ApoB100 in the blood essentially contributes to the innate immune recognition of S.aureus.

Conclusions: The present study identifies apolipoprotein B100 as an important serum protein able to inhibit the cytokine induction by staphylococcal LTA.

A 130 Role of pattern recognition receptors in endothelial cells response to dengue virus infection

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Background: Dengue virus (DENV) is a single stranded RNA virus that causes potentially life-threatening diseases known as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Evidences suggest that an exaggerated production of pro-inflammatory cytokines is involved in the vascular endothelium dysfunction that generates DSS. The aim of this study was to investigate the involvement of pattern recognition receptors in the production of inflammatory mediators by infected endothelial cells.
Observations: We analyzed gene expression changes in infected brain microvascular endothelial cells (BMEC) through real-time PCR. Our results showed that DENV infection stimulated the expression of both cytoplasmatic and endossomal pattern recognition receptors. MDA5 and RIG-I presented a considerable increase in their expression 48 hours post-infection, while TLR3 mRNA showed a slighter augment. RIG-I protein synthesis was accessed through immunoblotting, confirming that mRNA increase resulted in an augment in translation. Interestingly, flow cytometry experiments showed that the increase in RIG-I protein production was seen in both DENV-infected and bystander cells. Additionally, infection induced an augment in the expression of a variety of inflammatory mediators, including IL-6, IL-8, IL-12, RANTES and IFN-beta. To examine whether these mediators were produced via RIG-I signaling pathway, RNA interference experiments were conducted. The inhibition of RIG-I expression in infected cells leads to a significant reduction of the expression of these molecules, even though the virus yield did not change.
Conclusions: Our results suggest that RIG-I recognizes DENV in BMEC cells leading to the production of pro-inflammatory cytokines and interferons, which can contribute to the exacerbated immune response that causes DHF/DSS.

A 131 Altered host expression profile in early hepatitis B virus replication

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Background: Chronic carriers of Hepatitis B virus have increased risk of developing liver-associated diseases. Long-term inflammation resulting in abnormal liver regeneration, increased chances of insertional mutagenesis with prolonged residence of the virus, as well as the persistent trans-activation effects of HBx are thought to be causes of these diseases.
Observations: To investigate the viral-host interaction, the expression profile of early virus replication was compared between cells transfected with the replicative HBV plasmid construct at 4 hours and 48 hours post-transfection. Pathway analysis revealed that G-protein coupled receptor signaling, especially those involved in inflammatory responses is down-regulated. Interestingly, the expression of several protein family modules with uncharacterized functions was significantly up-regulated. These include multiple C2H2-type zinc-finger containing proteins, DENN domain-containing proteins, WD domain containing proteins as well as proteins containing the TPR domain. WD domain proteins form propeller-like structures with a central pore, while TPR proteins are likely scaffold proteins required for the assembly of large protein complexes. While C2H2-type zinc finger proteins are thought to be transcription regulators, their downstream targets are largely unknown. DENN domain proteins are putative GDP-GTP exchange factors for small G-proteins involved in vesicular transport, which is itself up-regulated in early HBV infection.
Conclusions: This suggests that HBV may evade immune surveillance during early infection and the consequence of HBV replication on its host cell requires further investigation. Studies on this may shed light on how chronic inflammation by persistent infection may eventually be brought about.

A 132 Pre-microRNA and mature microRNA in human mitochondria

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Background: Because of the central functions of the mitochondria in providing metabolic energy and initiating apoptosis on one hand, we hypothesized that some miRNA could be present in the mitochondria for post-transcriptomic regulation by RNA interference. We intend to identify miRNA localized in the mitochondria isolated from human skeletal primary muscular cells.
Observations: To investigate the potential origin of mitochondrial miRNA, we in-silico searched for microRNA candidates in the mtDNA. Twenty five human pre-miRNA and 33 miRNA aligments (E-value < 0.1) were found in the reference mitochondrial sequence and some of the best candidates were chosen for a co-localization test. In situ hybridization of pre-mir-302a, pre-let-7b and mir-365, using specific labeled locked nucleic acids and confocal microscopy, demonstrated that these miRNA were localized in mitochondria of human myoblasts. Total RNA was extracted from enriched mitochondria isolated by an immunomagnetic method from a culture of human myotubes. The detection of 742 human miRNA (miRBase) were monitored by RT-qPCR at three increasing mtRNA inputs. Forty six miRNA were significantly expressed (2nd derivative method $Cp > 35$) for the smallest RNA input concentration and 204 miRNA for the maximum RNA input concentration. In silico analysis predicted 80 putative miRNA target sites in the mitochondrial genome (E-value < 0.05).
Conclusions: This study demonstrated for the first time the presence of pre-miRNA and miRNA in the human mitochondria. Their origin should be further investigate to determine if they are imported from the cytosol and/or if they are partially processed in the mitochondria.

A 133 Regulated Drosha cleavage and RNA editing control processing of pri-miRNAs during Xenopus oogenesis

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Background: MicroRNAs (miRNAs) are ~21nt nucleotide long, single-stranded noncoding RNAs that regulate gene expression. Biogenesis of miRNAs is mediated by the two RNase III-like enzymes Drosha and Dicer. Processing by either enzyme has been shown to be affected by adenosine to inosine deamination type RNA-editing. Furthermore, miRNA processing has been shown to be regulated at many levels throughout development. Here we study miRNA biogenesis during maturation of Xenopus oocytes to eggs.
Observations: We show that processing of exogenous and endogenous pri-miRNAs is strongly enhanced upon maturation of oocytes to eggs. This developmental regulation of Drosha is neither transcriptionally nor post-translationally controlled. Instead, poly-A length addition boosts translation of Drosha mRNA upon oocyte maturation. Using activated Xenopus eggs for microinjection experiments we can show that RNA-editing can block pri-miRNA processing in vivo. This processing block is determined by the structural but not sequence changes introduced by RNA-editing.
Conclusions: Our studies show that cleavage of pri-miRNAs by Drosha is the rate-limiting step during Xenopus oogenesis. Translational activation of Drosha mRNA can relieve this block. Our studies show further, that structural changes introduced by RNA-editing can efficiently block Drosha cleavage in vivo.

A 134 Modulators of ADAR Activity

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Background: A to I editing catalyzed by ADARs is the most prevalent form of RNA editing. Editing deregulation has been linked to CNS diseases as depression and epilepsy. Recently, global hypoediting has been found in tumor tissues indicating a correlation between editing and cancer. Until now, no plausible mechanism regulating editing is known. Splicing mediated generation of different ADAR isoforms is transcriptional regulatory mechanism. However, new evidence suggests also post-translational regulation.
Observations: To identify potential cellular modulators of ADAR 2 activity a yeast-editing assay was established. In this assay, an amber stop codon located in a stem loop in the 5' region of the Ura3 gene can be edited into a tryptophan codon by ADAR2. Conversion of the amber codon to a tryptophan codon thereby allows Ura3 expression. However, addition of the drug FOA selectively kills the cells expressing Ura3. To identify cellular proteins that inhibit editing, a human cDNA library was transformed into the reporter strain. In the presence of FOA only colonies that fail to express Ura3, i.e colonies in which editing was inhibited could grow. A tissue culture reporter assay was established for tertiary screening of the potential inhibitors in the mammalian system. Three candidate proteins RPS14, SRSF9 and DDX15 were isolated from this screen as inhibitors of editing in mammalian cells. Further co-localization and co-immunoprecipitation studies are done in order to dissect the mode of interaction. RPS14 co-localizes and interacts with ADAR2. RPS14 and DDX15 both show editing inhibition on endogenous substrates.
Conclusions: Our results indicate that RPS14, SRSF9 and DDX15 are potential cellular inhibitors of ADAR2 mediated editing in mammalian cells, proving the functionality of the developed screening system.

A 135 Genome-wide determination of RNA stability defines functionality of long non-coding RNAs in mammals

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Background: Traditionally, RNA decay has been assessed by blocking global transcription with transcriptional inhibitors and subsequently monitoring ongoing RNA decay over time; however, inhibitor-mediated global transcriptional arrest has a profoundly disruptive impact on cellular physiology. Therefore, inhibitor-free method to measure RNA decay has been demanded.
Observations: We pulse-labeled endogenous transcripts with the uridine analog 5'-bromo-uridine (BrU) and then isolated the BrU-containing transcripts (BrU-RNA) by immunopurification. Measurement of the decrease in BrU-labeled RNAs over time was facilitated by deep-sequencing. We determined successfully half-lives for 11,052 mRNAs and 1,389 annotated ncRNAs. We named this novel method BRIC (5'-BRomo-uridine Immunoprecipitation Chase). Moreover, by analyzing the relationship between RNA half-lives and functional category, we found that half-lives of ncRNAs correlate with functional characteristics and propose a new category of ncRNAs those have relatively short-half lives. We named this novel class of ncRNAs, short-to-medium-lived intergenic non-coding transcripts (SLITs). To our knowledge, this is the first report to propose a new category of ncRNAs according to their half-life. These SLITs were found to include many well-known and functional lncRNAs. We also show that modulating the stability of short-to-medium-lived ncRNAs affects their functions. Finally, we identified novel functional lncRNAs involved in cell proliferation by analyzing a subset of SLITs.
Conclusions: We propose that RNA stability can be exploited for the identification of previously uncharacterized functional non-coding transcripts. Thus, determination of RNA half-lives under physiological conditions is very useful to study the functions of transcripts including many uncharacterized ncRNAs.

A 136 Jumonji domain containing protein 6 (Jmjd6): a lysyl-5-hydroxylase with impact on alternative splicing

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Background: Alternative splicing is a mechanism for the regulation of gene expression in mammalian cells. On the other hand alterations in splicing cause diseases. Cis-acting mutations usually affect splicing of a single gene. In contrast, mutations in conserved splicing regulators might cause missplicing of multiple genes and therefore have widespread consequences. Homozygote Jmjd6 knockout mice died prenatally with severe malformations in various different organs, like heart, lung and brain.

Observations: We were able to demonstrate that Jmjd6 can alter alternative splicing by posttranslational modifications on splicing associated proteins. In co-immunoprecipitation studies coupled to mass spectrometry, many splicing associated proteins were discovered as interaction partners of Jmjd6, e.g. U2 small nuclear ribonucleoprotein auxiliary factor (U2AF), Luc7-like1 and Luc7-like 2. Sequence comparison and crystal structure revealed Jmjd6 to be a member of the Fe(II) and 2-Oxoglutarate (2OG) dependent oxygenase family of enzymes. This large group of enzymes catalyses transfer of oxygen atoms on various different products, for example proteins, DNA, RNA or small molecules. They use Fe(II) as a cofactor and 2OG as a cosubstrate. We were able to identify a lysyl-5-hydroxylase activity of Jmjd6, which hydroxylates two particular Lys-residues in U2AF65. In addition, Jmjd6 was shown to change alternative splicing of some, but not all, endogenous and reporter genes used in our experiments. High resolution imaging showed localisation of Jmjd6 in nuclear speckles and the nucleoli.

Conclusions: The accurate mechanism of how the enzymatic activity of Jmjd6 can affect splicing needs to be elucidated. Jmjd6 seemed to influence alternative splicing events by post-translational modifications on splicing associated proteins and therefore might play a major role in mammalian embryonic development.

A 137 A comparative study of 5 pathogenic mutations affecting the mitochondrial tRNA-Ile gene

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Background: Pathogenic mutations of the mitochondrial (mt) genome can cause a variety of multisystemic as well as tissue specific diseases, even if they affect the same gene. Of the 17 described pathogenic base substitutions within the (mt)tRNA-isoleucine some were found associated with cardiomyopathy, while others caused ophthalmoplegia, encephalopathy, or multisystemic pathologies. As little is known about the genotype-to-phenotype relationship, we analyze selected mutants in a comparative approach.

Observations: We study the molecular and cellular consequences of five different pathogenic mutations of (mt)tRNA-isoleucine in a tissue culture model. For comparability of these five point mutations we use cytoplasmic hybrid cells carrying the nucleus of the 143B osteosarcoma cell line and the respective mitochondrial genome of the patients. Respiratory chain analysis revealed isolated or combined complex deficiencies in certain cases. We further found significant differences in the steady-state levels of the mutated tRNA-Ile and its precursors. We therefore studied the consequences of the five mutations on the processing of the precursor as well as the stability and resynthesis of the mutated tRNA molecule in the cell lines. Moreover, aminoacylation levels of the mutated tRNA-Ile by its cognate amino acid, as well as the quantitative and qualitative level of the 13 mitochondrially translated proteins and nuclear encoded subunits of the respiratory chain are under examination. Structure prediction programs suggest an alternative conformation for the tRNA-Ile carrying the mutation with the most severe phenotype.

Conclusions: Aside from the description and characterization of two hitherto undescribed (mt)tRNA-Ile mutations, the study may provide a basis for a better understanding of the molecular basis of the variable clinical outcome of mutations within the same gene.

A 138 Nuclear and mitochondrial ribonuclease P of Trypanosoma brucei

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Background: tRNAs are synthesized as immature precursors. Their 5' end is formed by the endonuclease RNase P. Traditionally, all RNase P enzymes were believed to be RNPs and to rely on RNA catalysis. However, human mitochondrial RNase P was recently shown to be composed of protein only. Still it is widely believed that such proteinaceous RNase P enzymes are confined to the simpler organellar systems mitochondria and chloroplasts. We characterized the nuclear and mitochondrial RNase P of Trypanosoma brucei.

Observations: The genome of T. brucei encodes neither an RNase P RNA nor any of the proteins typically associated with it. We identified two homologs of a subunit of human mitochondrial RNase P in the T. brucei genome. The two putative T. brucei proteinaceous RNase P enzymes were overexpressed in E.coli and purified to homogeneity by affinity chromatography. Both recombinant proteins had RNase P activity on their own, requiring neither an RNA nor additional proteins. YFP-tagged variants of both RNase P proteins were expressed in T. brucei. One of them is localized in the nucleus, whereas the other was found in the mitochondrion. Because all mitochondrial tRNAs of T. brucei are encoded by nuclear genes and imported into the mitochondrion, elucidating the biological role of mitochondrial RNase P will be most intriguing. Knock-down or gene deletion of

the two RNase P proteins should reveal their in vivo substrates and biological function beyond tRNA processing.

Conclusions: T. brucei uses a proteinaceous RNase P for the processing of nuclear encoded tRNAs. The homologous mitochondrial enzyme might be involved in different non-tRNA related RNA processing pathways.

A 139 Impact of RNA editing on miRNA maturation

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Background: microRNAs can be A to I edited by Adenosine Deaminases that act on RNA (ADARs). Around 16% of primary miRNAs are predicted to undergo A to I editing. Consequences of miRNA editing range from a change in target specificity (in case editing occurs within the seed region) to a different processing. More precisely, some edited miRNAs were shown to be blocked for either Drosha or Dicer cleavage during their maturation. Thus, miRNA editing can affect the abundance as well as the targeting of miRNAs.

Observations: However, to this point the consequences of miRNA editing have only been studied on a few miRNAs, whereas we aim with this study to explore the genome wide consequences of miRNA editing. ADAR deficient mice show severe phenotypes. An ADAR1 KO is embryonic lethal at day E12 – E12.5. Therefore we chose to use mouse embyos at E11.5 for our analysis. Sofar we generated libraries of mature miRNAs from wildtype, ADAR2-/- and ADAR1-/- /ADAR2-/- mouse embryos in 3 biological replicates and analysed miRNA abundance and also editing using the Illumina deep sequencing technology. Our first results show up- and also downregulation of many maure miRNAs in the ADAR deficient mice. The pool of highly up and downregulated miRNAs is enriched for edited miRNAs but not exclusively consisting of them. Further we see most of the effect on miRNA abundance already in the ADAR2-/- mice and not only in the double KO, which is completely editing deficient. Additionally, we were able to identify new edited mature miRNAs from this dataset. Futher analysis and more biological replicates will allow us to tell whether the abundance differences of mature miRNAs are caused by A to I editing.

Conclusions: The effect on miRNA abundance is already high in the absence of ADAR2, leading to the speculation that ADAR2 is the main editing enzyme for most miRNAs. Moreover, it is possible that an additional effect of ADARs on miRNA processing exists as also not-edited miRNAs are found to be deregulated.

A 140 Catalog of microRNA gene polymorphisms in livestock animals

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Background: MicroRNAs (miRNAs) are a class of non-coding small RNAs that regulate gene expression by altering translation of thousands of target genes. Genetic variants affecting miRNA pathways were associated with some livestock production traits. However, the data on miRNA genetic variability in livestock species are limited and remain fragmented among various databases and publications. A database integrating miRNA polymorphisms is needed and would allow more effective marker assisted selection.

Observations: We performed systematic genome-wide bioinformatics survey in livestock animals using online databases and tools (miRBase, ENSEMBL, AnimalQTLdb, and Biomart). We developed a comprehensive collection of miRNA SNPs in three major livestock species: pig, cattle and chicken. Genetic variability was present within pri-miRNA, pre-miRNA, mature, and seed miRNA regions. Polymorphic miRNA genes overlapped with several QTL associated with economically important traits in livestock. Several miRNA promoter variants possibly causing deregulation of transcription initiation were identified in the genomic regions of transcriptional control. We searched for pathways potentially affected by the altered expression of polymorphic miRNAs. Our results show, that most miRNA SNPs still need to be validated and most miRNA genes in livestock have to be systematically screened for polymorphisms. Polymorphisms with predicted impact to phenotypic traits were selected for experimental validation and association studies with production traits. Collated data can serve researchers as a starting point in testing more targeted hypotheses and designing experiments using species of choice for phenotypic screens.

Conclusions: In this study we systematically collected all miRNA SNPs of three livestock species in a single catalog which will contribute to the development of molecular markers in breeding programs. Upcoming data on miRNA genetic variability in other livestock species will also be included in the catalog.

A 141 MALAT1, a nuclear long noncoding RNA, suppresses transcription of p53-target genes through inhibition of p53 expression

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Background: MALAT1 (Metastasis Associated Lung Adenocarcinoma Transcript 1) is a nuclear long noncoding RNA. Previously, we found that MALAT1 promotes cell migration through regulation of migration-related genes, suggesting its importance in cancer development. In addition, we found that MALAT1 was involved in the repression of the expression of many genes, including p53-target genes, such as p21 and FAS. However, it is unknown whether MALAT1 regulates transcription through p53 pathway.

Observations: In this study, we found a novel function for MALAT1 in regulating gene expression through repression of p53 function. Up-regulation of p21 and FAS genes in MALAT1 knockdown cells was inhibited by PFT-alpha, a reversible inhibitor of p53. We also examined the p53 dependency of the expression of p21 and FAS by using p53-null H1299 cells and found that up-regulation of p21 and FAS by MALAT1 knockdown was not observed in p53-null cells. These results suggest that increased expression levels of these genes in MALAT1 knockdown cells

are dependent on p53 function. Further analysis revealed that protein and mRNA levels of p53 were up-regulated in MALAT1 knockdown cells. Because p53 has several alternative promoters, we investigated which promoter is activated in MALAT1 knockdown cells. Massive transcriptional start site (TSS) analysis, which can collect TSS information by combination of the oligo-capping method with a massively parallel sequencing technology, revealed that expression from P1 promoter, which can produce longest form of p53 mRNA, was up-regulated by more than 2-fold in MALAT1 knockdown cells.

Conclusions: Our data suggest that MALAT1 suppresses the expression levels of p53-target genes such as p21 and FAS through inhibition of p53 gene expression.

A 142 Structural basis for the methylation of A1408 in 16S rRNA by a panaminoglycoside resistance methyltransferase NpmA from a clinical isolate and analysis of NpmA interactions with 30S ribosomal subunit

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Background: NpmA, a methyltransferase that confers high level-resistance to aminoglycoside antibiotics was identified in an Escherichia coli clinical isolate. It belongs to the kanamycin–apramycin methyltransferase (Kam) family and specifically methylates the 16S rRNA at the N1 position of A1408. NpmA is plasmid-encoded and can be transferred between pathogenic bacteria; therefore it poses a threat to the successful use of aminoglycosides in clinical practice.

Observations: We determined the structures of apo-NpmA and its complexes with cofacor S-adenosylmethionine and reaction by-product S-adenosylhomocysteine at 2.4, 2.7 and 1.68 Angstrom, respectively. We generated 12 NpmA variants with alanine substitutions of evolutionary conserved amino acids and studied their ability to bind the cofactor, to methylate A1408 in the 30S subunit, and to confer resistance to kanamycin in vivo. Residues D30, W107 and W197 were found to be essential. We have also analyzed the interactions between NpmA and the 30S subunit by footprinting experiments and computational docking. Helices 24, 42 and 44 were found to be the main NpmA-binding site. Both experimental and theoretical analyses suggest that NpmA flips out the target nucleotide A1408 to carry out the methylation.

Conclusions: The results presented here will assist in the development of specific NpmA inhibitors that could restore the potential of aminoglycoside antibiotics.

A 143 Macro ncRNAs are a feature of all imprinted gene regions in the human genome and show tissue and cancer specific regulation

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Background: Recent whole-genome transcriptome studies revealed diverse classes of non-protein-coding (nc)RNAs and raised questions about the complexity and regulation of the genome. We use genomic imprinting as a model system to study the class of macro or long ncRNAs. The mouse Airn and Kcnq1ot1 imprinted macro ncRNAs function as in cis repressors of protein coding genes. In humans, 8 out of 27 imprinted regions express imprinted macro ncRNAs.

Observations: To determine if macro ncRNAs are universal features of all human imprinted gene regions we used a Human Imprinting Region Tilling Array (HIRTA) and RNA-seq technologies. We applied 20 normal and 23 cancer samples to HIRTA, developed a strategy for macro ncRNAs identification and mapped 111 novel transcripts. Bioinformatics approach independently showed non-protein-coding potential for 95% of the novel transcripts. Using ribosomal RNA depleted RNA-seq in fibroblast cells, we detected the known imprinted macro ncRNAs and validated ncRNAs mapped using HIRTA. We showed that novel macro ncRNAs are lowly expressed and tissue specific with up to 40% showing developmental regulation when compared fetal and adult: kidney, liver and brain, and about 10% showing regulation in differentiated human embryonic stem cells. Nuclear localization was found for 5/21 tested macro ncRNAs while 14/21 were both nuclear and cytoplasmic. Differentially methylated regions were characteristics of 2 CpG island promoters of novel macro ncRNAs. Analysis of allelic expression revealed 9 monoallelic or biased macro ncRNAs. Furthermore, 22 novel macro ncRNA transcripts were expressed exclusively in cancer samples.

Conclusions: In summary, all human imprinted gene regions express at least one macro ncRNA that may be imprinted and potentially play a role in gene regulation in normal or disease conditions in human. Cancer specific novel macro ncRNAs may represent a valuable starting point for biomarkers research.

A 144 Impact of telomere instability on biogenesis and expression of microRNAs

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Background: Telomere shortening likely contributes to chromosome instability (CIN) in vivo, a hallmark of cancer cells. Critical short telomeres enter into breakage-fusion-bridge (BFB) cycles and might play a role in the promotion of epithelial cancers through amplifications and losses of cancer genes. However, the contribution of such rearrangements to tumor progression remains uncharacterized.

Observations: We used an in vitro model of cell transformation to study the consequences of telomere dysfunction on the expression of tumor-related phenotypes. Human epithelial kidney cells immortalized at early passages by introduction of telomerase are free of CIN (CIN-), whereas exogenous expression at late passages leads to immortalization of karyotypically abnormal cells (CIN+). While CIN- HEK cells retain both morphology and expression markers typical of epithelial cells, CIN+ HEK cells display a fibroblast-like phenotype and enhance both migration and invasive capacities, suggesting that upon instability HEK cells underwent an epithelial-mesenchymal transition (EMT). Concomitantly, we observed a large-scale change in the miRNA (miR) expression profile that is independent of local rearrangements. Strikingly, changes in expression were more pronounced when levels of premature miR (pre-miR) were measured by RT-qPCR, while they were more discrete when measuring levels of mature miR (microarray). Nevertheless, changes in mature miR had a direct impact on mRNA expression and cell phenotypes.
Conclusions: Exhaustive profiles of pre-miR and miR in CIN- and CIN+ HEK cells were obtained with next generation sequencing. These results will allow us to identify new molecules expressed in CIN+ HEK cells, and to study correlation (maturation) between pre-miR and miR expression levels in these cells.

A 145 Nucleic acids – protein recognition: what we have learned from aptamers

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Background: Selection of aptamers from nuclei acid combinatorial libraries provides examples for spatial RNA or DNA structural arrangements recognizing by proteins. RNA-protein recognition example has taken from bacterial ribosome, and reveals RNA structure variations recognized by S7 – key protein of ribosomal small subunit assembly. DNA-protein recognition example belongs to G-quadruplex thrombin binding aptamer, and also reveals variations of DNA structures recognized by the single protein.
Observations: First example describes interactions of S7 with 16S rRNA and str mRNA. The methods were applied: comparative phylogenetic analysis, computer modeling of RNA and protein, UV-induced cross-linking, deletion analysis, and SELEX variations (SERF, SERW). Putative RNA recognition motif has been emerged. Thermodynamics were measured to evaluate enthalpy/entropy. Second example describes DNA G-quadruplex aptamers for human thrombin. Several G-quadruplexes were already developed by SELEX, and no direct structure based drug design was made yet. PDB G-quadruplexes were classified into several groups. For computer molecular dynamics simulations, force field parmbsc0 was adapted for GROMACS. The dynamics was studied with very long trajectories: total duration was tens of microseconds, the biggest known. Novel modular aptamer, RA-36, has been designed. Comparative studies with two known aptamers, 15-TBA and extended 31-TBA, were made. New thrombin activity assays, and calculation of kinetic parameters were developed. 15-TBA inhibits thrombin at high concentrations; 1 – 10 mkM, RA-36 and 31-TBA are effective at lower concentrations; 5 – 20 nM. The type of inhibiting is also different.
Conclusions: RNA motif for protein S7 recognition was found, explaining mechanism of feed-back translational regulation of bacterial str operon, and ribosomal biogenesis. Further improvements of molecular dynamics simulations of nucleic acids allowed tracing behavior of every nucleotide for DNA aptamers.

A 146 Elucidating molecular mechanisms underlying the formation of P bodies and stress granules

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Background: Upon environmental and nutrient stress mRNAs accumulate in cytoplasmic RNP granules, referred to as P bodies and stress granules. In yeast, RNP granule assembly is at least partially dependent on ‘prion-like’ aggregation domains. Prion-like domains are highly enriched in RNA-binding proteins and during RNP assembly they can functionally replace each other. The aim of this study was to investigate whether prion-like structures are involved in RNP granule formation.
Observations: Using yeast strains expressing fluorescent markers for P bodies and yeast prions, we followed P body formation and prion aggregation by live-cell imaging. We found that the dynamic properties of P bodies are unlike those of prion aggregates. For example, P body formation strongly increased upon stress, whereas fluorescent prion foci showed no changes with temperature increase. Treatment with low concentrations of aliphatic alcohols, which disrupt hydrophobic interactions, dispersed fluorescent P body foci. Prion aggregates on the other hand were unaffected by the same treatment. Using an in vitro aggregation assay we further found that the multimeric structures formed by P body proteins dissolved into their monomeric components in the presence of ionic detergents. In contrast, prion polymers are resistant to ionic detergents.
Conclusions: Our study demonstrates that P body assembly does not involve stable structures such as amyloids. The dynamic nature of P body and stress granule assembly rather suggests that RNP formation proceeds through a network of multivalent interactions between multidomain proteins and RNAs.

A 147 Serine/arginine-rich splicing factor 3 (SRSF3) regulates G1/S checkpoint and p53-dependent apoptosis

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Background: Serine/arginine-rich splicing factor 3 (SRSF3), a member of the SRSF family, regulates not only alternative

splicing, but also upstream events in transcription and downstream events, including translation and mRNA decay. SRSF3 exerts these wide-ranging roles in concentration-dependent manner. SRSFs must be maintained at a proper level, and its overexpression is suggested to facilitate tumor growth. Here we report potential roles of SRSF3 in G1/S transition and p53-dpendent apoptosis.

Observations: When HCT116 cells were treated with SRSF3 siRNA and subjected to FACS and microarray analyses, silencing of SRSF3 caused G1/S cell cycle arrest and apoptosis in association with changes in expression of the 833 gens by 2-fold. Pathway analysis with IPA ranked 'G1/S Checkpoint Regulation' as the top-scored pathway modified. qPCR and Western blotting showed decreased mRNA and protein levels of cyclin D1, cyclin D3, E2F1, and E2F7. Consequently, Rb was dephosphorylated in SRSF3-silenced cells. RNA immunoprecipitation followed by microarray analysis identified a number of SRSF3-interacting mRNAs including G1/S checkpoint regulater mRNAs (ccnd1, ccnd3, e2f1, and e2f7). High-throughput analysis of SRSF3-dependent alternative splicing and validation by RT-PCR demonstrated that SRSF3-scilencing specifically caused skipping of the hipk2 exon 8 that encodes a part of binding site to E3 ubiquitin ligase Siah1 (Δhipk2). Eventually, overexpressed ΔHIPK2 was resistant to proteasome digestion, and ΔHIPK2 alone was enough to phosphorylate p53 Ser15, 20, and 46 and to facilitate apoptosis. We confirmed that silencing of SRSF3-mediated and ΔHIPK2-dependent apoptosis did not occur in p53-null cells.

Conclusions: Silencing of SRSF3 may broadly down-regulate transcription of G1/S checkpoint regulators probably through suppressing RNA-elongation and facilitate skipping of the hipk2 exon 8 to produce a stable isoform. The present study uncovers potential roles of SRSF3 in regulation of cell cycle and apoptosis.

A 148 Regulation of monocyte pro-inflammatory activities through multiple targeting of TGF-beta signalling pathway components by the glucocorticoid-dependent microRNA 511

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Background: GC are pro-resolving systemic mediators with a key role in the control of the inflammatory response and in restoring tissue structure and homeostasis. miR are a new class of negative regulators, recently found to be also involved in the regulation of the inflammatory response and in establishment of a 'tolerant' phenotype in monocytes. However, whether GC regulate miR expression and what role eventually this event might have on monocyte biology during an inflammatory response is still unknown.

Observations: Here we report the induction at late time points in monocytes exposed to GC of miR-511, encoded by a pri-miR present in intron 5 of the mrc1 gene. When putative miR-511 targets, predicted by TargetScan, were functionally allocated to distinct signalling pathways using the IPA software, a total of 25 molecules involved in the TGF-beta signalling pathway, including receptors, adaptors, and transcription factors, were predicted to be inhibited by miR-511. This suggested a role for miR-511 in the GC-dependent negative regulation of the TGF-beta pro-inflammatory effect on monocytes, which includes induction of pro-inflammatory genes and down-modulation of IL-10 production, and, at the same time, its potential involvement in a negative feedback loop, as TGFbeta enhances monocyte response to GC. To put these hypothesis into a test, the monocytic cell line THP1 was constitutively transduced with lentiviral vectors over-expressing a scramble sequence (mock-THP1) or miR-511 (511-THP1). When exposed to TGF-beta, 511-THP1 showed a marked impairment in SMADs and MAP kinases activation, expression of TGF-beta target genes, and in other TGFbeta-dependent biological assays as compared to mock-THP1.

Conclusions: These results candidate miR-511 as a multi-targeting intracellular effector of the GC anti-inflammatory activity in monocytes exposed to TGF-beta.

A 149 The effect of inverted SINEs on gene expression

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Background: Alu elements are conserved and 300 nucleotide long sequence repeats. They belong to the SINE family of retrotransposons and are found abundantly in primate genomes. Alu elements are frequently found enriched in gene-rich regions, generally within noncoding segments of transcripts, such as introns and untranslated regions. About 70% of Alus have an inverted partner within 5 kilobases allowing the formation of extended double stranded structures.

Observations: To test whether such inverted Alu elements have an impact on gene expression, a number of reporter Renilla-luciferase constructs containing one, two, or three Alu elements, and an artificial double-stranded 3' UTR were created. These constructs were transiently transfected into different cell line such as primary mouse embryonic fibroblasts (MEFs). Our study shows that inverted Alu elements in 3' UTRs can dramatically suppress gene expression, in transiently transfected cells. For investigating the molecular mechanism of gene suppression by Alu elements, we tested different possibilities. By using knock out cell-lines we showed that A-to-I editing does not contribute to this phenomenon. Similarly, PKR and Dicer1 are not involved in this phenomenon. Our results also show that gene suppression by inverted Alu elements is independent of the primary sequence. In addition we compared mRNA levels for reporter genes with single and inverted Alu elements. The results show that inverted Alu mRNA levels are not reduced in comparison with single Alu containing mRNAs.

Conclusions: Inverted Alu elements in the 3' UTR of reporter genes dramatically reduce gene expression. This gene suppression is independent of ADAR mediated RNA-editing and most probably independent of the RNAi pathway.

A 150 Characterization of a novel antisense RNA overlapping the human p16/p14/p15 tumor suppressor gene cluster

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Background: Tumor suppressor genes (TSG) that limit cell proliferation and induce cellular senescence are frequently genetically mutated or epigenetically silenced in cancer. Macro or long non-coding (nc) RNAs have been shown to exert gene regulatory functions by attracting DNA methylation or active/repressive histone modifications to gene promoters. We hypothesize that similar to imprinted genes tumor suppressor genes could be epigenetically silenced in cis by overlapping antisense macro ncRNAs.
Observations: NimbleGen custom tiling arrays were hybridized with ds-cDNA from normal and cancer tissues to investigate macro ncRNAs in human imprinted and cancer gene regions. The p16/p14/p15 TSG cluster was found to be almost completely covered by transcription in some cancer cell lines but not in healthy control tissues. 5'RACE identified a promoter upstream of p16/CDKN2A. This antisense transcript provisionally termed p16p15AS was found to have a very short half-life and to be nuclear retained. Its length is still unknown but associations with the neighbouring genes MTAP or the recently discovered ANRIL, an antisense macro ncRNA silencing p15/CDKN2B, are being investigated. Analysis of cancer patient samples will show if p16p15AS expression correlates with p16/p14/p15 gene regulation. Additionally, we are using RNA-seq to investigate whether a general mechanism upregulates more macro ncRNAs in p16p15AS positive cells or if a specific mechanism induces only p16p15AS expression.
Conclusions: A detailed investigation of characteristics and function of p16p15AS could help to better understand the role of macro ncRNAs as genome-wide gene regulators. A macro ncRNA as a regulatory element for the TSGs p16/p14/p15 would provide an important therapeutic target or biomarker.

A 151 Mouse TDRD12 is an integral component of piwi interacting RNA (piRNA) silencing machinery in male germline

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Background: PIWI proteins and associated piRNAs provide a strong defence mechanism against transposons at the trancriptional and post-transcriptional level in mouse. Several other proteins have been implicated in the piRNA pathway, among which the helicases and tudor domain containing proteins are the most prevalent. TDRD12 protein is unique in the way that it contains both the tudor domains and the helicase domain. However, TDRD12 was never shown to be part of the piRNA silencing complex.
Observations: In this work, we demonstrate that TDRD12 is a part of the mouse piRNA silencing complex. Our immunoprecipitation (IP) experiment with anti-TDRD12 antibody using mouse adult testis lysate shows that, PIWI proteins (MILI and MIWI) and piRNA pathway components such as MVH, PL10, and TDRD1 associates with TDRD12. We also found that TDRD12 IP contains piRNAs which match in size with MILI and MIWI associated piRNAs further supporting that TDRD12 is part of piRNA silencing complex. Interestingly TDRD12 interaction with MILI, MIWI, MVH, and PL10 is RNA dependent, however, it's interaction with TDRD1 is RNA independent suggesting that it is perhaps directly interacting with TDRD1 protein. Further analysis of TDRD12 protein suggests that tudor domains are not involved in the binding with symmetrical dimethyl arginine (sDMA), a post-translational modification found in PIWI proteins that serves as a docking site for tudor domain containing proteins. This observation is further supported by the fact that TDRD12 tudor domains lack key aromatic residues which are responsible for sDMA binding and the interaction between TDRD12 and PIWI protein is sensitive to RNase treatment.
Conclusions: Taken together our results clearly suggest that TDRD12 is an integral part of the piRNA pathway in mouse, however the exact function of TDRD12 is not clear yet. We further explore the TDRD12 function in piRNA biogenesis using mouse and cell culture systems during mouse male germline development.

A 152 Nuclear import and substrate recognition by double-stranded RNA binding domains

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Background: A-to-I RNA editing by ADAR influences the stability, structure and localization of RNA molecules. hADAR1 is a nucleo-cytoplasmic shuttling protein containing three dsRBDs. The third dsRBD of ADAR1 is the first domain of this type for which NLS activity has been proven. This project aims at identifying the topological features of ADAR1-dsRBD3, influencing its NLS activity by determining its atomic structure using NMR spectroscopy in the presence and absence of dsRNA substrates.
Observations: Binding of dsRNA to ADAR1 interferes with nuclear transport of ADAR1 via Trn-1 which specifically recognizes the ADAR1-dsRBD3 in a RAN-GTP dependent manner. Mutagenesis results could not identify the specific amino acids responsible for the NLS activity but instead these residues span the entire dsRBD3. Furthermore, binding of dsRNA to the dsRBD3 impairs the interaction of ADAR1 with Trn-1 thereby preventing nuclear import. However, how the binding of RNA to ADAR1-dsRBD3 prevents binding of Trn-1 is not clear. This project will demonstrate the molecular basis of recognition of RNA substrates by ADAR1-dsRBD3 and their interference in nuclear import mediated by Trn-1. NMR spectroscopy will be used to identify how this dsRBD folds differently from other dsRBDs. The structural changes being induced in dsRBD3 upon binding of the dsRNA and whether RNA binding competes with Trn-1 will be determined by the chemical shift perturbations of the interacting surfaces of the dsRBD with either dsRNA or Trn-1. Depending on the NMR data, the specific amino acids of dsRBD3 will be tested by in-vitro mutagenesis by mimicking the change of residues in non NLS bearing dsRBDs.

Conclusions: By recognizing the necessary interaction surfaces crucial for nuclear import we will be able to predict other dsRBDs that act as NLSs. Possibly the nuclear import of many important regulators of RNA-metabolism such as Drosha, NF90, or TRBP found in the nucleus, is also regulated by RNA-binding.

A 153 Enoxacin is a cancer-specific growth inhibitor that enhances microRNA processing

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Background: MicroRNAs are small RNA molecules that regulate gene expression at the post-transcriptional level. The disruption of miRNAs and their processing machineries also contributes to the development of human tumors. A common scenario in carcinogenesis is emerging that shows that impaired miRNA production and/or downregulation occurs in many cancers. Despite the impact of miRNAs on cancer biology, miRNA-based cancer therapy is still in its early stages and mostly limited to target a single miRNA.
Observations: However, since most tumors show a global downregulation of miRNA expression relative to their normal counterparts and that several of these lost miRNAs have tumor-suppressor features, restoration of normal miRNA levels might represent an attractive approach in cancer therapy. Herein, we show that the small molecule enoxacin, a fluoroquinolone used as an antibacterial compound, enhances the production of miRNAs with tumor suppressor functions by binding to the miRNA biosynthesis protein TRBP. Our data indicate that enoxacin specifically inhibits the growth of a broad spectrum of cancer cells both in vitro and in vivo by enhancing the miRNA-processing machinery, particularly at the TRBP-mediated stage. The use of enoxacin in human cell cultures and xenografted, orthotopic and metastatic mouse models reveals a TRBP-dependent and cancer-specific growth-inhibitory effect of the drug. These results highlight the key role of disrupted miRNA expression patterns in tumorigenesis, and suggest a new strategy for restoring the distorted microRNAome of cancer cells to a more physiological setting.
Conclusions: Enoxacin has several advantages for manipulating miRNAs, as they are more easily delivered in human and experimental animal models, are more stable and cost less to manufacture. These results represent a new step toward the application of miRNA-based therapy in the treatment of human cancer.

A 154 Imprinted gene regulation by non-overlapping non-protein coding RNAs – a new Visceral Endoderm model

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Background: Genomic imprinting is an epigenetic process, which results in monoallelic, parental-specific gene expression. In many imprinted clusters, some genes show imprinted expression only in extra-embryonic tissues. Imprinted expression of all genes in a cluster in all tissues is controlled by the same regulatory non-coding (nc) RNA. However, extra-embryonic lineage (EXEL) specific and multiple lineages (ML) imprinted expression has been proposed to depend on different downstream mechanisms.
Observations: A model system to study EXEL imprinted expression is required that is suitable for genetic manipulations of the imprinted locus and is not contaminated with embryonic or maternal tissues. The majority of previous studies used placenta as a model of EXEL imprinted expression, but we show that it is contaminated by maternal tissue and that this can exaggerate maternal allele bias. To develop an alternative model, we characterize imprinted expression of known placental-specific imprinted genes in the visceral yolk sac (VYS) the middle extra-embryonic membrane in mice. The VYS is a bilaminar membrane derived entirely from the embryo and consists of a visceral endoderm (VE) layer (primitive endoderm origin), and a visceral mesoderm (VM) layer containing blood islands (epiblast origin). We developed a more efficient method to enzymatically separate the two layers and show that EXEL imprinted expression is limited to the VE layer. Genes known to show EXEL imprinted expression in placenta also exhibit it in VE. Lastly we report our attempts to develop an in vitro system for studying EXEL imprinted expression by differentiating ES cells to VE in several culture conditions.
Conclusions: We identify VYS as an improved model to study EXEL imprinted expression and show that imprinted expression localises to the VE layer. We present a new method to efficiently isolate the VYS layers enabling isolation of a pure population of cells showing EXEL imprinted expression.

A 155 Toward development of a platform to analyze RNA modifications in the translational response of microbial pathogens to chemical mediators of inflammation

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Background: The goal of our studies is to define the role of translational control in the stress response of pathogenic bacteria to exposure to reactive nitrogen and oxygen species arising at sites of inflammation in humans. The strong association between bacterial infections and cancer is illustrated with H. pylori and gastric cancer, with one mechanistic link involving activation of macrophages and neutrophils leading to generation of reactive oxygen and nitrogen species that cause mutagenic and cytotoxic damage to host cells.

Observations: Using LC-MS/MS technology, we discovered that the ~25 non-canonical RNA modifications present in tRNA in every organism undergo reprogramming in the cellular stress response, which leads to selective translational of stress response proteins. These observations have motivated efforts to define the role of RNA modifications in other non-coding RNA species ranging from 22 nt microRNA to large ribosomal RNA species over 2000 nt. In order to analyze the functions nucleoside modifications in these RNA species, there is a need to isolate them in purified form. To this end, we developed a multidimensional HPLC system to purify all major forms of non-coding RNA in eukaryotic and prokaryotic cells. As proof of principle, we successfully separated all major species of RNA using tandem online size exclusion chromatography coupled with reverse phase ion-pairing chromatography in a human lymphoblastic leukemia cell line and Escherichia coli. Each RNA species was then quantified using a multi-well format, size-weighted RiboGreen fluorescence assay. The purified RNA species were then subjected to LC-MS/MS analysis of the spectrum of RNA modifications.

Conclusions: In conclusion, we have developed a platform of RNA purification and LC-MS/MS analysis of RNA modifications in an effort to define the role of the modifications in microbial responses to exposure to chemical mediators of inflammation. This approach will provide insights into the mechanisms by which microbial pathogens evade the human innate immune system.

A 156 The poly (CAAU) region of CUP1 5'-UTR appears to be a novel translation regulatory element from yeast Saccharomyces cerevisiae

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Background: Untranslated leader regions of mRNA influence various steps of protein biosynthesis in eukaryotic cells. The role of 5'-UTR in regulation at post-transcriptional level is especially interesting. The regulation can be implemented by binding of regulatory proteins to the specific elements in mRNA upstream initiation codon. Here we describe a novel regulatory element from untranslated leader region (5'-UTR) of yeast Saccharomyces cerevisiae CUP1 mRNA.

Observations: For this research we have created the set of plasmids that bear reporter gene under control of CUP1 or GPD promoter with additional sequence of CUP1 5'-UTR. Transcription rates were estimated using reverse transcription real time PCR. We have shown that the additional CUP1 5'-UTR between regular promoter and the reporter gene cause the decrease in the level of mRNA. For each case we have estimated the level of reporter protein production. We have found that the additional CUP1 5'-UTR increases the production of the protein comparatively to the basic level. As for GPD promoter we have found that the addition of this particular sequence was enough to turn the reporter gene translation into CUP1 – like way. All potential secondary structures 5'-UTR are not stable and characterized by the formation of single-stranded loops incorporating tandem repeats (CAAU) and highly A – enriched. We have proposed that poly(CAAU) region of CUP1 5'-UTR is responsible for the translation enhancement via interaction with regulatory proteins. It has been confirmed by gel-shift experiments.

Conclusions: The poly (CAAU) region of CUP1 5'-UTR appears to be translation enhancer which regulates production of CUP1 protein in yeast S. cerevisiae. It contributes to our understanding of gene expression regulation at the post-transcriptional level.

A 157 Spatial and temporal restriction of long 3´ UTR extensions during Drosophila embryonic development

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Background: Messenger RNA 3' end formation has emerged as a highly regulated process that impacts transcript stability, localization and translatability.

Observations: Here we report that a subset of RNA processing genes (including ago1 and brat) exhibit a synchronous lengthening of their 3' termini during embryogenesis, resulting in 3' UTRs that are up to 20-fold longer than those found in typical Drosophila mRNAs. These large 3' extensions appear at 4-6 hours after fertilization, and several genes acquire additional phased extensions ~8 hours later during development. We show that the 3' UTR extensions are selectively expressed in neuronal tissues and contain recognition sequences for the translational control protein Pumilio, which also exhibits the 3' lengthening phenomenon documented in this study.

Conclusions: These findings suggest a previously unknown mode of post-transcriptional control in the Drosophila nervous system.

A 158 GPR120 mediates a anti-inflammatory and anti-apoptotic response from Alfa linolenic (w3) and Oleic (w9) Fatty Acids in the Hypothalamus of Diet-induced Obese Rodents

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Background: In obesity, the hypothalamic function is disrupted by local inflammation and apoptosis of neurons involved in the control of feeding and thermogenesis, mediated by saturated fatty acids. Omega 3 and 9 can revert it, but the mechanism remains unknown.We evaluate the properties of these fatty acids (FA) as protective factors against diet-induced inflammation and apoptosis of the hypothalamus and the specific mechanism of action.

Observations: Obese and diabetic male Wistar rats and Swiss mice receive w3 or w9 or BSA via icv infusion into 3rd ventricule of hypothalamus for 5d, or by diet for 2mo. Feeding behavior was measured. Molecular, histological and biochemical parameters were analyzed. The pro- and anti-inflammatory proteins TNFa, JNK, IL6, IL10; the insulin and leptin pathways proteins IR, IRS1/2, AKT, FOXO, JAK, and STAT; pro- and anti-apoptotic proteins BAX, Bcl-2, proteins involved on unsaturated fatty acids mechanism of action GPR120, TAK, TAB, b-arrestin, and neuropeptides controllers of feeding POMC, CART, NPY e AgRP were evaluated by immunoblot and RT-PCR. For the immunehistochemical analysis, the antibody F4/80 was utilized to detect macrophages and the omega pathway proteins to test the mechanism hypothesis. Both animals treated with w3 or w9 by icv or oral had improvements on food intake and blood glucose profile. They reduced the inflammatory and apoptotic status, improved the neuron sensitivity to insulin and leptin, and increased the expression of POMC. GPR120 activated by FA interrupted inflammation by deubiquitinated TAK1 and TAB1/2, involved on this cascade by b-arrestin activity.

Conclusions: A possible new mechanism of action from these FA in the control of food intake could be evaluated: The hypothalamic cell survival was increased by w3 and w9, and all the effects together resulted in the control of the hyperphagic state, reducing the body weight and glycemic levels.

A 159 Variability and expression of human NAD salvage enzymes

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Background: Nicotinamide adenine dinucleotide (NAD) is a cofactor in redox reactions and a substrate for NAD-consuming enzymes that rely on specific biosynthetic pathways to maintain cellular NAD levels. NAD can be synthesized de novo or salvaged from different precursors. Nicotinamide phosphoribosyltransferases (NAMPT) and nicotinic acid phosphoribosyltransferases (NAPRT) are key enzymes in salvage pathways and are attractive targets for the development of novel anticancer therapies.

Observations: We have characterized the genetic variability and expression pattern of human NAMPT and NAPRT1. Sequencing of these genes has uncovered several novel single nucleotide polymorphisms including two missense mutations located in conserved regions of NAPRT1. The structural impact of the mutations at the enzyme level is shown using the yeast Npt1p as a model. In addition, we have observed that both genes are expressed in all tissues tested and the presence of potential new transcripts is also shown.

Conclusions: Mutations or altered expression of NAMPT and NAPRT1 are associated with disease, thus the functional relevance of the mutations and transcripts found is being addressed. Due to their importance in NAD biosynthesis characterizing these enzymes will help to understand their role in health and disease.

A 160 Sucrose metabolism and its role in the development of the cyst nematode Heterodera schachtii

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Background: The obligate plant-parasitic cyst nematode Heterodera schachtii induces highly specific feeding cell system in plant roots called syncytium. From this feeding structure it obtaines all needed nutrients and thus energy. Among different metabolites, sucrose is the major transport sugar in the phloem of Arabidopsis, and therefore expected to be the major sugar imported into syncytia.

Observations: In order to make fructose and glucose available, sucrose needs to be degraded by a sucrose synthase (SUS) or an invertase (INV). To elucidate whether these sucrose-breakdown enzymes are involved in this process and play a role in the maintenance of the nematode feeding structure, we analyzed the development of H. schachtii in different sus and inv Arabidopsis T-DNA lines. Three double and one quadruple mutant of sus and one double mutant of cinv were bulked, and checked for the T-DNA insertion.These lines and the wild type Col-0 were grown under sterile conditions, J2 juveniles of H. schachtii were inoculated on the plants. Further, after two weeks the number of females and males was counted, additional parameter such as female size and number of eggs per cyst were also estimated. From all tested lines two of them showed a significant increase in the number of female nematodes per root centimeter. The total infection rate was increased by a half in all assayed lines, while parameter like number of eggs per cyst and female size were only significantly higher in cinv1/2.

Conclusions: In this work we found that most of the mutants show an increase in the infection rate. Further the neutral invertase mutant additionally shows a increase in the female size and egg production. These results suggest that the lack of cleavage of sucrose is beneficial for the nematode development.

A 161 Histone H1 controls variant surface glycoprotein silencing in African trypanosomes

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Background: African trypanosomes (Trypanosoma brucei) escape the immune system by expressing at their cell surface a single type of variant surface glycoprotein (VSG). Although there are hundreds of VSG genes, only one is active at a time. Silent VSGs display a more condensed chromatin than the active VSG. In many eukaryotes and in T. brucei, histone H1 is involved in chromatin condensation in vitro. In this work we studied the role of histone H1 in VSG expression.
Observations: First we characterized the histone H1 gene family in T. brucei 427 strain by PCR and sequencing. We identified the three histone H1 variants that were described in the T. brucei 927 strain genome database. Each variant has a unique 5'UTR and N-terminal encoding domain. Using qRT-PCR, we observed that variants are transcribed at different levels, suggesting they may have different functions. Using histone H1-tagged cell lines we observed that one of the variants localizes mainly at the nuclear periphery, whereas the other histone variants are more evenly distributed in the nucleus. Downregulation of all histone variants by RNA interference showed that histone H1 is required for normal growth. Importantly, using a luciferase reporter strain, we detected that loss of histone H1 leads to derepression of a silent VSG (4- to 10-fold), indicating that histone H1 plays a role in VSG transcriptional control in T. brucei. We will discuss the contribution of histone H1 to maintain the different chromatin conformations at active/silent VSGs and its role in VSG gene switching.
Conclusions: We showed that histone H1 is necessary to maintain VSGs silenced in Trypanosoma brucei. Different expression levels and nuclear distribution of the three histone H1 variants suggests that variants are not functionally redundant.

A 162 Unexpected subcellular localization of mammalian Pc2/CBX4 protein

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Background: Polycomb proteins are a set of conserved key regulators of transcription in metazoan organisms. The Polycomb 2 protein (Pc2/CBX4 protein), a component of the polycomb repressive complex 1 (PRC1), is one of five mammalian paralogs of Pc protein in Drosophila. Moreover, a Pc2 protein has, contrary of the other CBX proteins, SUMO E3 ligase activity. In mammalian cells, a Pc2 protein co-localizes with other members of PRC1 complex and is situated into nuclear domains called PcG bodies.
Observations: Aim of our study was to determine the subnuclear localization of polycomb proteins at light and electron microscopy level and their relationship to various nuclear components. In contrast to expected nuclear localization of polycomb proteins we found out that a Pc2 protein is also situated in the cytoplasm. In mouse liver as well as in murine and human cell lines, the Pc2 protein has punctate patern in the cytoplasm close to the nucleus. Using the specific organelle markers, a Pc2 protein was localized into peroxisomes. The peroxisomal localization of the Pc2 protein was confirmed also by electron microscopy approach accompayned with high-pressure freezing, cryosubstitution and post-embedding immunogold labeling. This approach, due to its better resolution, allowed us to localize the Pc2 protein into the peroxisomal matrix rather than its membrane or crystalloid core.
Conclusions: All to date known functions of polycomb proteins take place in the cell nucleus. However, our results on peroxisomal localization of the Pc2 protein propose that PcG proteins could function even outside the nucleus. The possible function of the Pc2 in the cytoplasm is a subject of our future study.

A 163 Tightly coupled hierarchical transcription cascades generate anti-phasic expression of circadian clock genes in Neurospora

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Background: Circadian clocks control rhythmic expression of 5-20% of eukaryotic genes. Genes controlled by the White Collar Complex (WCC), the core transcription factor of the circadian clock of Neurospora, peak in the morning. Mechanisms and components generating rhythmic gene expression with different phases are not known.
Observations: Previously, we have identified transcription repressor csp1 as a direct target of the WCC. Expression of CSP1 protein and RNA are rhythmic and peak during the morning. Biochemical analysis of CSP1 revealed that newly synthesized CSP1 exists in a transient complex with the corepressors RCM1/RCO1 (orthologues of SSN6/TUP1 of S.cerevisiae) that promotes phosphorylation of the N-terminus of CSP-1. Hyperphosphorylated CSP1 is rapidly degraded via the ubiquitin ligase UBR1 and its ubiquitin conjugase RAD6. The exchange of phosphorylated residues in the N-terminus of CSP1 by alanine results in loss of interaction with UBR1 and stabilizes CSP1. Interaction of the phosphorylation site mutant with the RCM1/RCO1 is enhanced suggesting a critical role of phosphorylation in the function and stability of CSP1. RNA-seq and ChIP-seq experiments showed that CSP1 represses hundreds of genes in Neurospora. Luciferase reporter constructs revealed that genes controlled by CSP1 are rhythmically expressed and peak in the evening, i.e. in anti-phase to morning-specific genes directly controlled by WCC. The phosphorylation site mutant of CSP1 does not support rhythmic expression of the luciferase reporter.
Conclusions: CSP1 as a short-lived morning-specific transcription repressor that is rhythmically regulated by the WCC. CSP1 controls rhythmic expression of second-tier genes with an evening phase. Phosphorylation and rapid turnover of CSP1

ensure coupling of CSP1 abundance and function to the activity of WCC.

A 164 Understanding the crosstalk between ubiquitination of H2B and methylation of H3-lys79 on the nucleosome core

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Background: Dot1 methylates lysine 79 of histone H3 on the nucleosome core. It has a unique fold among histone lysine methyltransferases and mediates mono-, di- and trimethylation in an uncommon distributive manner. Dot1 is conserved from yeast to humans and hDot1L is involved in a subset of MLL-rearranged leukemias. One of the regulatory mechanisms of Dot1 is its stimulation by ubiquitination on histone H2B. However, the underlying mechanism of this trans-histone crosstalk is still under debate.
Observations: Using data obtained by quantitative mass spectrometry and yeast genetics we have developed a mathematical model for in vivo H3K79 methylation in yeast and determined the rate constants for the different methylation events. Using this model we investigated the regulation of K79 methylation by ubiquitination of the C-terminus of H2B by the E2/E3 enzymes Rad6/Bre1. The specific loss of H3K79me3 observed in cells lacking Bre1 fits the model only when all three rate constants are reduced. Thus, a lack of histone H2B ubiquitination slows all three methylation steps, not just trimethylation, and mimics a wild-type cell with a lower Dot1 expression level. This idea was confirmed by genetic epistasis analysis. Two opposing models have been proposed for the mechanism of this crosstalk: ubiquitin could open up the chromatin to increase accessibility (wedge model) or it could increase the activity of Dot1 (bridge model). To address this problem, we are generating yeast strains expressing conditional histone-ubiquitin fusions to test if these fusions can take over the role of endogenous H2Bub. This system will be used to study the plasticity of the crosstalk between H2Bub and H3K79 methylation.
Conclusions: We have elucidated the kinetics of H3K79 methylation by Dot1 in vivo and found that H2B ubiquitination acts as an enhancer of overall Dot1 activity. We are developing tools to study the underlying mechanism of this trans-histone crosstalk and test the two opposing models that currently exist.

A 165 Molecular Recognition of DNA: A tool for epigenetic DNA transcription regulation

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Background: Pyrrole-Imidazole (PI) polyamide molecule was originally designed from structures of natural DNA binding molecule, such as Distamicine and Diocarbamicine and has been discovered as a synthetic molecule which recognizes the minor groove of Watson-Click base pair of double-stranded DNA in a sequence-dependent manner. In order to regulate histone acetylation in specific genomic region, we tested conjugates between this synthetic DNA binding molecule and Histone Deacetylase (HDAC) inhibitor.
Observations: We have synthesized a library of sequence-specific PI polyamide conjugates attached with the potent HDAC inhibitor Suberoylanilide hydroxamic acid (SAHA), termed SAHA-PIP and screened their effect on the expression of tumor suppressor genes and Yamanaka factors. Chromatin immunoprecipitation analysis revealed SAHA-PIP inducible acetylation of Histone H4 and H3 in the promoter region of p16, Oct-4 and Nanog. SAHA-PIP showed significant induction against c-Myc, Oct-3/4, Sox-2, and Klf-4, respectively in MEF but did not show significant induction of tumor suppressor p16. We also confirmed only SAHA did not show such induction, which implicated the role of PI polyamide conjugates in the induction of histone acetylation in specific genomic regions. Furthermore, a significant change in MEF morphology was demonstrated with our hit SAHA-PIPs.
Conclusions: Those data suggests their possible use to regulate histone modification in a genomic sequence specific manner and promote agents for reprogramming efficiency in order to generate iPSCs, although the endogenous tumor suppressor gene expression was not observed.

A 166 Regulation of stem cell maintenance in the Drosophila ovary via the chromatin-remodelling factor ISWI

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Background: In Drosophila ovary, the ATP-dependent chromatin-remodeling protein ISWI plays a crucial role to maintain germline stem cells (GSCs), but how ISWI interfaces with the genetic circuitry controlling GSC self-renewal remains unknown.
Observations: To investigate the activities of ISWI in controlling stem cell self-renewal and to carry out a detailed genetic analysis we screened mutantions in some group of genes for their ability to genetically modify ISWI GSC defects, using FLP-mediated recombination approaches Our preliminary study has showed that mutations in genes playing a role in cell cycle regulation enhance self renewal defects observed in ISWI mutant GSCs resulting ovarian tumor phenotype, while mutations in other ISWI interactors restored those defects.
Conclusions: Our preliminary work indicate that the mechanism by which ISWI plays a role in the control of self-renewal and proliferation can be influenced by a variety of nuclear factors that modulate ISWI activity.

A 167 Mechanism of Retinoic acid-induced transcription: epigenetic changes, DNA oxidation and formation of chromatin loops

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Background: Retinoic acid (RA), the active derivative of vitamin A, plays a pivotal role in regulation of development, omeostasis and differentiation of adult tissues. The biological activity of RA is mediated by its binding to retinoic acid receptors (RARa, RARb and RARg) that function as hetero-dimers with retinoid X receptors Following hormone binding, the receptor induces covalent modifications at the N-terminal tails of nucleosomal histones and assembles on chromatin an active transcription complex.
Observations: Despite extensive studies on RA-induced transcription, it is not known if there is a common set of histone modifications, essential for RA-induced transcription. We find that upon activation of RA receptor by the hormone, on the RARE-promoter chromatin of caspase 9 and cyp26a genes, both histone H3 lysine 9 and lysine 4 undergo a rapid and transient demethylation, catalyzed by lysine specific demethylase, LSD1 and JMJ-domain containing demethylase, D2A. The action of these 2 demethylases is essential for the assembly of the transcription initiation complex. The combined actions of the FAD oxidase (LSD1) and Fe + + dioxygenase (JMJD2A) set off an oxidation wave, that modifies the DNA locally (dG oxidation and single strand nicks) and recruits the enzymes (APE1-OGG1-UDG) involved in base excision repair (BER). The recruitment of these enzymes on the RARE and promoter triggers the formation of specific chromatin loop(s) juxtaposing the RARE with the 5' transcription start site and the 3' end of the caspase 9 and cyp26a genes. The receptor bound on the RARE governs the 5' and 3' end selection of the sites, directing the productive transcription cycle of RNA polymerase II.
Conclusions: The transient demethylation of histone H3, the DNA bases modifications and the formation of specific chromatin loops of the transcribed genes are causally linked, because inhibition of demethylation by a dominant negative LSD1 mutant prevents the looping of chromatin and compromises transcription.

A 168 Impact of genomic inversions on co-expressed gene clusters

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Background: Chromosomal inversions have been found in prokaryotes as well as eukaryotes and its rate varies among different lineages. We aim to study the phenotypic and transcriptional effects of inversions within blocks of co-expressed genes, such as the DAL cluster. The species belonging to the S. cerevisiae 'sensu stricto' group compared to the S. castellii present different orientation of the genes within this cluster and I aim to understand whether this difference have an evolutionary adaptive origin.
Observations: We constructed six inverted and non-inverted strains using the cre-loxP system. Among them were the strains with single gene inversion, triple gene inversion and a strain mimicking the inversions present in S.castellii. We then analyzed the fitness of wild type, inverted and non-inverted strains in allantoin containing media that is responsible for triggering the expression of the DAL genes. Interestingly it was observed that wild type S.castellii grows much slower then S.cerevisiae on allantoin containing media. The inverted strains in fitness assays showed a decline in growth rate as compared to the non-inverted and wild type strains. The expression studies of the DAL cluster in different strains carrying different inversions showed a reduction of the expression of the DAL4 gene. We also showed that DAL4 anti-sense RNA (SUT614) is much highly expressed in both inverted and non-inverted strains as compared to the mRNA in allantoin containing media. Apart from studying the affects of gene inversion we also studied the affect of small sequence insertions in DAL metabolic cluster.
Conclusions: The DAL2 expression remains unchanged however DAL4 is down-regulated which can explain the reduced fitness of the strains since the DAL4 gene is involved in the allantoin uptake. The DAL genes are interlinked therefore inversion of one gene changes the expression of neighbouring genes as well.

A 169 Fine structure of the 'PcG body' in human U-2 OS cells established by correlative light-electron microscopy

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Background: Polycomb group (PcG) proteins are a set of conserved, essential regulatory factors that repress the transcription of their target genes. At the fluorescence level, the proteins of the Polycomb repressive complex 1 (PRC1) are detected in two forms – diffuse one and as intensely fluorescent foci termed PcG bodies. A PcG body is believed to be a nuclear body and is situated into the interchromatin compartment. However, an ultrastructural description of the PcG body has not been reported to date.
Observations: To establish the ultrastructure of PcG bodies in human U-2 OS cells stably expressing recombinant polycomb BMI1-GFP protein, we used correlative light electron microscopy (CLEM) implemented with high-pressure freezing, cryosubstitution and on-section labeling of BMI1 protein with immunogold. CLEM approach allowed us to clearly identify fluorescent PcG bodies, not as distinct nuclear bodies, but as nuclear domains enriched in separated heterochromatin fascicles. Importantly, high-pressure freezing and cryosubstitution allowed for a high and clear-cut imunogold BMI1 labeling of heterochromatin structures throughout the nucleus. The density of immunogold labeled BMI1 in the heterochromatin fascicles corresponding to fluorescent 'PcG bodies' did not differ from the density of labeling of heterochromatin fascicles outside of the 'PcG bodies.' Further, we focused on compaction aspect of the chromatin accumulated into PcG foci and their behaviour in hyperosmotic environment. Our preliminary data show that with increasing hypertonic load

and incubation time the PcG foci reduce their size gradually up to total disappearing and this process is reversible.
Conclusions: We were able to identify the 'PcG body' with the locally higher spatial accumulation of heterochromatin structures not bearing the characteristics of a nuclear body. Now, we focus our effort on the compaction properties of the chromatin concentrated into the PcG foci.

A 170 Relation of factors influencing genomic uracil appearance and Drosophila development

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Background: Drosophila lacks a significant member of uracil-DNA glycosylases and statio-temporally down regulates dUTPase expression in larval tissues that undergo programmed cell death during metamorphosis. We examined whether this phenomenon is enough for uracil-DNA emergence, having role in development.
Observations: Immunostaining and RT-qPCR showed that dUTPase protein is present only in proliferating tissues in embryo and imaginal discs of larvae. We proved that Drosophila-related Replication Element (DRE) found in the dUTPase promoter is responsible for this expression pattern. We also confirmed that Drosophila cells tolerate and interpret uracil-rich DNA, supposedly due to the lack of detectable uracil-DNA glycosylase activity. By a newly developed qPCR based method we detected increased level of uracil in the Drosophila larval genome, except for imaginal tissues. dUTPase silencing increases uracil content of the imaginal progenitor genome of larvae as well. However, silencing resulted in developmental block and lethality in early pupa.
Conclusions: We confirmed that uracil-DNA appears in Drosophila life cycle and correlates with programmed cell death during development. Results suggest an unknown factor that does not tolerate increased genomic uracil in dUTPase silenced pupa.

A 171 OTUB1 sequesters UBC13 and inhibits DNA-damage dependent ubiquitination

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Background: DNA double-strand breaks (DSBs) are a potent threat to genome integrity. DSBs elicit a signaling cascade that modifies the chromatin surrounding DSBs first by ATM-dependent phosphorylation and then by regulatory ubiquitination. The early steps of DSB-induced ubiquitination are catalyzed by the RNF8 and RNF168 E3 ubiquitin ligases, and the E2 ubiquitin conjugating enzyme, UBC13 (UBE2N).
Observations: Here we report that OTUB1, a deubiquitinating enzyme, is a negative regulator of DSB-induced chromatin ubiquitination. We made the surprising observation that OTUB1 suppresses RNF168-dependent poly-ubiquitination independently of its catalytic activity. OTUB1 is able to do so by directly binding to UBC13, the cognate E2 enzyme for RNF168. OTUB1 inhibits the ability of UBC13 to catalyze the attachment of ubiquitin to another ubiquitin moiety but it does not interfere with the formation of the ubiquitin-UBC13 thioester intermediate or with the discharge of ubiquitin from UBC13. Chromatin ubiquitination in response to DSBs is therefore kept in check by OTUB1 via a non-canonical mode of action by a deubiquitinating enzyme.
Conclusions: OTUB1 inhibits UBC13-dependent DNA damage response signaling in its catalytic activity-independent manner.

A 172 Cell Cycle-Specific Repair of UV-Induced DNA Damage in Human Melanoma

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Background: A multitude of investigations over the years has clearly shown that nucleotide excision repair (NER) represents a critical front-line defence against both melanoma and non-melanoma skin cancers by removing highly-genotoxic solar UV-induced photoproducts from DNA. Nonetheless, a recent authoritative study has revealed that a majority of cultured human melanoma strains in fact exhibit normal capacity to repair UV-damaged DNA.
Observations: Our group previously developed and utilized a flow cytometry-based NER assay to demonstrate that functional ataxia telangiectasia and rad3-related (ATR) kinase is required for removal of UV-induced DNA photoproducts during S phase, but completely dispensable for this repair process during either G0/G1 or G2/M. We now report that among 17 human melanoma strains evaluated using this assay, 14 exhibited profoundly reduced repair capacity exclusively during S, whereas only 3 manifested normal repair during all phases. Moreover defective S phase-specific NER in melanoma strains correlated significantly with decreased levels of phosphorylated histone H2AX.
Conclusions: Our overall data indicate that abrogation of ATR signaling underlies defective cell cycle-specific regulation of NER in cultured human melanoma strains. This finding potentially harbours critical implications for our understanding of melanoma development and treament.

A 173 Regulation of type I interferon gene expression by the DEAD box helicase DDX3X

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Background: Sensing of a pathogens initiates signalling events that lead to the expression of type I interferons (IFN-I). Signals emanating from many pathogen recognition receptors merge in the activation of TANK binding kinase 1 (TBK1). TBK1 subsequently phosphorylates critical serines in the regulatory domains of the interferon regulatory factors 3 and 7 (IRF3, IRF7), which are transcriptional activators of IFN-I genes.

Observations: We previously described the RNA helicase DDX3X as a TBK1 substrate and an important player in TBK1 dependent IFN-I production. Studies in mouse embryonic fibroblasts lacking DDX3X underscore the importance of DDX3X in pathogen recognition, showing that DDX3X and TBK1 act synergistically to stimulate IFN-I production. This synergism depends on TBK1-mediated phosphorylation of DDX3X. We identified two specific phospho-sites as functionally relevant for TBK1-dependent signalling but not for signalling by the closely related kinase IKKi. Surprisingly, cotransfection of DDX3X and a constitutive active form of IRF3 or IRF7 in TBK1/IKKi double-deficient fibroblasts revealed that DDX3X has an additional, kinase- independent role in the regulation of IFN-I synthesis. Using fibroblasts lacking IRF3 and IRF7 we found that IRF7 is superior in transporting the stimulatory effect of DDX3X on IFN-I gene expression.
Conclusions: Taken together we identified DDX3X as an important player for the regulation of IFN-I genes after pathogen recognition. IFN-I gene stimulation by DDX3X occurs both dependent and independent from phosphorylation by TBK1 and is prominently transported to the IFN-I promoters by IRF7.

A 174 Impaired IFNalpha and IL-2 signaling in PBL of breast cancer patients

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Background: Although pSTATs in tumors have oncogenic potential, STAT dysregulation has not been extensively investigated in peripheral blood lymphocytes (PBL) of breast cancer patients. Alterations in JAK-STAT pathway in cancer patients contribute to immunosuppression and inadequate antitumor immune response.
Observations: In this study level and activation of pSTAT1, 3 and 5 were investigated in PBL of breast cancer patients and controls after in vitro treatment with IFNalpha and IL-2 by Western blotting. PBL of patients and controls were also analyzed for percentage NK cell and NK cell activity by using Flow cytometry and 51Cr-release assay, respectively. Our results indicate that untreated PBL of breast cancer patients, compared to controls, express lower level of pSTAT1, 3 and 5. IFNalpha induce pSTAT1, similar to induction of pSTAT3 and pSTAT5 by IL-2, in controls and in patients, although the effect in patients is lower. PBL and CD3-CD16 + NK cells of patients compared to controls have also significantly lower baseline level of IFNgamma. IL-2 significantly increases the production of IFNgamma in both PBL and CD3-CD16 + NK cells in breast cancer patients and in controls, while IFNalpha does not significantly increase the production of IFNgamma in patients. We found that NK cell activity of patients is significantly lower compared to controls, suggesting association of NK cell activity and STAT activation in these patients.
Conclusions: The obtained findings indicate the possibility to augment STAT signaling in PBL of breast cancer patients by Th1 cytokines, that could by improving anti-tumor response show clinical benefit in these patients.

A 175 In vivo enzymology: parallel characterisation of dUTPase mutants by quantitative structural biology and phenotype analysis in Mycobacterium smegmatis

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Background: Thymidine biosynthesis is an essential metabolic pathway, as dTTP is one of the nucleotide building blocks of DNA. To fulfill the task of dTTP synthesis, three major pathways exist in humans, while in mycobacteria only one of these is present, which involves the action of the dUTPase enzyme (dut). dUTP is constantly produced through the pyrimidine biosynthesis and can be incorporated into DNA. To prevent DNA uracilation and subsequent cell death, dUTPase is required to eliminate excess dUTP.
Observations: All mycobacterial genomes encode dUTPases (dut) with over 85% sequence identity and a mycobacterium-specific surface loop absent in the human dUTPase. Using Mycobacterium smegmatis as a fast growing model organism for Mycobacterium tuberculosis, we demonstrated that dUTPase knock-out results in lethality. This mutant lethal phenotype can be reverted by complementation with the wild-type dUTPase. These findings enabled us to investigate the in vitro already well-known reaction mechanism of dUTPases in the living cell. We analyzed four enzyme mutants with different, in vitro characterized enzyme activity in M. smegmatis. Importantly, the mutant dUTPase gene lacking the genus-specific loop was unable to complement the knock-out phenotype, although deletion of the loop-motif had no major effect on dUTPase enzymatic properties in vitro. In additon, mutant dUTPase constructs that have drastically lower in vitro enzyme activity than the loop-deleted one were able to complement the lethal phenotype. We also investigated the viability of these mutant dut encoding strains by monitoring their growth and treating them with different DNA-damaging agents or general antibacterial drugs.
Conclusions: Our results provide formal genetic proof that dut essentiality in M. smegmatis is at least partially transmitted through the mycobacterium-specific motif. Therefore we propose that targeting this genus-specific site will potentially yield an efficient and specific antimycobacterial treatment.

A 176 Imaging SpoIIIE proteins in live sporulating Bacillus subtilis bacteria by PALM microscopy

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Background: Under adverse conditions, B. Subtilis produces endospores to enhance its survival. The process of sporulation starts by the formation of an asymmetrically located division septum that leads to the formation of two unequally sized

compartments: the mother-cell and the forespore. The septum traps about 30% of the chromosome to be transferred into the forespore. The remaining (3Mbp) are then translocated by a mechanism involving the formation of SpoIIIE DNA translocase complexes at the septum.

Observations: The mechanisms of translocation, particularly the control of the directionality, still remains unclear and various models have been proposed so far. Each of these models predicts very different distributions of SpoIIIE proteins at the sporulation septum. We used a home-made PALM microscopy setup (Photo-activated Localization Microscopy) to investigate proteins localization in live-sporulating bacteria deposited on agar pads. By using this technique, single SpoIIIE-EosFP fusion proteins can be localized within single bacterium with a precision as low as 30 nm. Our results show that the two SpoIIIE complexes responsible for independently translocating the two chromosomal arms form a single and stable complex at the septal membrane, with a characteristic size below the resolution of our microscope (< 30 nm). We also observed that this SpoIIIE complex is preferentially found on the mother cell side of the septum, the complex/septum distance being 40nm in average. This is the first time that such asymmetric localization of SpoIIIE proteins has been observed directly.

Conclusions: These observations are inconsistent with models suggesting that SpoIIIE is required on either side of the septum to form a DNA conducting pore. However, they support a model by which SpoIIIE assembly is mediated by specific interactions between proteins and small chromosomal sequences called SRS.

A 177 Cloning and purification of a putative repressor protein from Arthrobacter nicotinovorans pAO1

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Background: The pAO1 megaplasmid of A. nicotinovorans consists of 165 ORF's related mainly to nicotine degradation, uptake and utilization of carbohydrates, amino acids and sarcosine. The putative sugar catabolic pathway consists of 11 ORF's organised as a single operon and coding for an ABC-type sugar-transport system and several putative oxidoreductases and dehydrogenases. The current work is focused on orf32, a putative PdhR related protein, most probably involved in the control of the whole operon.

Observations: Directional gene cloning using degenerated primers as well as protein expression and protein purification by IMAC were performed according to standard methods. Gel permeation chromatography (GPC) was performed on a 16/20 Sephadex 200 pg (Amersham Biosciences) column attached to Pharmacia LKB FPLC system. The column was first calibrated using bluedextran, aldolase (158 kDa), ovalbumine (47 kDa), and chimiotripsinogen (25 kDa). The aprox. 700 kb orf32 gene was cloned in the pH6EX3 plasmid vector and the gene product purified to homogenity as a 29 kDa His-tagged recombinant protein. As indicated by GPC, it consists of a monomeric protein with a native molecular weight of 32 kDa. The specific UV/Vis spectra showed only a single peak at 280 nm common for all proteins and did not indicated the presence of any colored cofactors. This is in good agreement with the fact that PdhR-family proteins contain a winged helix-turn-helix (wHTH) domain responsible for DNA binding, and not a Zn-finger or any other metal containing domains.

Conclusions: The orf32 gene of Arthrobacter nicotinovorans pAO1 encodes a monomeric 32 kDa protein containing no metal ions. Due to its position and orientation, it is most probable the repressor protein for the whole putative carbohydrate catabolic operon.

A 178 Distribution of the Human Papilloma Virus types in patients with high grade dysplastic lesions of the cervix

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Background: Evaluation of the Human Papilloma Virus (HPV) type's distribution in patients with dysplastic lesions of the cervix.

Observations: 40 patients with persistent HPV cervicitis and high grade dysplastic lesion of the epithelium were divided into three groups according to the grade of the dysplastic lesion of the cervical epithelium: group A(n = 14) with mediocre dysplasia, group B (n = 21) with grade dysplasia and group C (n = 5) with Ca in situ. In all study groups, The HPV type's distribution was estimated. Methods: We used assay based on the reverse hybridization principle for the identification of 28 different genotypes of the HPV by detection of specific sequences in the L1 region of the HPV genome. The assay uses SPF10 primer set for amplification of HPV genotypes and a set of primers for the amplification of the human HLA-DPB1 gene to monitor sample quality and extraction Results: In group A predominated HPV 16 (4/14; 28.6% and HPV 31 (n = 4/14; 28.6%); in group B also predominated HPV 16 (13/21; 61.9%) and HPV 31 (3/21; 14.3%); in group C again predominated HPV 16 (4/5; 80.0%).

Conclusions: HPV 16 was the most predominant type in patients with high grade dysplastic cervical lesions and its presence was increasing as the lesion was going higher, from 28.6% in mediocre dysplasia to the 61.9% in gravis lesion and 80% in Ca in situ.

A 179 Novel signaling pathway governing the biofilm formation in Bacillus subtilis

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Background: Spore-forming bacterium Bacillus subtilis forms complex communities called biofilms in response to signals that remain poorly defined. Biofilm formation depends on the synthesis of an extracellular matrix that holds the constituent cells together. The matrix production is indirectly regulated by the transcriptional regulator Spo0A. The level of phosphorylat-

ed Spo0A is controlled by a network of kinases that respond to different environmental and physiological signals that we aim to elucidate.
Observations: We found that a combination of glycerol and manganese (GM) strongly promotes biofilm formation, as well as biofilm associated sporulation and pigment production in B. subtilis. Furthermore, we demonstrate the significant effect of GM in other bacteria too, such as Bacillus licheniformis and Bacillus cereus. We further show that the transcription of the major operons responsible for the matrix production in B. subtilis was enhanced strikingly as a response to GM. We also found that the major kinase for sensing the GM effect is a histidine kinase KinD. The phenotype of ΔkinD appears to be similar to that of phosphorelay mutants: Δspo0F, Δspo0B or Δspo0A, indicating that the signal is sensed by KinD and transduced via the phosphorelay to Spo0A. The ΔkinD phenotype was suppressed by ΔabrB mutation, confirming that AbrB is downstream of KinD and phosphorelay in the signaling pathway. We further show that the kinase KinD harbors unique CACHE domain as a sensor for the C3/C4 small molecules, and that the kinase KinD directly senses glycerol or its derivative by this extracellular domain.
Conclusions: We discovered a novel signaling pathway for matrix production and biofilm formation in B. subtilis. We further provide evidence indicating that this signaling pathway is highly conserved in Bacillus.

A 180 Investigating cardiac miRNA regulatory networks for novel insight into molecular mechanisms of cardiotoxicity

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Background: MicroRNAs (miRNAs) are small non-coding regulatory RNAs found in all multicellular organisms. Since their discovery in 2001, there has been impressive progress in miRNA research, and a great deal is now known about the biosynthesis of miRNAs and their regulatory function in messenger RNA degradation and translation.
Observations: The potential roles of microRNAs in cellular responses to xenobiotic stress, the development of pathophysiological changes and other toxicological phenomenon such as susceptibility/resistance to xenobiotics are currently the focus of active research. In particular, it has recently been demonstrated that miRNAs play a fundamental role in cardiac differentiation and heart development. The profiling of miRNAs in the context of drug safety assessment can add value by providing 1) novel insight into molecular mechanisms of toxicity and 2) novel safety biomarkers, especially when measured in body fluids such as plasma.
Conclusions: In the present work, we have used an integrated approach for the identification of miRNA target genes using a combination of miRNA and mRNA profiles to highlight transcriptional regulatory networks underlying cardiotoxicity signals.

A 181 RCD-8: a new function for 'mRNA murderer'

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Background: Processing bodiies are the cellular structures, which have critical roles in the mRNA degradation and post-transcriptional gene silencing. One of the scaffold proteins of this structure is RCD-8. Depletion of RCD-8 from cells by siRNA-mediated knock-out prevents DCP1a and DCP2 from accumulating in P-bodies and affects the mRNA degradation. Previously we have shown that RCD-8 is a novel binding partner of CoA Synthase,which is responsible for CoA synthesis in cells.
Observations: The formation of the CoASy/RCD8 complex was shown to be induced by stimulation of cells with growth factors. We found that some cellular stresses strongly reduce the interaction between CoASy and RCD-8. Subcellular fractionation and immunoblot analysis revealed the localization of RCD-8 on mitochondria, but after stress treatment its relocalise to the cytoplasm. We propose that CoASy serves as a repository of RCD-8 on mitochondria and therefore might be involved in regulating mRNA life-span. We found that RCD-8 interacts with CoASy via the C-terminal domain, which is responsible for the localization of this protein to PB. Also we were interested whether the interaction CoASy/RCD-8 could affect enzymatic activity of CoASy. Unexpectedly we have observed a strong inhibition of CoASy activity by RCD-8. Moreover, the inhibitory effect of RCD8 showed a concentration-dependent profile. No inhibition of CoASy activity was detected in the presence of RCD-8 mutant, lacking the C-terminal domain which mediates the interaction with CoASy. The inhibitory effect of RCD8 on CoASy was also observed on His-dPCoAK domain.
Conclusions: We showed that RCD-8 strongly affects the activity of CoASy in vitro, which can represent a novel possible mechanism of regulation of CoA biosynthesis in mammalian cells.

A 182 When bacteria get the flu and sharpen their knives: a novel RNA maturation pathway to activate the CRISPR immune system

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Background: In bacteria and archaea, CRISPR (clustered, regularly interspaced short palindromic repeats)/Cas (CRISPR-associated proteins) constitutes an adaptive RNA-based defence system against invading phages or plasmids wherein spacer/repeat-derived short CRISPR RNAs (crRNAs) silence the foreign nucleic acids in a sequence-specific manner. The maturation of

crRNAs represents a key event in CRISPR activation, however details about the mechanisms involved are lacking.

Observations: We have analyzed the CRISPR/Cas system II in our model organism Streptococcus pyogenes. Differential sequencing RNA sequencing of S. pyogenes led to the discovery of tracrRNA, expressed as trans-encoded small RNA species that display 24 nucleotide perfect complementarity to the CRISPR repeats. We show that tracrRNA directs the processing of CRISPR pre-crRNA into crRNAs by the activities of the widely conserved endogenous RNase III and the CRISPR-associated protein, Csn1. All these components are essential to protect S. pyogenes against prophage-derived DNA. Furthermore, we show evidence that tracrRNA and pre-crRNA of the CRISPR/Cas system II from other bacterial species (Listeria innocua, Neisseria meningitidis, Streptococcus mutans and Streptococcus thermophilus) are expressed and co-processed. Thus, the type II CRISPR/Cas system including tracrRNA seems to be constitutively activated to target and affect the maintenance of invader genomes to protect bacteria against cell death (lytic phages, e.g. S. thermophilus). Importantly, the system would also be a bottleneck in the acquisition of new virulence genes by horizontal gene transfer (lysogenic phages, S. pyogenes).

Conclusions: Our study reveals a novel pathway of small guide RNA maturation and the first example of a host factor (RNase III) required for bacterial RNA-mediated immunity against invaders. Our work further highlights the remarkable diversity and complexity of the RNA-guided mechanisms of the CRISPR/Cas system.

A 183 Nonsense mediated RNA decay deficiency triggers autoimmune-like response in Arabidopsis thaliana

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Background: Nonsense mediated RNA decay (NMD) is an evolutionarily conserved RNA quality control mechanism that eliminates transcripts containing nonsense mutations. NMD has also been shown to affect expression of a number of genes, and inactivation of this pathway is lethal in higher eukaryotes. However, despite relatively detailed knowledge of the molecular basis of NMD, our understanding of its physiological functions is still limited and the underlying causes of lethality are unknown.

Observations: In this study we examined the importance of NMD in plants by analyzing an allelic series of Arabidopsis thaliana mutants deficient in the core NMD components SMG7, UPF1, and UPF3. We found that impaired NMD elicits a pathogen defense response which is proportional to the extent of NMD deficiency. We also demonstrate that developmental aberrations and lethality of the strong smg7 and upf1 alleles are caused by constitutive pathogen response upregulation. Disruption of pathogen signaling suppresses the lethality of the null upf1-3 allele and growth defects associated with SMG7 dysfunction. Interestingly, infertility and abortive meiosis observed in smg7 mutants is not coupled with impaired NMD suggesting a broader role of SMG7 in cellular metabolism. Finally, we show that NMD efficiency decreases upon plant infection with the bacteria Pseudomonas syringae leading to stabilization of alternatively spliced transcripts.

Conclusions: Our results uncover that the primary physiological consequence of NMD deficiency in plants is an upregulation of the pathogen response. We further demonstrate that NMD efficiency responds to bacterial infections. We therefore suggest that NMD plays a regulatory role in the plant immune response.

A 184 Premature stop codon-containing tra2beta4 mRNA isoform regulates cellular senescence

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Background: The transformer 2beta (tra2beta) gene contains an ultraconserved element having premature stop codons (PTCs) in exon 2 and generate five mRNA isoforms (tra2beta1-5). Functional Tra2beta protein, a crucial regulator of alternative splicing, is encoded by tra2beta1 isoform lacking the exon 2. The tra2beta4 isoform harboring exon 2 is decomposed through nonsense-mediated mRNA decay (NMD), while this PTC variant is specifically expressed under oxidative stress. We report here a novel function of tra2beta4 mRNA isoform.

Observations: Using siRNAs selectively targeting tra2beta1 or tra2beta4 mRNA isoform, we found that reduction of Tra2beta protein induced apoptosis, whereas tra2beta4 siRNA targeting exon 2 doubled cellular size and triggered cellular senescence in a human colon cancer cell line (HCT116), which were assessed by appearance of beta-galactosidase activity and by induction of senescence markers, such as p21, p27, and dephosphorylated Rb. Compared with early-passage human diploid fibroblast (WI-38), senescent WI-38 cells decreased tra2beta4 isoform expression in parallel with a increase in p21 levels. We investigated whether tra2beta4 mRNA was involved in the induction of p21. Silensing of tra2beta4 mRNA did not change p21 mRNA stability and translation. We also found that treatment with tra2beta4 siRNA increased the promoter activity of the p21 gene without changing p53 expression, and that -163 bp of the p21 5'-flanking region was crucial for this regulation. Finally, using RNA FISH, we confirmed that tra2beta4 mRNA was retained in the nucleus and escaped from the NMD surveillance. In situ hybridization with an exon 2-specific probe revealed that tra2beta4 mRNA was specifically overexpressed in human colon cancer tissues.

Conclusions: We show that silencing of PTC-containing tra2beta4 mRNA isoform facilitates p53-independent cellular senescence through activating transcription of the p21 gene. Our results suggest that the novel function of tra2beta4 mRNA isoform may be associated with malignant transformation of colon cancer cells.

A 185 Permutations of sRNA sections for in silico RNA-RNA interaction study and chaperone function prediction

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Background: sRNA-mRNA interaction is modulated in numerous ways by Hfq, a hexameric sRNA-binding protein. DsrA sRNA is thought to bind to the leader region of the RpoS stress-sigma factor mRNA in order to regulate its translation. However, when run through interaction characterization software RNAup, the MFE site does not match that found in vitro. Hfq's involvement as a chaperone is not well understood, but here we investigate the possibility that it is a structure modulator.
Observations: The use of thermodynamic parameters is supported through two experiments: 1) Experiments from Majdalani 1998 are performed in silico. A correlation with R^2 = .72 is found between ΔGsm- ΔGm1m2 (energy of binding of sRNA DsrA to the mRNA rpoS, minus accessibility of mRNA site due to secondary structure) and the amount of expressed rpoS; 2) 24 experimentally shown Hfq-dependent pairs are analyzed in 'RNAup' to compare binding sites on the mRNA found in literature to the theoretical values, returning an average sensitivity of 0.89 and an average PPV of 0.89. Outlier DsrA/rpoS had sensitivity 0.50 and PPV 0.65. Base pairs on the 5' end of Hairpin I of DsrA are studied permutationally. When the hairpin is destabilized, RNAup returns the exact DsrA/rpoS pairing region shown experimentally. This implies that in order for DsrA to bind to rpoS, the first hairpin must be destabilized to increase the site's accessibility. Melting curve analyses are run for DsrA Domain I (containing Hairpin I and adjacent single-stranded regions) and find that increased concentrations of Hfq result in lower melting temperatures. 1microM Hfq lowers Tm from 62˚C to 54˚C, and 1.5 microM Hfq lowers Tm a further 3˚C.
Conclusions: A permutational study offers a deeper understanding of how important regions of sRNA and mRNA are to their interaction with one another. Here, the method finds that Hfq melts hairpin I of DsrA in its rpoS interaction. The method could be used to study other RNA pairs and chaperones.

A 186 Mature to immature microRNAs ratio in cultured rat cardiomyocytes under anoxia-reoxygenation

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Background: The violent evolution of knowledge concerning RNA interference phenomenon has became from its discovery to its application as therapeutics strategy in some fields of medicine which became possible due to obtained information about the changes of microRNAs (miRs) expression in experimental models of diseases. Such progress is also very perspective for cardiology, therefore understanding of involvement of miR into intimate mechanisms underlying pathogenesis of heart disease is of great interest.
Observations: We have evaluated the changes of mature (m) and immature (im) miR-1, -208a, and -29a in rat neonatal cardiomyocytes (CMC) culture under under anoxia-reoxygenation (AR), using Real-Time PCR. The different directions and extents of changes for m and im miRs that are reflected by the m to im miRs ratio have attracted our attention. It was significantly increased upon AR (0.5 hour/1 hour) for miR-1 and -208a, returning to control level at AR group (0.5 hour/24 hours), while for miR-29a this ratio had the progressive tendency to decrease under AR. Coefficient of determination R2 was used to evaluate the degree of dependence of m miR from level of its im forms. For miR-1 this parameter decreased from 59.2% in control to 26.4% at AR (0.5/1) and to 5.1% at AR (0.5/24). For miR-208a it is close to such for miR-1 – 60.6%, and decreases to 0.2% in AR (0.5/1) group but returns to the control level upon AR (0.5/24). The same situation is observed for miR-29a: even though initial R2 for this miR is much lower than for miR-1 and miR-208a (39.8%), it has similar to miR-208a pattern of changes – it decreases to 18.3% at AR (0.5/1) and comes back to control level in AR (0.5/24) group.
Conclusions: Discrepancy between level of m and im miRs is peculiar to CMC under AR, reflecting in changes of m to im miRs ratio and of dependence of m on im miRs level. It supports the idea that posttranscriptional regulatory mechanisms of miRs level may play the role in response of CMC to AR.

A 187 Coupled pre-mRNA and mRNA dynamics unveil operational strategies underlying transcriptional response to stimuli

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Background: Transcriptional responses to extracellular stimuli involve tuning the rates of transcript production and degradation. Yet, no simple method is available allowing for inference of the time-dependent rates of transcript production and degradation.
Observations: Here we show that the time-dependent profiles of these rates can be inferred, on a transcriptome-wide scale. This advance is made possible by analysis of intronic and exonic probe sets from Exon arrays, which allow simultaneous measurements of precursor mRNA (pre-mRNA) and mature mRNA profiles. In three different experimental systems (i.e. an EGF stimulated human mammary epithelial cell line, LPS stimulated primary murine dendritic cells, and in retinoic acid stimulated human embryonic stem cells), we observed that genes with similar mRNA profiles often exhibit marked differences in the amplitude and onset of their production rate. The latter is characterized by a large dynamic range, with a group of genes ex-

hibiting an unexpectedly strong transient production overshoot, thereby accelerating their induction and, when combined with time-dependent degradation, shaping transient responses with precise timing and amplitude.
Conclusions: The method of analysis of pre-mRNA and mRNA dynamics presented herein allows to determine the transcript production and degradation strategies underlying transcriptional response to stimuli.

A 188 Ppargc1a gene expression is repressed in liver of rats treated with high fat diet and a single low dose of Streptozotocin

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Background: Type 2 diabetes mellitus implies deregulation of various metabolic processes, being the maintenance of glycemia one of the most important. Many genes seem to be involved in the deregulation of this particular process. Therefore, the aim of this study, was to evaluate the gene expression of Ahsg and Ppargc1a genes in the liver of rats with hyperglycemia induced by high fat diet along with a low single dose of streptozotocin.
Observations: 210-240 g female rats were feeding with a high fat diet (58% of Kcal, n = 5) or a control diet (n = 5) for three weeks. At day 14, animals fed with high fat diet were injected with a low dose of streptozotocin (35 mg/kg) and the control group rats were injected only with the vehicle. Plasmatic glucose, triglycerides and total cholesterol levels were measured at the beginning, at day 14 and at the end of treatment. Body weight and food consumption were measured each two days. Two weeks after the end of treatment, the rats were appropriately killed and then the liver was surgically removed and frozen in liquid nitrogen. Total RNA from liver was isolated using TRIzol reagent, treated with DNase I, and finally converted to cDNA in a reverse transcription reaction. The gene expression analysis of Ahsg and Ppargc1a genes were performed using SyBR Green – Real time PCR and the comparative Cq method, using three reference genes. The rats fed with high fat diet and treated with streptozotocin showed higher values of plasmatic glucose (11.26 – 15.36 vs. 4.82 – 6.30 mM, $p < 0.01$) and a minor expression of Ppargc1a versus the control group (2-fold less expressed, $p < 0.05$).
Conclusions: The repression of Ppargc1a gene may be an important contributing process in the establishment of chronic hyperglycemia, probably through deregulation of gluconeogenesis. However, further studies need to be performed in order to clarify the role of Ppargc1a deregulation in liver glucose homestasis.

A 189 Expression of ATP Synthase F0 Subunit 6 Gene in Nile Tilapia During Temperature Acclimation

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Background: Physiological mechanisms adjusting cold and heat tolerance have gained interest. Temperature is one of the most important environmental stressor that strongly affects physiological functions in fish. The role of metabolic regulation, skeletal muscle fibers mitochondrial content modifications, concomitant with alterations in oxidative metabolism that occur during the development of muscle fiber and response to environmental temperature stimuli should be studied.
Observations: Different temperature acclimation (22°C and 32°C) affects were examined for 7 days on the mitochondrial properties exemplified by ATP Synthase F-type gene expression in tilapia fish Oreochromis niloticus using the relative transcriptional activity of this gene (RT-PCR). ATP synthase F0 gene has remarkably stable expression in response to elevated temperature; in contrast this gene was up-regulated in case of low temperature exposure compared to ß-actin. ATP synthase F0 was identified and characterized, its cDNA is composed of 1139 bps with a 1079 bps open reading frame. The predicted gene product is 353 amino acid with molecular weight of 39.75 kDa., and Signal peptide probability equals 0.973. ATP synthase F0 subunit 6 shares high identities with the same gene from other fishes and invertebrates.
Conclusions: ATP gene has a stable expression in response to high temperature but up-regulated in low temperature exposure compared to ß-actin. ATP cDNA is composed of 1139 bps, the predicted gene product is 353 amino acid, it shares high identities with the same gene from other fishes and invertebrates.

A 190 Impact of cadmium on the level of hepatic metallothioneins and selected enzymes in experimental rat model

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Background: Cadmium, one of the most toxic heavy metals, is widely used in many industries. Cadmium enters to the human body particularly by ingestion of contaminated food or by passive inhalation of tobacco smoke. In the organism causes formation of reactive oxygen species (ROS). Rats represent the model organisms for assesing the bioavailability of cadmium from contaminated nutrient.
Observations: Rats of strain Long Evans (LE), Spontaneously hypertensive rat (SHR) and Brown Norway (BN) were subjects of the application of cadmium as $CdCl_2$ in drinking water or by injection into peritoneum. In the case ob Brown Norway, we applied also diet with real samples of soil or hay from poluted areas. In plasma, specific enzymatic activity of glutathionreductase was increasing with the amount of applied cadmium for all three strains, whereas more complex dependence has been found for activity of glutathion S-transferase. Liver metallothionein connectrations, determined with adsorptive chronopotentiometry using modified Brdicka reaction, were correlated ($r = 0.9645$) with the liver cadmium content found by ICP-OES.

Direct noncompetitive ELISA for determination of metalothioneins liver and plasma metallothionein was introduced.
Conclusions: Bioavailability and subsequent resorption through gastrointestinal tract is in the case of cadmium so minimal, that it does not increase synthesis of metallothioneins.

A 191 Transcription Factor Target Search Strategy at the Single Molecule Level in Eukaryotic Cells

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Background: Transcription is the mechanism by which information encoded in genes is transmitted to an mRNA template. This process is initiated by the binding of a transcription factor (TF) to a gene promoter. Within a genome of 3.2 billion base pairs, TFs have to be fast and efficient in finding their specific gene promoters (of ~10bp length). Screening or random probing of targets is a highly inefficient process. So how do transcription factors execute their task of target search in a crowded nucleus?
Observations: To address this question, we use a non-native doxycycline-deactivable Tet Repressor (TetR) DNA binding domain integrated into a human cell line (U2OS) as the target site. By transfection with TetR tagged with the photo-convertible DENDRA2, we are able to visualize their target search dynamics in the nuclei of live cells by the sptPALM technique: low UV irradiation causes stochastic conversion of the DENDRA2 from the green to the red state at an event probability low enough to make out single molecules. Observations of the target search of TetR exhibits three different behaviours: (I) trajectories that cross the entire nucleus, screening it predominantly with a fast motion; (II) immobile molecules, and (III) an intermediate behaviour, with fast motion being confined in subparts of the nucleus. When doxycycline is added, disabling DNA binding properties, there is a drastic decrease in the population of immobile molecules. From a target search point of view, this indicates a strategy combining the three-dimensional global and local search with a one-dimensional local screening of the DNA.
Conclusions: TFs seem to have a well established strategy of target search, consisting of combinations of different search strategies in the nucleus at the dimensional level. Observations as such are in agreement with recent theories of nuclear fractal organization.

A 192 Rrn7, an RNA polymerase I transcription factor, is required for RNA polymerase II-dependent transcription directed by core promoters with a HomolD box sequence in Schizossaccharomyces pombe

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Background: The region in promoters, which specifies the transcription machinery, is called core promoter, displaying core promoter elements (CPE) necessary for establishment of a preinitiation complex and the initiation of transcription. The classical CPE is the TATA box. In fission yeast Schizosaccharomyces pombe a new CPE, called HomolD box, was discovered. Collectively, 141 ribosomal protein genes encoding ribosomal proteins and more than 60 other housekeeping genes display a HomolD box in the promoter.
Observations: Here we show that transcription directed by the HomolD box requires the RNAPII machinery including the general transcription factors (GTFs). Most intriguingly, however, we identify by DNA affinity purification Rrn7 as the protein binding to the HomolD box. Recombinant Rrn7 showed specific binding activity as determined by EMSA. Rrn7 is an evolutionary conserved member of the RNAPI machinery involved in transcription initiation of core rDNA promoters. It is part of the core factor (CF), which also contains Rrn6 and Rrn11. Therefore, we investigated, whether Rrn7 independently binds to the Homol D box or, whether the CF complex is needed for binding. With EMSA assays and using a fission yeast strain expressing epitope-tagged TAP-Rrn7 and HA-Rrn11, we found that Rrn7 can bind independently to the HomolD box. Finally, we studied protein interactions performing pull down and immunoprecipitation experiments in order to explain how Rrn7 recruits the complete RNAPII machinery. We discovered that Rrn7 interacts with several GTFs such as Tbp1.
Conclusions: Taken together, our results suggest that Rrn7 is an excellent candidate to be involved in the coordination of rDNA and ribosomal protein gene transcription during ribosome synthesis and, therefore, offer a new perspective to study conservation and evolvability of regulatory networks in eukaryotes.

A 193 A Powerful Ubiquitous Activity of Bombyx mori Heat Shock Protein 70 Promoter

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Background: For stable germline transformation of Bomyx mori, the promoter of the Bombyx cytoplasmic actin 3 (BmA3) was used to choose to drive the EGFP in piggyBac vector. The BmA3-EGFP marker was found to be useful for the screening of G1 transgenic individuals at the larval stage, but not detectable in embryonic stages. We isolated B. mori heat shock protein70 gene (bHsp70) which is showing high transcriptional activity from embryonic stages, and investigated characteristics as constitutive promoter.
Observations: To identify more powerful promoter than previously reported BmA3 promoter, we isolated 9 clones that show stronger signal compared to BmA3 by dot blot hybridization. Among these 9 clones, we focused on one clone which has high amino acid homology (94%) with hsp70 gene of Trichoplusia ni. This clone, named bHsp70 was ubiquitously expressed in all tissues and developmental stage of B. mori, and up-regulated by thermal and ER stress. As result of promoter assay using dual luciferase assay system, we found the highest transcription activity region (-1003/+147) in the 5'-flanking region of bHsp70 gene,

which has about 264 fold more intensive promoter activity than BmA3 promoter. Transcriptional activity of bHsp70 promoter under heat shock condition (42 , 4 hr) was increased over 2 fold than normal condition. The bHsp70 promoter was normally regulated in Bm5, Sf9, and S2 cells. Moreover, the bHsp70-EGFP marker was detected ubiquitously in embryonic, larval, pupal, and adult tissues of transgenic silkworm. Therefore, it is possible to screen transgenic individuals before the G1 larval stage using this promoter, because the bHsp70-EGFP marker was expressed in embryos.

Conclusions: As above results, we suggest that bHsp70 promoter may be used more effectively for the large-scale production of biologically active recombinant proteins and screening of transgenic silkworm.

A 194 High resolution imaging of RNA polymerase II nuclear organization

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Background: RNA polymerase II (Pol II) is the molecular complex responsible for transcribing all messenger RNAs in eukaryotes. A non-homogenous distribution of Pol II in the nucleoplasm and potential organization in dedicated transcriptional organized units had been hypothesized. Nuclear foci of Pol II accumulation have been reported demonstrating the existence of such structures. However a single-molecule level, high-resolution characterization had not been reported previously.

Observations: Here, we have used sub-diffraction localization microscopy (PALM and STORM), and report a super-resolution picture of Pol II in the nucleus of both fixed and live human cells. Nuclear accumulations of Pol II in foci over a wide scale of sizes are observed, and with 3 dimensional imaging in the nucleus we measure Pol II local concentrations that span a vast dynamic range. In light of transcription initiation and the formation of a pre-initiation complex, our results show that Pol II can be a limiting component or far in excess depending on the spatial location of a particular gene.

Conclusions: Our approach is not limited to the case of Pol II and can be expanded to other nuclear proteins and transcription factors. Our results provide a new framework for understanding gene regulation in eukaryotes, which can now take into account local concentrations of the transcriptional machinery.

A 195 A TATA-less promoter that contains HomolD-box directs transcription through DDB1, RECQL and RNAPII transcription machinery

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Background: Most human promoters lack TATA-box and contain other core promoter elements. One of such core promoter element is HomolD-box, which was first described in promoters of ribosomal protein coding genes in the fission yeast Schizossacharomyces pombe. The transcription mechanism of HomolD box-containing promoters is not known. The purpose of this study is to determine the transcription mechanism of HomolD-box in human.

Observations: As model of TATA-less HomolD-box containing promoter we choose the human gene promoter from ATP6V1H, an ATPase subunit. This 58 bp core promoter was cloned in pUC19, fused to a G-less cassette reporter and was used for in vitro transcription assay. The chemically synthesized promoter oligonucleotide was used for EMSA assay. The in vitro transcription assay with this promoter is dependent on HomolD-box since transversion of only two nucleotides abolished transcription. HeLa nuclear extract has specific binding activity to HomolD in EMSA assay. By stepwise purification through different ion exchange columns and affinity chromatography, we purified two proteins from HeLa nuclear extract: DDB1 and RECQL (DNA damage-binding protein 1 and ATP-dependent DNA helicase Q1 respectively). These proteins showed specific binding activity to HomolD-box in EMSA assay and were required for in vitro transcription. Additionally, general transcription factors TFIID, TFIIH, TFIIE, TFIIB and TFIIF and RNA polymerase II were also required.

Conclusions: Collectively, these data suggest that HomolD box-containing TATA-less promoters require the RNAPII machinery and the proteins DDB1 and RECQL.

A 196 Cisplatin action on phospholipids composition of rat liver chromatin

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Background: It is well known that the eukaryotic nuclei are the sphere of lipids active metabolism. Although the nuclear membrane is accepted as the main place of the lipids localization, nearly 10% of nuclear lipids are discovered in chromatin fraction. The ability of chromatin phospolipids to regulate DNA replication and transcription was already demonstrated. It seems also possible that chromatin phospholipids may be participated in realization of cisplatin antitumor effects.

Observations: The 24-hour in vivo effect of cisplatin on rat liver chromatin phospholipids was investigated. The phospholipids of rat liver chromatin were fractionated by microTLC technique. The quantitative estimation of fractionated phospholipids was carried out by computer program FUGIFILM Science Lab. 2001

Image Gauge V 4.0. The alteration of total phospholipids content as well as the quantitative changes among the individual phospholipids fractions in rat liver chromatin after in vivo action of cisplatin was established. The total content of chromatin phospholipids was significantly decreased after the cisplatin action. Four from five individual phospholipids fractions were markedly changed after the drug action. Two cholin-content phospholipids, particularly phosphatidylcholine and sphingomyelin exhibit diversity in sensitivity to this drug: the increase of sphingomyelin content accompanied by quantitative decrease of phosphatidylcholine. The quantity of cardiolipin was markedly increased while the amount of phosphatidylinositol was decreased after the cisplatin treatment. The phosphatidylethanolamin content remained unchanged after the drug action.

Conclusions: It seems that high sensitivity of chromatin phospholipids exhibited to cisplatin action may play an important role in antitumor effects of this drug.

A 197 In fission yeast, Rrn7, a member of the pol-I machinery, binds in vivo to pol-II promoters containing a HomolD box

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Background: Mechanisms of RNA polymerase-II (pol-II) dependent transcription directed by TATA box containing promoters are the most studied. However, over 85% of the promoters do not contain a TATA-box. In S. pombe, pol-II dependent transcription directed by a new element, called HomolD box, has been investigated. Rrn7, a member of pol-I core factor (CF), participates in the HomolD-binding complex, but this has not been corroborated in vivo. It is not known if other CF members participate in this complex.

Observations: In this work we study by an in vivo approach the binding activity of Rrn7 and Rrn11, a CF member, to the HomolD box containing promoter. For this purpose, we immunoprecipitated chromatin using a strain expressing epitope-tagged Rrn7-TAP and Rrn11-HA Immunoprecipitation (IP) of TAP-Rrn7 followed by PCR using specific primers for the HomolD box promoter revealed the association of TAP-Rrn 7 with this promoter region. In contrast, IP of HA-Rrn11 followed by PCR showed that Rrn11 did not associate with the HomolD promoter region. However, when the IPs are followed by PCR using specific primers for a putative HomolD core promoter of an rDNA transcription unit, TAP-Rrn7 as well as HA-Rrn11 coprecipitate the core promoter region of the rDNA unit, thus indicating that Rrn7 and Rrn11 bind to the rDNA core promoter. The results presented here clearly demonstrate that TAP-Rrn7, but not HA-Rrn11, associates specifically with the HomolD box promoter of the ribosomal protein gene, whereas no association of Rrn7 can be detected with the classical TATA-box containing promoter.

Conclusions: In conclusion, this study shows for the first time the association of Rrn7 and a HomolD box core promoter in vivo. And, it demonstrates, that Rrn7 associates with a HomolD core promoter independently of its partner, Rrn11, in the CF complex.

A 198 Intermediates of nucleosome disassembly investigated on a single molecule level

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Background: Nucleosomes are the smallest compaction unit of chromatin in the cell nucleus. They consist of short stretches of DNA wrapped around a core of histone proteins, two (H2A H2B) dimers and one (H3-H4)2 tetramer. The compaction makes most genomic DNA inaccessible to protein binding and nucleosomes must open to allow full DNA access.

Observations: We studied nucleosome dissociation on a single molecule level using fluorescence correlation spectroscopy (FCS) and single molecule Förster resonance energy transfer (spFRET). Here we show evidence that the first step in nucleosome disassembly is the opening of the (H3-H4)2 tetramer/(H2A-H2B) dimer interface. Only after this initial opening the histone subunits are released from the DNA: first the H2A-H2B dimers and, last, the (H3 H4)2 tetramer. We estimate that the previously uncharacterized intermediate state, in which all subunits are bound to the DNA while the interface between the histone subunits is opened, is populated at 0.2 – 6% under physiological conditions.

Conclusions: The existence of the open intermediate state could be significant in vivo for factor-mediated histone exchange, as well as for regulating DNA accessibility to the transcription and replication machinery.

A 199 Hepatitis B virus genotypes in Egyptian patients

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Background: HBV has a circular and partially double stranded DNA genome of 3.2 kb containing four overlapping open reading frames .There are eight genotypes of hepatitis B virus (A-H) and four serotypes ayw, ayr, adw and adr are recognized. Genotyping can be accomplished based on a partial sequence of HBV genome such as the pre-S or S gene. Several methods have been developed and used for HBV genotyping. This study was undertaken to determine the HBV genotypes in Egyptian patients.

Observations: Clinical history and liver function tests were determined to distinguish between acute and chronic HBV infection. Specific PCR to detect four main genes (HBVC, HBVC/PC, HBV pre-S1/pre-S2 and HBV universal region). HBV genotypes were determined in 17 patients who had acute forms of liver disease (AH) and in 23 patients with chronic active hepatitis (CAH). Genotypes were determined using INNO-LiPA methodology which is based on the reversed hybridization principle, Restriction fragment length polymorphism (RFLP) method and Hepatitis B conserved region (980 bp) of 10 patients were sequenced automatically for further confirmation of restriction fragment length polymorphism.This study showed that HBV infections in

patients are attributed predominantly to viral genotypes D and C that constituted (57.5% and 22.7%), respectively of the total infections. In addition, Genotype E and Genotype F constituted (15% and 5%) of the total infections.
Conclusions: These findings show the distribution of HBV C, D, E and F genotypes in Egyptian patients. Furthermore, our results indicate a markedly high prevalence of genotype D and serotype ayw infections in subjects with CAH while the genotypes C and F mostly encountered in AH.

A 200 Physiological response and gene expression of Vibrio parahaemolyticus under multiple stress conditions

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Background: Vibrio parahaemolyticus is a food borne pathogen found in coastal regions. This pathogen leads to the symptoms of diarrhea and abdominal cramps due to uncooked seafood. Previous researches have investigated on the relationship between stresses of environment and how the bacterium overcomes the low temperature or stomach acids before it successfully invades human gastrointestinal. However, most of the studies focused on single stress when they are actually various stresses in the environment.
Observations: Hence, in our present study, we mocked the multiple stresses which Vibrio paraheamolyticus encountered to understand the behaviors difference under the treatment of one or more stress. Previous studies had demonstrated that Salmonella have higher acid resistant ability under acidic(pH4) and cold(4 or 20) stress after acid adaptation at pH5. However, in this study, we found that Vibrio parahaemolyticus have lower acid resistance after acid adaptation which totally opposed to the previous studies. Our results also showed that Vibrio parahaemolyticus has higher acid resistance ability in low temperature but showed no difference on acid resistance after cold adaptation. Thus, we further investigate the underlying reasons of Vibrio parahaemolyticus showing higher acid tolerance under chill environment. We used RT-PCR to observe the RNA expression levels and found that VP0081, rpoS, oxyR, rfaH and VP2446 have differential expressions pattern under multiple stresses. However, rpoS and rfaH mutants of Vibrio parahaemolyticus showed weaker acid resistant under low temperature. We assumed that rpoS and rfaH may play important role of regulatory mechanisms involved in stress responses.
Conclusions: Our results demonstrated that Vibrio parahaemolyticus has higher acid resistance in low temperature and on the other hand, rpoS and rfaH mutants have lower acid resistance in low temperature. RT-PCR showed that VP0081, rpoS, oxyR, rfaH and VP2446 have different expressions under multiple stresses.

A 201 Relationship of TTC1139 and TTC1138 in biofilm formation by Thermus spp.

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Background: Thermus thermophilus HB27 is a gram-negative filamentous eubacteria, and isolated from hot spring in Japan. The optimum temperature is about 60 . It was able to grow as matrix-enclosed multicellular communities called biofilms. Thermus thermophilus HB27 were analyzed by proteomic analysis in order to identify proteins involved in biofilm formation.
Observations: Two-dimensional gel electrophoresis revealed distinct and reproducible different protein expression pattern between biofilm cells and planktonic cells, and one of them is the TTC1138 encoding protein. According to the data base of NCBI, function of TTC1138 is a two component system regulator gene. The archetypal two-component signal transduction systems include a sensor histidine kinase and a response regulator, while TTC1139 that form an operon, encodes a sensor histidine kinase. In order to know whether TTC1139 can phosphorylate TTC1138, we constructed TTC1139 over expression strain and purify TTC1139 by the Ni2+ affinity chromatography. Observe the TTC1138 expression and phosphorylation assay. On the other hand, to identify the TTC1138 regulated genes, we constructed a TTC1138 mutant strain, and TTC1138 over expression strain. The biofilm formation ability will be test by those strains.
Conclusions: We predicted that TTC1139 can phosphorylate TTC1138, and biofilm formation will be regulated by TTC1138.

A 202 Diversity of the genus Micromonospora isolated from the rhizosphere region of Avecennia marina and Rhizophora mucronata of mangrove ecosystem and its functional characterization

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Background: Actinomycetes resources under extreme condition including high and low temperature, high and low pH, high salt concentration have attracted the attention of microbiologist. The Mangroves ecosystem is a unique coastal ecosystem with soils with high salt and low O2 content. Diversity of micromonospora inhabiting this, saline, partially anaerobic environment is useful as it provides clue of the microorganism and their adaptability in such habitats.
Observations: The strains belonging to the genus Micromonospora were isolated from the rhizosphere region of two different mangrove ecosystem by using different pre treatments (Dry/Dilute, Dry/Dilute/Heat, Dry/Dilute/SDS, Dry/Dilute/Phenol, Dry/ Freeze/Dilute) and by using different media (Soil extract agar, Starch casein agar, Oatmeal agar, Actinomycetes isolation agar, Asparagine agar). According to our isolation protocol Dry/Dilute treatment in soil extract agar and Oatmeal agar were found to be more suitable for the isolation of Micromonospora from the mangrove ecosystem and it was predominantly present in the isolation plates using these treatments.The strains also were screened for the presence of nifH genes encoding Dihydrogenase reductase enzyme. Among the 283 Micromonospora strains screened, 16 were nif H positive, 100 strains were tested for presence of polyketide synthase gene, 43 harbours type I PKS gene and 18 having type II PKS. Antimicrobial activities of these strains against plant pathogen are in process. These isolated

strains also produced extracellular enzymes like chitinase (14 strains), cellulase (47 strains).
Conclusions: From this study it can be concluded that the genus Micromonospora has naturally inhabited this ecosystem and plays a major role in plant growth promotion by fixing the atmospheric nitrogen in the form ammonia to balance the nitrogen level in this nitrogen limiting ecosystem.

A 203 Molecular Epidermiology of Rotavirus patients with Acute Gastroenteritis in Northern Gyeonggi-Do

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Background: Rotavirus is the major cause of severe diarrhea among infants and young children. Rotaviruses, belonging to a genus of double-stranded RNA viruses in the family Reoviridae, infect the mature villus epithelial cells of the small intestine, often leading to fever, vomiting, and diarrhea in children.
Observations: This study was carried out to investigate molecular epidermiology of rotaviruses in northern Gyeonggi-Do. During 3 years surveilence (August 2007 through July 2010) for patients with acute gastroenteritis in northern Gyeonggi-Do, 347 out of 5,872 stool samples were selected as rotavirus-positive specimens by means of antigen-capture enzyme-linkes immunosorbent assay(ELISA). The peak seasons of rotavirus infection were winter and spring, from December to May. To survey genotyping of rotavirus was performed the RT-PCR using 137 samples out of 347 rotavirus-positive samples. The predominant genotypes were confirmed as G3P[8](47 samples, 34.3%) followed by G1P[8](31 samples, 22.6%), G2P[4](20 samples, 14.6%), G4P[6] (12 samples, 8.76%), G9P[8](6 samples, 4.38%).
Conclusions: In this study, the distribution and genotype of rotavirus would provide the useful information to establish the preventing strategy of rotavirus and of developing vaccines which will be used in northern Gyeonggi-Do.

A 204 Utilization of glucose as carbon source for production of single cell protein in high salt conditions

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Background: The production of SCP from low cost carbon sources is the most promising breakthrough of biotechnological innovations. This will certainly increase the availability of affordable quality proteins and reduce dependence on animal proteins.
Observations: In this study, the effect of different factors (carbon source concentration, type of phosphorus source, phosphorus source concentration, type of nitrogen source, nitrogen source concentration and temperature) on the single cell protein production form glucose as carbon source by Haloarcula sp. IRU1 was investigated in extreme conditions.
Conclusions: Taguchi experimental design revealed that optimal conditions for single cell protein production are: glucose 8% (w/v), tryptone 0.8% (w/v), NaH2PO4 0.016% (w/v) and temperature 55 C. Taguchi approach resulted in evaluating the main and interaction effects of the factors. The results showed that Haloarcula sp. IRU1 can be used satisfactorily to produce single cell protein in different conditions (high salt and high temperature conditions).

A 205 Effect of Phosphate Availibility on Expression of Selected Rice Protein Kinases During Symbiosis with Piriformospora indica

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Background: Phosphate is an important nutrient which affects the symbiotic interaction between fungus and host roots. The nutritional requirements of plants control the extent of fungal colonization but the molecular mechanisms are yet to be understand. Early phase of molecular crosstalk between symbiotic partners involves the specific plants protein kinases. These protein kinases employed in various molecular and cellular events which may involved in enhanced nutrient acquisition by host plants.
Observations: In present study, we used rice (Oryza sativa var.indica) and fungus Piriformospora indica as a experimental model system for symbiotic partner. We attempted to study the effect of phosphate concentration on some selected rice protein kinases in response to P. indica infestation by transcript analysis. The colonized plant roots which were grown in low and high phosphate condition, collected on different early stage of interaction. The expression level of some selected rice protein kinases (Lectin Protein Kinase and LRK1 Protein Kinase) was performed by Real time PCR. The reduced fungal colonization in host roots were observed in high P condition as compare to low P condition. In gene expression study, we obsereved that the expression level of lectin protein kinase gene was higher at 3 dai as compare to LRK1 protein kinase in low P condition. While expression level of LRK1 protein kinase was higher at 6 dai in high phosphate condition.
Conclusions: The differential expression of selected protein kinases Lectin PK and LRK1 PK indicate that the phosphate availibilty regulates the expression of these protein kinases during early mycorrhizal symbiosis.

A 206 Histone hyperacetylation around the inflammatory gene promoters is associated with E. coli induced early immune response in mastitic mice mammary tissue

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Background: Mastitis is a multietiological complex disease, which is defined as inflammation of parenchyma of mammary glands. Bacterial infection is the predominant cause of mastitis, though fungal, viral and mycoplasma infections also have been reported. Based on severity of the disease mastitis can be classified into- subclinical, clinical and chronic forms. Till date several mastitic pathogens have been studied extensively but the host response to the infection is poorly understood.
Observations: Epidemiological studies have shown that E. coli is the 2nd largest mastitic pathogen after S. aureus. Lactating mice was challenged with a field isolate of E. coli through intramammary inoculation. Histopathological examination of H&E stained sections showed severe infiltration of polymorphonuclear neutrophils (PMNs) and mononuclear inflammatory cells in the alveolar lumen and also in interstitial space and necrosis of alveolar epithelial cells after 24h. Western blot and immunohistochemical analysis of the mice mammary tissues have shown significant hyperacetylation at histone H3 and H4 at selective residues of both mammary epithelial cells and migrated inflammatory cells. Quantitative real-time PCR and genome-wide gene expression profile in the E. coli infected mice mammary tissue revealed differential expression of genes related to inflammation, immunity, antimicrobial peptide expression, acute phase response and oxidative stress response, whereas expression levels of milk protein lactoferrin did not alter significantly. ChIP assay from the paraffinized tissues have shown selective enrichment of hypercetylated histones at the promoters of overexpressed immune genes.
Conclusions: To summarise, E. coli infection in the mice mammary tissue leads to histone hyperacetylation at the immune gene promoters which is pre-requisite for expression of inflammatory genes to mount drastic immune response. Histone hyperacetylation was observed in the clinical mastitis cases, indicating a novel therapeutic target.

A 207 In situ reverse transcription polymerase chain reaction and confocal laser scanning microscopy of Newcastle disease virus (NDV) in 4T1 xenotransplant breast cancer Balb/c mice

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Background: Cancer is a leading cause of death around the world. WHO estimates that 84 million people will die of cancer between 2005 and 2015. Breast cancer is the most frequently diagnosed cancer in women. Intratumoral infusion is currently the most common method for viral gene delivery in cancer treatment. The purpose of the present study is to investigate the dissemination of NDV-AF2240 in liver and brain during intratumoural injection in 4T1 xenotransplant breast cancer BALB/c mice.
Observations: In situ reverse transcriptase polymerase chain reaction (in situ RT-PCR), polyclonal chicken antibody and goat anti-chicken antibody conjugated with fluorescein isothiocynate (FITC) using confocal laser scanning microscopy (CLSM) were carried out to detect the NDV-AF2240 in tumor, liver and brain during intratumural injection in 4T1 xenotransplant breast tumor in female BALB/c mice. A total of 200 female BALB/c mice were divided randomly into 10 cancerous groups consisting 20 mice per group. The mice were initially induced with 104 4T1 cells, NDV-AF2240 and tamoxifen co-culture. Cancerous groups were divided into cancer control (CC), cancer treated with 0.5 microgram/ml tamoxifen citrate (CT), cancer treated with 8, 16, 32 and 64HA units of NDV-AF2240 respectively named C/NDV8, C/NDV16, C/NDV32, C/NDV64, cancer treated with 8, 16, 32 and 64HA units of NDV-AF2240 and tamoxifen respectively as CT/NDV8, CT/NDV16, CT/NDV32 and CT/NDV64 daily for four weeks. In situ RT-PCR and CLSM were successfully used to detect the NDV-AF2240 in xenotransplant 4T1 tumor cells, liver and brain. The virus disseminated into liver and brain during intratumural injection.
Conclusions: In tumor tissue the virus were found in the cells, whereas, in the brain and liver were found mainly in the blood vessels.This study contributes to understand the systemic dissemination of NDV-AF2240 and contributing to the effort of treatment and suppression of the disease.

A 208 Synthesis of PEGylated Nanospheres encapsulating doxorubicin and mitomycin c for their specific macrophage targeting, reduced toxicity and enhanced antileishmanial activity

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Background: Leishmaniasis is a life threatening tropical disease caused by Leishmania sp., a protozoan parasite. No effective vaccine is yet available against this parasite and its control relies primarily on chemotherapy. The search for new strategies to overcome toxicity, parasitic resistance and enhanced uptake of drug still remains as the current goal. Our research is focused on study of encapsulated doxorubicin and mitomycin c, antileishmanial drugs for their efficient delivery to macrophages.
Observations: The biodegradable polymer MPEG-PLA (methoxy-poly (ethylene glycol)-b-poly (lactic acid)) was synthesized under intermittent N2-flux at 1300C and characterized by H1-NMR and

FTIR. Doxorubicin and mitomycin c loaded polymeric nanoparticles (MPEG-PLA NPs) were prepared by nanoprecipitation method with an entrapment efficiency of 90 ± 5.5%. The mean diameter of spherical drug carriers was found to be 30 ± 10nm with a surface area of 14.491 sq.m/g as characterized by TEM, AFM and BET surface area analyzer respectively. Invitro release profile of drugs suggested fairly slow release of 92 ± 3% drug after 240 h. Encapsulation of the drug leads to enhanced uptake by macrophage compared to free drug as monitored by confocal microscopy. Cytotoxicity studies via MTT assay showed that encapsulation of doxorubicin and mitomycin c into NPs nullifies cellular toxicity on Human embryonic kidney cell line (HEK-293), adrenal medulla cell line (PC12) and macrophages itself without affecting their antilieshmanial activity.

Conclusions: Thus, the output of this study shows efficient delivery of antilieshmnial drugs (doxorubicin and mitomycin c) to macrophage, the parasite proliferation site, with decreased toxicity and enhanced antilieshmnial activity.

A 209 Rationale based, de novo design of dehydrophenylalanine containing antibiotic peptides and systematic modification in sequence for enhanced potency

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Background: Increased microbial drug resistance has generated a global requirement for new anti infective agents. As part of an effort to develop new, low molecular mass peptide antibiotics we used a rationale based minimalist approach to design short, non hemolytic, potent and broad spectrum antibiotic peptides with increased serum stability. These peptides incorporated a non natural amino acid alpha- beta dehydrophenylalanine, and were designed to attain an amphipathic structure in helical conformations.

Observations: Peptides containing alpha- beta dehydrophenylalanine (del Phe) amino acids were synthesized. del Phe residues are present in many naturally occurring antibiotic peptides like albonoursin. Presence of del Phe in peptides has been shown to increase the helicity of the peptides and increase the relative stability of peptides towards proteolytic degradation. An eleven residue peptide VS1 was used as the lead compound and its properties were compared with three series of derivates obtained by N-terminal amino acid addition (12-14 residues), systematic Trp substitution and peptide dendrimerization. The Trp substitution approach underlined the optimized sequence of VS2 in terms of potency, faster membrane permeation and cost effectiveness. VS2 (two Trp substituted variant of VS1) was found to exhibit good antimicrobial activity (5-25 micromolar) against both, Gram negative bacteria E.coli and Gram positive bacteria S. aureus. VS2 showed negligible hemolysis, non cytolytic activity and has the ability to permeate and depolarize the bacterial membrane. Lysis of E.coli inner membrane by the peptide VS2 was also confirmed by Scanning and Transmission Electron Microscopy.

Conclusions: A combination of small size, presence of unnatural amino acid, high antimicrobial activity, insignificant hemolysis and relative proteolytic resistance provides fundamental information towards the de novo design of an antimicrobial peptide useful for the management of infectious disease.

A 210 Isolation and characterization of antimicrobial peptides from marine microalgal symbiotic bacteria

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Background: The associated bacterium was isolated during the exponential phase of micro algae by serial dilution method. The bacteriocin producing strains were screened by agar overlay method. The bacteriocin producer strain was inoculated in Marine zobell broth and incubated for 72 hrs and isolated the bacteriocin like inhibitory substance by centrifugation at 8000 rpm for 10min.The crude bacteriocin substance thus obtained was subjected for characterization.

Observations: The Zobell's broth and MRS broth have equally supported to the bacteriocin production and its activity the maximum yield of bacteriocin was obtained in the incubation period of 48hrs. The crude bacteriocin showed the best activity at the pH range of 4-8 and it was found to be thermally stable at 60°C for 30min and became completely inactive when the temperature reached 110°C. The addition of 0.5-4% of NaCl had activity and among which the 2.5% maximized the biogenic activity. The activity against the pathogen plated on Muller Hinton Agar medium increased with decrease concentration of agar. The molecular weight of the bacteriocin was found to be 12kDa and 32KDa.

Conclusions: This bacteriocin producing new strain of vibrio sps MMB2 was used as probiotic in aquaculture industry.

A 211 In silico screening of SdiA inhibitors from Melia dubia stems against uropathogenic E.coli quorum sensing

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Background: Abstract Urinary tract infections (UTI) are one of the most frequent infectious diseases encountered all over the world and that causes enormous morbidity and mortality. Uropathogenic Escherichia coli is the major cause of UTI and it is most frequently isolated from 50-90% of UTIs. The realization that E. coli and many other pathogens regulates much of their virulence factors by means of signal molecules in a process denoted quorum sensing gave rise to a new 'drug target rush'.

Observations: In this study, the potentiality of five different extracts of Melia dubia stem for quorum sensing inhibitory activity were investigated against uropathogenic Escherichia coli (UPEC). The following assays were performed, cell density, swarming motility, protein, protease, hemolysis, hemagglutination, hydrophobicity and biofilm inhibition. The biofilm, hemolysis, swarming motility were inhibited by 45.71%, 12.97% and 33.33% respectively when the media was supplemented with 30 mg/ml of ethanolic extract. The remaining parameters

were also found to be declined in ethanolic extract as compared to control. By docking analysis we found that the binding free energy of the ranked ligands is as follows -6.12, -5.95, -4.66, -4.53 and -4.51. The ligand showed best activity is 4, 6, 10, 10-Tetramethyl-5-oxatricyclo [4.4.0.0 (1,4)]dec-2-en-7-ol, since it can effectively bind to the active site of SdiA.

Conclusions: The overall results suggested that the ethanolic extract have the potency to attenuate the quorum sensing of uropathogenic E. coli. Further investigation is desired to study the effectiveness of the ligand by in vitro and in vivo strategies.

Poster Abstracts Session B

Monday 12 September 17:15–18:45

B 001 Galanin inhibits hair follicle growth

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Background: Galanin, a trophic factor of the central and peripheral nervous system, has been shown to have a widespread distribution in the skin, including the interfollicular and follicular epidermis. However, the exact localization and the role of galanin in the hair follicle (HF) has not been investigated. Therefore, we have determined the cellular localization of galanin in human HFs and the effects of galanin on normal growing human scalp HFs in organ culture.
Observations: Immunohistochemistry was performed on cryosections of human female scalp skin. Anagen HFs were isolated and cultured up to 9 days and treated with 100 nM galanin. Staining for Ki-67, TUNEL and Masson-Fontana were used to analyze proliferation, apoptosis and staging of the HFs. Quantitative real-time PCR (qRT-PCR) was performed with RNA from HFs to assess the mRNA expression of galanin and galanin receptors. Galanin-like immunoreactivity (galanin-LI) was detected in the outer root sheath and inner root sheath (epidermal origin) of the HFs. Galanin treated organ-cultured normal human scalp HFs revealed less proliferation of hair matrix keratinocytes compared to untreated controls. Galanin also reduced the duration of the hair growth phase (anagen) after 5 and 9 days in vitro. The reduced hair shaft elongation was accompanied by the development of a catagen-like morphology in hair bulbs of HFs treated with galanin. qRT-PCR analysis revealed expression of GALR2 and GALR3 but not GALR1 indicating a non-GALR1 mediated function of galanin on HF growth.
Conclusions: Thus, we present for the first time that human HFs are a source and target of galanin. The effect of galanin on organotypic HF cultures indicates that galanin plays a role in HF growth. Therefore, galanin has to be included in the list of neuropeptides regulating hair follicle physiology.

B 002 Drebrin E regulates apical epithelial cell morphogenesis

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Background: The organization of polarized cells characterized by the asymmetric distribution of polarity complexes, cytoskeleton and organelles is the main feature of intestinal epithelium. Cell polarity is maintained by the balance between the apical complexes Crumbs3A (Crb3A), Par-3/Par-6 and the basal complex Scribble. These complexes are essential for monolayer integrity and prevent cell over-proliferation. Our main objective is to understand how the Crb3A complex is involved in apical morphogenesis.
Observations: Caco-2/TC7 cells are able to differentiate (expression of differentiation markers and apical brush border organization) and are a useful model of polarized cells. We have transiently depleted Crb3A partners in human intestinal epithelial Caco-2/TC7 cells (derived from a colonic adenocarcinoma) expressing GFP::Crb3A or GFP::Crb3AΔERLI (Crb3A deleted of its ERLI motive). These cells behave like wild-type cells for polarity and differentiation markers expression and localization. We have also shown that GFP::Crb3 constructs are localized at the apical domain. The Crb3A complex is maintained as GFP::Crb3A interacts with endogenous Pals1 and PATJ (two other members of the Crb3A complex). We have uncovered an interaction between GFP::Crb3A and Drebrin E by co-immunoprecipitation. Our team has shown that Drebrin E (an Actin-binding protein) controls apical morphogenesis of Caco-2/TC7 cells by organizing the Actin-based apical cytoskeleton. We are now exploring the role of Drebrin E in regulating the expression of apical proteins including the Crb3A complex.
Conclusions: We have pointed out an interaction between GFP::Crb3A and Drebrin E and that Drebrin E is involved in apical morphogenesis. Our goal is to understand the mechanisms involved in the regulation of the expression of apical proteins and how Drebrin E might control their expression and trafficking.

B 003 Planar polarity and the orientation of cell divisions in Drosophila

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Background: Tissues can grow in a particular direction by controlling the orientation of cell divisions. This phenomenon is evident in the developing Drosophila wing epithelium, where the tissue becomes elongated along the proximal-distal axis.
Observations: We show that orientation of cell divisions in the wing requires planar polarisation of an atypical myosin, Dachs. Our evidence suggests that Dachs constricts cell-cell junctions to alter the geometry of cell shapes at the apical surface and that cell shape then determines the orientation of the mitotic spindle. Using a computational model of a growing epithelium, we show that polarised cell tension is sufficient to orient cell shapes, cell divisions and tissue growth.
Conclusions: Planar polarisation of Dachs is ultimately oriented by long-range gradients emanating from compartment boundaries and is therefore a mechanism linking these gradients with the control of tissue shape.

B 004 Mechanical and molecular requirements for bleb and lamellipodium formation in migrating cells

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Background: Protrusion formation at a cell's leading edge is an important step during cell migration. Studies in development and cancer cell motility show that blebs are a common alternative to the well characterized actin polymerization-based

lamellipodia or filopodia, particularly in three-dimensional environments. However, the biochemical pathways that control bleb formation and the physical mechanisms of blebbing-based motility are largely unknown.

Observations: To address these questions, we used Walker 256 carcinosarcoma cells, which can be selected based on the adhesiveness of the substrate to generate two distinct sublines that form either blebs or lamellipodia. By comparative analysis, we found that cellular mechanical properties like cortical tension as well as molecular signals (e.g. Rac1 signaling) dictate the type of protrusion formed by a cell. Moreover, changing the balance between the protrusive forces led to a switch in protrusion formation, a behavior observed in metastasizing cancer cells facing pharmacological or physical barriers. In parallel, the mechanisms of force transmission during blebbing-based motility were investigated using migrating blebbing Walker cells. Our data suggest that these cells may use other mechanisms than classical transmembrane coupling to generate and transmit forces necessary for locomotion.

Conclusions: In summary, we show that the two sublines of Walker 256 carcinosarcoma cells can be used as a model system for comparing pressure driven and actin-polymerization-based protrusions and for investigating the mechanisms of blebbing-based motility.

B 005 In Vitro Dissection of the Functional Interplay between Core Components of the Microtubule Plus End Tracking Network

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Background: Microtubules (MTs) are cytoskeletal filaments that switch between phases of growth and shortening. A diverse group of proteins dynamically localizes to growing MT ends, thereby controlling MT dynamics and spatial organization. End Binding proteins (EBs) have been identified as autonomous plus end tracking proteins (+TIPs) that recruit a variety of non-related proteins to growing MT ends. These proteins bind EBs via a CAP-Gly domain or a linear SXIP motif.

Observations: The large dynactin subunit p150 glued contains a GAP Gly domain and might mediate end tracking of dynactin/dynein. Several mutations within the CAP-Gly domain of p150 glued that weaken EB1, but not MT binding, are linked to severe neuronal disorders which suggests an important role for p150 glued MT plus end localization. RNAi studies in cultured cells revealed that p150 glued end tracking relies on both EB1 and CLIP-170, another CAP-Gly protein. Using an in vitro TIRF microscopy based MT plus end tracking reconstitution assay with purified proteins we show that EB1 alone, – but not CLIP-170 -, is necessary and sufficient to target CAP-Gly domain containing fragments of p150 glued to growing MT ends. p150 glued is expressed in two major splice variants. We show here that these two isoforms differ strongly in their MT lattice affinity whereas both are targeted to growing MT ends by EB1. We further demonstrate that SXIP motifs interfere with EB1 mediated plus end tracking of p150 glued and CLIP-170 and vice versa.

Conclusions: We studied here competitive and cooperative effects between +TIPs directly at the MT end in a defined in vitro system. We shed new light on the molecular mechanism of p150 glued end tracking and show that the two major classes of EB dependent +TIPs compete for EB binding.

B 006 Alpha-Synuclein nucleates short microtubules

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Background: Alpha-Synuclein (syn) is the major component of Lewy body, the cytosolic inclusions associated to Parkinson's disease, and its mutation have been linked to genetic forms of the disorder. Although largely studied, syn physiological role is still unclear. In vitro, syn is unfolded, but it acquires a secondary structure upon interaction with the partners. It is also reported the capacity of syn to induce microtubule (MT) polymerization, but a detailed analysis of kinetic parameters still lacks.

Observations: Here, we decided to undertake an in depth evaluation of the effects of syn on tubulin polymerization kinetics. Because standard protocol failed in reproducibility, we decided to incubate syn in the presence of tubulin, just to allow syn folding. Circular dichroism spectra show that upon pre-incubation syn acquires an alpha-helix conformation. Thus, we performed tubulin polymerization following pre-incubation with WT and mutated syns, and the analyses of kinetic parameters suggest that syns promote MT nucleation, but they seem to reduce MT mass at the end of polymerization. By using fluorescence microscopy, we evaluated the number and the length of MTs polymerized in presence of syns, at the very beginning and at the end of the polymerization. Accordingly to the already published data, mutated syn causes tubulin aggregation instead of MT polymerization. On the contrary, at both time points, WT syn promotes the polymerization of a higher number of MTs, confirming its capacity in nucleating MTs. Moreover, MTs polymerized in the presence of WT alpha-syn are shorter in respect to control MTs, reconciling the apparent reduction of MT mass due to the physics of the system.

Conclusions: Our results underscore the ability of alpha-syn to nucleate short MTs, as usually are MTs present at synaptic terminal, the cell compartment where syn resides. Thus, we shed light on a new physiological role for alpha-syn as a feasible axonal MT nucleating structures.

B 007 Alpha-Catenin recruits Vinculin to Cadherin junctions in a force dependent manner: Does it play a role during morphogenesis?

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Background: Cell-cell junctions (CCJs) mediated by Cadherins are mechanosensory structures. An essential step for this function is the Vinculin recruitment to the CCJs in a force dependent manner. It was proposed that the recruitment of Vinculin is depending on its binding to alpha-Catenin. The specific contribution of junctional Vinculin to the mechanosensory process is difficult to evaluate because this protein is also present in cell-extracellular matrix adhesions (CEMAs).
Observations: We hypothesized that blocking the binding of Vinculin to alpha-Catenin, a protein absent from CEMAs, could interfere with Vinculin function specifically in CCJs. To this end, we replaced the Vinculin interaction domain of alpha-Catenin, with the homologous domain from Vinculin (its closest homolog), obtaining an alpha-Catenin/Vinculin hybrid. This construct fully restored the formation of CCJs in several alpha-Catenin negative cell lines. Interestingly, actomyosin contraction induced by HGF was not able to induce the recruitment of Vinculin to the junctions in these cells, while Vinculin at CEMAs was not affected. Although it is already known that mechanosensing is involved in migration, tissue remodelling and polarization during development, the function of Cadherin mechanosensing during morphogenesis is still not clear. So, we aim to use the hybrid construct as a tool to study the relevance of the Cadherin mechanosensing in vivo. Because heart development is impaired when expression of junctional proteins such as VE-Cadherin, alpha-Catenin and Vinculin is reduced, we are now analyzing zebrafish heart morphogenesis for early defects caused by the loss of junctional Vinculin.
Conclusions: The results in vitro suggest that force dependent recruitment of Vinculin to CCJs specifically can be prevented by an alpha-Catenin mutant that does not perturb CCJs per se. This allows us to design in vivo experiments that determine the role of Cadherin mechanosensing during morphogenesis.

B 008 Characterization of Banderuola, a novel regulator of asymmetric cell division in Drosophila melanogaster

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Background: Asymmetric cell division is a process whereby a progenitor cell can generate daughters with a different fate. The underlying molecular mechanisms have been widely studied in the Drosophila Sensory Organ Precursor (SOP) cells, that generate the 4 different cell types forming the external sensory organs (bristles) through 2 rounds of asymmetric cell division. This is achieved through the partitioning of the protein Numb, that represses Notch signaling in the daughter cell where it is segregated.
Observations: To gain a better insight of this process, we used tissue specific RNAi to score the effects of the knockdown of genes on the morphology of the bristles. We have screened a library of 22,247 transgenic Drosophila RNAi lines, and identified 130 genes potentially involved in asymmetric cell division. To identify the genes involved in the asymmetric localization of Numb, we developed a live time-lapse cell-imaging assay based on a GFP reporter. Among the 130 candidates screened and not previously thought to play a role in asymmetric cell division, there were three genes whose knockdown reproducibly affected Numb localization. We have confirmed the observed phenotypes by independent secondary RNAi lines and have started investigating the function of one of the identified genes, which encodes a previously uncharacterized protein. Loss-of-function studies suggest that the protein, which we named Banderuola, specifically acts in establishing an axis of cell polarity for asymmetric cell division. Co-Immunoprecipitation experiments show that Banderuola binds to Discs-large (Dlg), a membrane-associated guanylate kinase acting in many cell-polarity events.
Conclusions: Our study has identified Banderuola, a new component of the machinery of asymmetric cell division. As its binding partner Dlg acts in several polarity events, including synaptogenesis and tumor formation, our studies may be relevant for a variety of biological processes.

B 009 Remodelling of adherence junction proteins and actin filament in cell sheet by mechanical stimuli

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Background: It has been shown that focal adhesion and actin stress fibers are the key structures in mechano-sensing in cells. On the other hand, recent studies suggest that focal adhesion and stress fibers are not present on soft substrate, indicating that some other mechanism are present as mechano-sensor inside soft tissues. In cells that are not attached to substrates, it is probable that cell-cell junction proteins play important role in mechano-sensing.
Observations: Here we induced mechanical stretch on NIH 3T3 cells that are not adhered to the surface and studied the effect of stretch on the actin filament remodelling. Because we did not use enzymatic digestion while preparing the cell-sheet, cell-cell adhesion remained intact leaving artificial tissue like structure without substrate attachment. When RFP-actin expressing cells were observed under the real-time confocal microscope, filamentous actin structure was not observable when cells were not attached to the substrate. However, when cells were stretched, actin filament structure became visible parallel to the direction of the stretch. When GFP-beta-catenin was observed, patchy structure was formed upon stretch, which was not present when cells were not under tension.
Conclusions: These results indicate that remodelling of actin filament occur by the application of mechanical stimuli, which may involve adherence junction proteins.

B 010 Quantitative proteomics of the integrin adhesome reveals a myosin II-dependent recruitment of LIM domain proteins

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Background: A hallmark of integrins is their ability to connect cells to the extracellular matrix and transfer chemical and mechanical signals across the plasma membrane. Force generated by myosin II enables cells to sense substrate stiffness and to induce growth and maturation of integrin adhesions. In this study, we performed the first quantitative proteomic analysis of the effect of myosin II activity on the molecular composition of the integrin associated sub-proteome (adhesome).
Observations: Using a new in vivo crosslinking method to preserve adhesion-associated proteins at integrin adhesion sites combined with quantitative mass spectrometry, we identified 890 proteins enriched upon cell attachment to fibronectin (integrin-mediated) versus PLL-coated (non-specific adhesion) substrates. Mechanical cues mediated by myosin II induce the maturation of newly formed nascent focal adhesions (NAs) into mature focal adhesions (FAs). This transition can be inhibited by blocking myosin II activity with the small molecule blebbistatin. Using our proteomics workflow we examined the compositional differences of FA versus NA, which were induced with blebbistatin treatment. We identified 358 proteins whose recruitment to integrin adhesions was significantly reduced upon myosin II inhibition. Strikingly, the most dramatic intensity reductions were seen for proteins containing zinc finger-type LIM domains. The protein family domain enrichment for LIM in the blebbistatin sensitive clusters was highly significant. Using TIRF-microscopy we validated the proteomics result by quantifying the dynamic translocations of LIM-domain and control proteins upon blebbistatin perfusion.
Conclusions: The finding that recruitment of almost all LIM domain-containing FA proteins depended on myosin II activity suggests that LIM domains can function as tensions sensors within proteins.

B 011 Cytoskeletal dynamics in TNT formation and functioning in endothelial cells

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Background: Tunneling nanotubes (TNT) are long protrusions of the cells with either actin or actin and tubulin filamens. The proposed function of TNTs is the cell-to-cell trafficking of organelles and signals.
Observations: Recently we have found TNT-like structures in human microvascular endothelial cells (HMEC-1). The identified TNTs were studied with fluorescence and environmental scanning electron microscopy (ESEM). Lysosomes were identified in the intercellular nanotubes between endothelial cells as a plausible cargo. The observed TNTs contained both actin and tubulin. Nevertheless, the exact function of each of these cytoskeletal components remained unclear. The cells were treated either with actin-depolymerizing agents, or actin filament stabilizing substances. Moreover, the role of Rho GTPases (RhoA, Rac1, Cdc42) in the TNT formation has been studied. Not only actin, but also tubulin dynamics was in the focus of the presented study. For all treatments we have examined the structural (number of TNTs, length etc.) as well as functional (localisation of lysosomes) nanotube characteristics.
Conclusions: The experimental approach, which is described in the presented study, is an important tool to characterize tunneling nanotubes and to elucidate the mechanisms of TNT formation and functioning.

B 012 Dissecting roles of crucial microtubule regulators by peptide aptamers

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Background: Microtubules (MTs) play essential roles in cellular processes. Conserved Drosophila proteins, Mini spindles (Msps) and EB1, regulate MT dynamics. However, their functions in non-dividing cells are not clear. To find out their functions in post-mitotic cells, I use peptide aptamers which are protein reagents designed to bind and disrupt protein interactions inside cells. These peptide aptamers can also be used to discover drug targets.
Observations: I screened a peptide library of random composition by yeast two-hybrid to find those interacting with Msps. So far, I found two peptides from 12 million screened which interact with Msps C-terminus and none out of 40 million peptides screened which interact with the N-terminus. To identify peptide aptamers for EB1, I screened the random peptide library encompassing SxIP motif, which is known to be essential but not sufficient for binding of numerous proteins to EB1. So far, ~300 interactors were found after screening a library of 5 million peptides. Sequencing of the peptides revealed that certain amino acids occurred more frequently at some positions in vicinity of the SxIP motif than other amino acids. I will further select biologically active aptamers that can disrupt Msps and EB1 functions to test the roles of Msps and EB1 in different cell types.
Conclusions: I concluded that the N-terminus of Msps may not be as easily druggable as the C-terminus. Also, some of the amino acids in SxIP proximity are favored at certain positions influencing the binding strength of proteins containing such a motif to EB1.

B 013 Regulation of axonal branching by BDNF signalling

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Background: The formation, refinement, and maintenance of neural circuits require exquisite control of axonal growth, guidance and branching. While axonal growth and guidance have been extensively studied, much less is known about the mechanisms controlling axonal branching, despite its importance in allowing neurons to make connections with several targets. Furthermore, it has been proposed that axonal branching may play a major role in neuronal regeneration following spinal cord injury.
Observations: We have recently identified Sprouty3 as a new negative regulator of signalling downstream the Brain Derived Neurotrophin Factor (BDNF). Sprouty3 is expressed specifically in the trigeminal and in spinal motor and sensory neurons in a BDNF-dependent manner. Biochemically, Sprouty3 does not regulate MAPK or Akt activity downstream BDNF. However, using live imaging of spinal cord explants and calcium indicators, we were able to show that Sprouty3 prevents BDNF-dependant calcium spikes. Interestingly, loss-of-function experiments in Xenopus embryos reveal that Sprouty3 represses specifically axonal branching in motor neurons in vivo. Furthermore, expression of Sprouty3 in mouse cortical neurons reduces the number of branches induced by BDNF, indicating that Sprouty3 function may be conserved in mammals. Time-lapse DIC imaging of spinal cord neurons in culture showed that knockdown of Sprouty3 expression leads to an increase in the number and the stability of filopodia. Finally, we have characterised a promoter driving expression of transgenes specifically in Xenopus motor neurons, allowing us to study axonal branching in vivo.
Conclusions: We have uncovered a new negative feedback loop downstream of BDNF signalling to control its ability to induce axonal branching. We are currently exploring further the cellular and molecular mechanisms by which BDNF regulates axonal branching in vivo.

B 014 Modelling epithelial plasticity in pancreatic ductal adenocarcinoma

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Background: Pancreatic Ductal Adenocarcimoma (PDAC) is a highly lethal cancer type in which activating Kras mutations drive Pancreatic Itraepithelial Neoplasia (PanIN) to develop invasive PDAC. Evidence is accumulating that PanIN lesions develop from the acinar compartment undergoing Acinar to Ductal Metaplasia (ADM). Our goal is to understand pancreatic acinar plasticity and how it eventually originates tumor precursor lesions.
Observations: In order to understand acinar epithelial plasticity we employ a combination of mouse models and acinar epithelial explants in 3D collagen cultures. We work with the well-established KrasG12D-based conditional mouse model (named CK). This model shows the complete spectrum of clinically relevant preneoplastic lesions. Acinar epithelial explants from wt mice undergo ADM after activation of EGF signaling. As expected, acinar epithelial explants lacking EGF-receptor were not able to undergo ADM ex vivo. The RAS-related C3 botulinum substrate 1 (Rac1) is a Ras effector molecule that is known to regulate actin rearrangements. Acinar explants lacking Rac1 do not undergo ADM ex vivo after activation of EGF signaling. Interestingly, acinar explants from CK mice undergo rapid ADM ex vivo without external activation of EGF signaling. We observe impairment of ADM in CK acinar explants in which Rac1 was knocked out. This result is well in agreement with reduced ADM observed in CK mice crossed with Rac1fl/fl mice. Most interestingly CK acinar explants in which EGF-receptor was knocked out, show impaired ADM ex vivo. This phenotype could be reversed by mechanical stress of the 3D acinar explants.
Conclusions: We use acinar epithelial explants to adress specific questions about the molecular mechanisms underlaying ADM in PDAC. The results shown here suggest: (1) a role of Rac1 in actin-dependent plasticity during ADM. (2) The requirement for EGF-receptor in ADM driven by constitutive active Ras.

B 015 Formin Diaph1 and the Arp2/3 complex are necessary to form the submembranous actin cortex

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Background: The contractile actin cortex has a fundamental role in cell morphogenesis, motility, and cytokinesis. It enables the cell to resist mechanical forces and maintain cell shape. The formation of lamellipodia and filopodia have been characterized, but the formation of the actin cortex has remained elusive. We have used M2 melanoma cell blebs as a model to study how the cell cortex reforms. In this study we show that two actin nucleators are needed for cortex formation.
Observations: First we asked which actin nucleators are expressed in M2 cells and found that 8 different formins, Spire1 and the Arp2/3 complex are expressed. To gain further insight into their identity, we performed proteomic studies on blebs separated from M2 cells as these can still reform a well-defined actin cortex. Mass spectrometry analysis of the non-soluble fraction of separated blebs revealed that only two nucleators, the formin Diaph1 and the Arp2/3 complex (all seven subunits), were associated with the cortex. Localisation studies showed that constitutively active Diaph1 as well as Diaph1 speckles localise to the cortex of cells. The Arp2/3 complex activator WAVE and cortactin showed a cortical enrichment, but the Arp2/3 complex itself appeared diffuse. Diaph1 knockdown resulted in formation of bigger blebs, suggesting a weaker cortex. Arp3 knockdown was lethal and only few cells survived. However, treating M2 cells with the Arp2/3 complex inhibitor CK-666 led to the formation of cortical defects. When simultaneously depleting Diaph1 and the Arp2/3 complex, the cellular cortex disappeared, suggesting that together they provide the majority of the F-actin in the cortex.
Conclusions: Taken together our results show that the formin Diaph1 and the Arp2/3 complex have a fundamental role in actin cortex nucleation in mammalian cells.

B 016 Differential interfacial tension controls specific adhesion of germ layer progenitors

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Background: During gastrulation, progenitor cells of the three germ layers (ectoderm, mesoderm and endoderm) segregate from each other to form the different germ layers. This segregation behavior can be reconstituted in mixed cultures of primary progenitor cells. Differences in the adhesive and tensile properties have been shown to regulate progenitor cell segregation in culture. However, the interplay between those two properties in progenitor cell segregation remains poorly understood.
Observations: We have used a dual micropipette assay to characterize the adhesion of the germ layer progenitors based on the cell-cell contact shape, separation force, and the spatiotemporal distribution of cytoskeletal and adhesion molecules. We found that myosin activity positively regulates the size and strength of cell-cell contacts. Consistently, ectoderm cells exhibiting the highest cortical tension of all three germ layer progenitor cell types also form the largest and strongest cell-cell contacts. Myosin functions in controlling cell-cell contact size and strength by localizing preferentially to the cell-medium interface and thereby pulling on the edges of the cell-cell contact and expanding it.
Conclusions: Using a simple theoretical model, we estimate that the difference of tension between the cell-cell- and cell-medium-interface is mostly responsible for setting the contact size and strength, while the adhesion energy is negligible.

B 017 Impact of pre-mRNA editing on Filamin A function

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Background: Adenosine to inosine RNA editing is highly conserved mechanism which induces post-transcriptional modifications in RNAs. Screening for editing sites in transcriptomes revealed conserved sites in the pre-mRNAs encoding Filamin A, where it induces Glu to Arg amino acid substitution. So far, little is known about the biological function of these events. Therefore, we generated Filamin A editing deficient mice to explore the function of RNA-editing at the organismic and cellular level.
Observations: Phenotypic and behavioral analysis of FlnA editing deficient mice revealed reduced body weight and slower movement in the open field assay indicating possible disorders in locomotor, exploratory, or anxiety-like behavior. Editing of FlnA is highest in brain, heart, and bladder indicating that editing might play an important role in these tissues. Ongoing deep sequencing of mouse organs samples at different developmental stages will allow us to determine the complete developmental and tissue specific editing profile of FlnA. It has been shown that filamin A interaction with actin mediates the induction of cell protrusions such as dendritic spines, lamellipodia, or fillipodia. Therefore, dendrite morphology of cultured primary hippocampal neurons was determined. Our data show no difference in branching but significantly reduced number of dendritic spines in neurons of FlnA-editing deficient animals. Dendritic spines are the major sites of informational processing in the brain, and filamin A is known to interact with numerous spinal receptors. Therefore, additional memory and learning assays, as well as colocalization and receptor trafficking studies are planned.
Conclusions: Our study indicates that Filamin A editing is dispensable for mice viability. However, Filamin A editing influences behavior and cellular outgrowth. Future studies are aimed to determine the molecular mechanisms underlying the observed phenotypes.

B 018 Functional and Spatial Analysis of C. elegans SYG-1 and SYG-2, Orthologs of the Neph/Nephrin Cell Adhesion Module Directing Selective Synaptogenesis

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Background: The assembly of specific synaptic connections represents a prime example of cellular recognition. Members of the immunoglobulin superfamily are ancient proteins in both mammals and invertebrates, where they constitute a trans-synaptic adhesion system. The correct connectivity patterns of the IgSF proteins nephrin and Neph1 are crucial for the assembly of functional neuronal circuits and the formation of the kidney slit diaphragm, a synapse-like structure forming the filtration barrier.
Observations: We utilized the nematode C. elegans model for studying the requirements of synaptic specificity mediated by nephrin-Neph proteins. In C. elegans, the nephrin/Neph1 orthologs SYG-2 and SYG-1 form intercellular contacts strictly in trans between epithelial guidepost cells and neurons specifying the localization of synapses. We demonstrate a functional conservation between mammalian nephrin and SYG-2. Expression of nephrin effectively compensated loss of syg-2 function in C. elegans and restored defective synaptic connectivity further establishing the C. elegans system as a valuable model for slit diaphragm proteins. Next, we investigated the effect of SYG-1 and SYG-2 trans homodimerization respectively. Strikingly, synapse assembly could be induced by homophilic SYG-1 but not SYG-2 binding indicating a critical role of SYG-1 intracellular signalling for morphogenetic events and pointing towards the dynamic and stochastic nature of extra- and intracellular nephrin-Neph interactions to generate reproducible patterns of synaptic connectivity.
Conclusions: In summary, our findings corroborate that C. elegans is a useful tool for investigating fundamental nephrin/Neph protein functions. Furthermore, we present novel insights into the mechanisms of SYG-1 and SYG-2 homotypic adhesion properties and intracellular functions.

B 019 Mechanisms of Pom1 concentration gradient formation for coordinating cell growth with mitosis

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Background: Concentration gradients regulate many cell biological and developmental processes. In rod-shaped fission yeast cells, polar cortical gradients of the DYRK family kinase Pom1 couple cell length with mitotic commitment by inhibiting a mitotic inducer positioned at midcell. However, how Pom1 gradients are established is unknown.
Observations: We show that Tea4, which is normally deposited at cell tips by microtubules, is both necessary and, upon ectopic cortical localization, sufficient to recruit Pom1 to the cell cortex. Pom1 then moves laterally at the plasma membrane, which it binds through a basic region exhibiting direct lipid interaction. Pom1 autophosphorylates in this region to lower lipid affinity and promote membrane release. Tea4 triggers Pom1 plasma membrane association by promoting its dephosphorylation through the protein phosphatase 1 Dis2.
Conclusions: We propose that local dephosphorylation induces Pom1 membrane association and nucleates a gradient shaped by the opposing actions of lateral diffusion and autophosphorylation-dependent membrane detachment.

B 020 Drosophila aPKC is required for mitotic spindle orientation during symmetric division of epithelial cells

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Background: aPKC-Par6 is required for spindle planar orientation in mammalian tissue culture epithelial cells but not in vivo in chicken neuroepithelial cells. This suggests the cortical cues necessary for spindle planar orientation in dividing epithelial cells are not conserved.
Observations: One of the major problems with studying the epithelial functions of aPKC is the fact that strong alleles of apkc show a complete loss of tissue integrity. We took advantage of a temperature-sensitive allele of apkc (apkcts) to generate graded reductions in apkc activity. From this work, we concluded aPKC regulated planar orientation of the mitotic spindle in symmetrical epithelial divisions and was required for efficient exclusion of Pins, a known regulator of spindle orientation, from the apical domain of mitotic epithelial cells. Furthermore, since spindle misorientation can potentially cause a rotation of the division cleavage plane, epithelial cell extrusion, and subsequent apoptosis, we also showed that a non-lethal reduction in aPKC activity could lead to tissue overgrowth and tumor development if there was inhibition of apoptosis and compensatory cell proliferation.
Conclusions: Our data suggest the cortical cues necessary for spindle planar orientation in dividing epithelial cells are conserved between Drosophila and mammalian cells, and are likely to be similar to the ones known to be important for spindle apicobasal orientation during neuroblast asymmetric cell division.

B 021 Coordination between microtubules and intermediate filaments during astrocyte migration

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Background: Cell division, differentiation and also cell migration involve the coordinated regulation of the different cytoskeletal elements. Evidence for a crosstalk between microfilaments and microtubules is increasing. However, whether and how intermediate filament organization is coordinately controlled remains unclear. We have investigated the implication of microtubules and associated proteins in intermediate filament organization during astrocyte migration.
Observations: Primary astrocytes mainly express Glial Fibrilary Acidic Protein (GFAP), vimentin and nestin, which are essential for migration. During astrocyte migration, intermediate filaments reorganize to form a polarized network which is coextensive with microtubules in cell protrusions. Microtubule depolymerization abolishes intermediate filament elongation. Intermediate filaments colocalize with microtubule-associated APC. We show that APC act as a major molecular bridge between Intermediate filaments and microtubules. It nteracts directly with the two cytoskeletons through its Armadillo repeats and its basic region. As a consequence, APC depletion by siRNA, as well as overexpression of the vimentin-binding or microtubule-binding region of APC, dramatically perturbs the coupling between Intermediate filaments and microtubules, leading to a collapse of the intermediate filament network.
Conclusions: Together with its function in actin and microtubule regulation, this novel role of APC in the regulation of intermediate filaments provides a better understanding of how cellular cytoskeletons are coordinated during cell migration and bring new insights on APC achieves tumor suppressor functions.

B 022 Understanding the establishment of epithelial polarity upon cell division

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Background: A central feature of epithelial tissues is that the cells are highly polarized along the apical-basal axis. Yet, epithelial cells divide and create a novel long and polarized interface between the two daughter cells at each division, where a new adherens junction (AJ) is assembled. The mechanisms coupling cell division and the formation of a long and polarized interface are currently unknown.
Observations: To bring insight into this question, we use as a model system Drosophila pupal epithelia, which allow both the use of powerful genetic tools and in vivo confocal imaging of the intact epithelia. We are currently exploring three aspects: (1) how cytokinesis occurs and whether the position of the midbody is important for the polarization of the new interface; (2) what is

the dynamics of appearance of the different polarity complexes at the new interface and how E-cadherin (E-cad) molecules are recruited to the new AJ and (3) how a long interface is formed after cytokinesis. We show that cytokinesis can occur either symmetrically or asymmetrically in different epithelia, and that this correlates with both the structure of the contractile ring and the organization of apical-basal polarity before cytokinesis. We also show that E-cad is an early polarity marker to appear at the new interface and that pre-existing AJs might be its main source. Another finding is that actin is intensely recruited as the new interface is being formed, what could contribute to the generation of a long interface after cytokinesis.
Conclusions: This work should contribute to a better understanding of the mechanisms by which epithelial tissues preserve homeostasis during proliferation.

B 023 Willin and Par3 cooperatively regulate epithelial morphology through aPKC-mediated ROCK phosphorylation

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Background: Apical domain constriction is an important process for regulating epithelial morphogenesis. Epithelial cells are connected via apical junctional complexes (AJCs) that are lined with circumferential actomyosin cables. The contractility of these cables is regulated by Rho-associated kinases (ROCKs). However, it remains to be clarified how ROCK is regulated to maintain proper contractility of the circumferential actomyosin.
Observations: Here, we report that Willin (a FERM-domain protein) and Par3 (a polarity-regulating protein) cooperatively regulate ROCK-dependent apical constriction. We found that Willin recruits aPKC and Par6 to the AJCs, independently of Par3. Simultaneous depletion of Willin and Par3 completely removed aPKC/Par6 from the AJCs, inducing apical constriction. The aPKC loss upregulated the level of AJC-associated ROCKs, explaining why Willin/Par3 depletion induced the constriction. We finally found that aPKC phosphorylates ROCK, and this process suppresses the junctional localization of ROCK.
Conclusions: This system functions to prevent ROCK from localizing at AJC, thereby allowing cells to retain the normally shaped apical domains. Thus, we uncovered the Willin/Par3-aPKC-ROCK pathway to maintain normal epithelial apical morphology.

B 024 Mechanical stress in the developing Drosophila wing disk

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Background: The development of an organ or a tissue is the result of coordination between morphogen signaling and mechanical stress. Here we investigate the connection between signaling and tissue stress using the Drosophila wing disk as model system.
Observations: During the development of the wing disk, the number of cells increases by two order of magnitude while the cell cycle length increases from 4.5 hours to 33 hours due to the lengthening of G2 phase. Theoretical models argued that the control on cell proliferation and on the final size of an organ could be based on mechanical forces. To investigate these relationships we measured the stress distribution in the wing at different times during the development of 3rd instar larvae. We assessed the mechanical properties of the wing tissue by measuring geometrical parameters such as the apical cell shape, as well as the tension of the apical cell junctions using laser ablation.
Conclusions: We found that the stress is anisotropic in the tissue and its spatial pattern changes within the development. We are investigating the role of Dpp, expressed at the A/P boundary and Wingless (Wg), localized on the D/V boundary. Along the boundaries, cell shape and the stress direction are peculiar.

B 025 ADAMTS-1 regulating migration, invasion, and invadopodia formation in breast cancer cells

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Background: ADAMTS-1 (a disintegrin and metalloprotease with thrombospondin motifs) is a member of the ADAMTS family of metalloproteases. This enzyme is related to pathological processes such as inflammation and cancer. In spite of ADAMTS-1 biological relevance, the mechanisms underlying its involvement in tumor biology remain elusive. Here we studied the role played by ADAMTS-1 regulating migration and invasion of malignant mammary cell lines (MDA-MB-231 and MCF7).
Observations: In vivo, qPCR showed that ADAMTS-1 mRNA decreased in breast cancers compared to normal tissue. Silencing of ADAMTS-1 by siRNA enhanced migratory activity of MDA-MB-231 cells. MDA-MB-231 cells with reduced expression of ADAMTS-1 exhibited increase in migration and invasion. Cancer cells depend on invadopodia to invade extracellular matrix. Invadopodia are membrane protrusions with enzymes required for extracellular matrix degradation. Silencing of ADAMTS-1 increased invadopodia in MDA-MB-231 cells, as shown by fluorescent degradation substrate assays. Cortactin and MT1-MMP are important invadopodia proteins. Immunoblot of cells transfected with ADAMTS-1 siRNA showed augmentation of cortactin and MT1-MMP. ADAMTS-1 effects may be related to VEGF, growth factor involved in migration and invasion. MDA-MB-231 cells with silenced ADAMTS-1 showed increase of VEGF concentration in conditioned medium. This conditioned medium induced HUVEC tubulogenesis. Furthermore, MBA-MB-231 showed high expression of VEGF receptor (VEGFR2) compared to MCF7 cells. We infer that ADAMTS-1 effects in tumor invasiveness are related to VEGF and VEGFR expression.
Conclusions: We suggest that decrease of ADAMTS-1 stimulates migration, invasion and invadopodia formation in breast

cancer cells. These effects are probably regulated by VEGF and VEGFR.

B 026 Correlative live cell imaging and electron tomography: insights into how actin is organized to push in migrating cells

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Background: Cell movement is initiated by the extension of cytoplasmic leaflets termed lamellipodia, formed by the unidirectional growth of actin filament networks. An essential component of these networks is the Arp2/3 complex, which promotes branching of actin filaments in vitro. Conventional EM studies have further identified high density branched arrays of actin filaments in lamellipodia, from which it has been concluded that protrusion is based on short, stiff filaments pushing from branch points.
Observations: We have re-investigated the structure of lamellipodia by electron tomography in vitreously frozen cells and cytoskeletons as well as in cells chemically fixed and embedded in negative stain after imaging them live in the light microscope. In the tomograms, the helical organization of actin filaments in lamellipodia is preserved, indicative of a high degree of structural preservation. By tracking filaments in 3D we now identify actin branch junctions in lamellipodia and show for individual filaments, that the distance between branch junctions and the cell membrane is highly variable, up to at least 1micrometer. Image analysis reveals a close structural correlation between actin branch junctions in cells with that obtained for branches formed from the Arp2/3 complex and actin in vitro. Using an intracellular wound repair model we demonstrate that initiation of lamellipodia involves branching from the sides of pre-existing 'mother' actin filaments recruited to the wound edge. Current work focuses on localizing the Arp2/3 complex by immunoelectron microscopy and determining the modulation in actin branch distribution according to the protrusion and signaling status of the lamellipodium.
Conclusions: We show that side branching on actin filaments is required to initiate lamellipodia and end branching to maintain the network and that pushing is not performed by arrays of short filaments, but by filaments of variable length extending from branch junctions which treadmill with the actin network.

B 027 Sperm cell crawling in the nematode Caenorhabditis elegans

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Background: Cell motility is important in biological processes, such as the immune response. Actin is implicated in most amoeboid cell movement, but Caenorhabditis elegans sperm cells lack actin, and their motility is driven by the Major Sperm Protein (MSP) cytoskeleton. Both MSP and actin form filament systems, but there is no similarity between them.
Observations: On the one hand we test how cell tension affects movement and cytoskeleton dynamics. To enable these analyses, we create transgenic worm strain carrying sperm with a fluorescent labeled cytoskeleton. We relax or tense the cell membrane with osmotic shock and we observe the effect on rugosity and speed of the sperm cell. We demonstrate that speed decreases when tension decreases. Thus the tension's decrease reduces the organization of the lamellipodium. Overall we propose that membrane tension optimizes motility by stream-lining polymerization in the direction of the movement. On the other hand, in vitro systems where cellular actin polymerization is recreated on the surface of a bead, give us actin comet tails and movement. Our goal is to apply this technology to the MSP motility system. The first step is to identify the activator of MSP polymerization (MPOP) which appears to be a membrane-bound protein that contains a phosphorylated tyrosine residue in its active form. We perform immunoprecipitation from sperm cell extracts with an anti-phosphotyrosine antibody, followed by mass spectroscopy to identify MPOP. We obtain some candidates and we will confirm them with RNAi on worms.
Conclusions: Once the activator(s) is in hand, we will produce MSP comets in sperm cell extracts. These comets will permit a characterization of MSP-based movement. In the long term, the comparison of MSP and actin based movement should lead to a better understanding of the fundamental principles cell motility.

B 028 Delivery of the cytokinetic signal to the plasma membrane – analysis of the RhoGEF Ect2

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Background: Cytokinesis is the final stage of cell division and leads to the birth of two individual daughter cells. During the process of cytokinesis, the ingression of cleavage furrow divides the cytoplasm of the mother cell.
Observations: In animal cells, cytokinesis is controlled by activation of the small GTPase RhoA, which initiates the formation of the contractile ring at the equatorial cell cortex during anaphase. It is well established that the mitotic spindle determines the position and activity of the contractile machinery at the plasma membrane. However, the precise mechanism by which the microtubule-associated signalling complexes control the cytokinetic machinery at the membrane is poorly understood. The conserved RhoGEF protein epithelial cell transforming sequence 2 (Ect2) is essential for cleavage furrow formation and RhoA activation at the equatorial cortex. Ect2, through its N-terminal tandem BRCT domains, interacts with the centralspindlin subunit MgcRacGAP leading to Ect2 recruitment to the spindle midzone. In its C-terminal region, Ect2 contains a guanine nucleotide exchange factor domain and a pleckstrin homology domain, the detailed function of either remain to be understood. We are using genetic, cell biological, and biochemical assays to address how Ect2 initiates cytokinesis at the plasma membrane in a spatially and temporally controlled manner.

Conclusions: Our analysis will provide new insights into the molecular mechanism that delivers the cytokinetic signal to the equatorial cortex during anaphase in animal cells.

B 029 On the correlation between the timing of the embryonic cell cycle and the temperature niche of an organism

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Background: Temperature is one of the fundamental abiotic factors that defines the habitat of ectotherm species. Free-living nematodes such as C. elegans are directly affected by changes in temperature. Hence they have evolved to live and reproduce despite fluctuations in temperature. Understanding how organisms can cope with varying temperature and how they adapt to new temperature niches is of particular importance with the immanent onset of global warming.

Observations: We study the effect of temperature on the reproductive fitness of C. elegans and C. briggsae. C. elegans can reproduce over a 15°C temperature range from about 10-25°C. C. briggsae isolates are also fit over a 15°C temperature range but the maximum temperature at which they can survive is higher in isolates from warmer climates. We observed that early embryogenesis is the part of the life cycle that is most temperature sensitive (at both high and low temperatures). We focused on the effect of temperature on the first asymmetric cell division of the embryo. We found that the rate of cell cycle progression, including polarity establishment, adjusts immediately to changes in temperature and that all cell cycle events remain synchronized across the viable temperature range. We also observed that at low temperature phases of the cell cycle become asynchronous and that this disorganization leads to death. These findings indicate that all aspects of an asymmetric cell division need to respond to changes in temperature to the same extent in order to survive in a habitat with fluctuating temperatures.

Conclusions: We propose that the mechanism underlying the rate of the cell cycle gives rise to the temperature niche of an organism. This suggests, that ectotherms can adapt to a different climate by fine-tuning the molecular clock that defines the rate of cell cycle progression.

B 030 An acto-myosin network driving chromosome positioning during early mitosis

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Background: Actin and myosin II play important roles during various stages of cell division. Previous studies focused on the role of cortical acto-myosin flow in centrosome separation and on acto- myosin ring contraction during cytokinesis. In addition a role for actin and myosin has been proposed within a putative spindle matrix that could bear the forces generated by microtubules and microtubule-based motors during mitotic chromosome separation.Direct evidence for such a spindle matrix has been missing.

Observations: Here we show by a combination of life cell imaging and electron microscopy that during interphase actin forms an extensive cytosolic network that holds the nucleus in place and keeps it in a pre-stressed state. During premature chromosome condensation (PCC) after treatment with the phosphatase inhibitor calyculin A, myosin II and actin contracted into a compact network around the nucleus. Inhibition of myosin II or depolymerization of actin completely blocked PCC. During mitosis, myosin II and actin form a joint three-dimensional network, which is connected to the cell cortex. This network assembles into a cage-like structure around chromosomes during prophase. Depolymerization of actin at this stage blocked mitotic progression and prevented formation of a metaphase plate. During metaphase we observed myosin II rings around the spindle poles and as motile minifilaments of myosin II along the spindle.

Conclusions: In summary we propose that a cytosolic acto-myosin network positions and mechanically stabilizes whole nuclei during interphase, as well as individual chromosomes during mitosis. This network is especially important for chromosome positioning during early mitosis.

B 031 Knockdown of a Potassium Channel Suppresses Tumorigenesis by Inducing ROS-Mediated Cell Cycle Arrest

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Background: Our work focuses on identifying factors critical to the cancer cell senescence.

Observations: In the present study, we demonstrate that knockdown of a K+ channel Kir2.2 induced growth arrest without additional cellular stress in cancer cells. Kir2.2 knockdown also induced senescence-associated beta-galactosidase (SA-beta-Gal) activity in multiple cancer cell lines derived from different tissues, including prostate, stomach, and breast. Interestingly, knockdown of Kir2.2 induced a significant increase in reactive oxygen species (ROS) that was accompanied by cell-cycle arrest, characterized by significant up-regulation of p27, with concomitant down-regulation of cyclinA, cdc2, and E2F1. Kir2.2 knockdown cells displayed increased levels of PML bodies, DNA damage (gamma-H2AX) foci, senescence-associated heterochromatin foci (SAHF), mitochondrial dysfunction, secretory phenotype, and phosphatase inactivation. Conversely, over-expression of Kir2.2 decreased doxorubicin-induced ROS accumulation and cell growth inhibition. Kir2.2 knockdown-induced cellular senescence was blocked by N-acetylcysteine, indicating that ROS is a critical mediator of this pathway. In vivo tumorigenesis analyses revealed that tumors derived from Kir2.2-knockdown cells were significantly smaller than those derived from control cells ($P < 0.0001$), and showed a remarkable increase in senescence-associated proteins, including SA-beta Gal, p27, and PAI-1.

Conclusions: Therefore, we propose that Kir2.2 knockdown induces senescence of cancer cells by a mechanism involving ROS accumulation that requires p27, but not Rb, p53, or p16.

B 032 Investigating functions of CENP-A N-tail in mitosis

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Background: The histone variant CENP-A (CenH3) is the epigenetic factor responsible for centromere determination. It allows the recruitment of a handul of centromeric proteins, and thus acts as the primary foundation for the kinetochore. It comprises an unstructured amino-terminal domain to which no precise function has yet been assigned, although it is established that the mere presence of that domain is required for proper centromere function and thus successful completion of mitosis.
Observations: Wa have established several cell lines stably expressing GFP-tagged CENP-A constructs, allowing us to perform pseudogenetic experiments by si- or shRNA-mediated silencing of the endogenous CENP-A. Our results show a dramatic increase of mitotic defects and plurinuclear cells when cells express only the globular domain of CENP-A; this is in accordance with the litterature and confirms the importance of the amino-terminal tail. More importantly, a similar increase of mitotic defects is observed when cells express a full-length, but non-phoshatable, CENP-A. Moreover, cells expressing a chimeric protein H3-CENP-A (amino-terminal tail of H3 fused to the globular domain of CENP-A) don't exhibit mitosis defects except when Serine 10 and Serine 28 of the H3 tail are mutated to Alanine.
Conclusions: Our results show the involvement of the phosphatable serine 7 of CENP-A in the successful completion of mitosis, and may suggest that the role of the whole amino-terminal tail of CENP-A could be reduced to this single phosphorylation event.

B 033 FRET-biosensor-based RNAi screen reveals PP1-mediated stabilization of anaphase chromosome segregation

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Background: Aurora B is a conserved mitotic kinase that translocates from chromosomes to the central spindle at anaphase onset. A spatial gradient of Aurora B substrate dephosphorylation on segregating chromosomes suggests an important function of yet unknown Aurora B-counteracting phosphatases.
Observations: Based on a FRET biosensor for Aurora B substrate phosphorylation (Fuller et al., Nature, 2008), we designed an assay to detect regulators of Aurora B phosphorylation on mitotic chromatin in human cells. Using automated live cell microscopy and the CellCognition machine learning software (Held et al., Nature Methods, 2010), we screened a siRNA library targeting a genome-wide set of phosphatases for altered Aurora B substrate phosphorylation. We identified two PP1 regulatory subunits, Repo-Man and Sds22, to be essential for timely dephosphorylation of Aurora B sites on chromatin during anaphase. Depletion of Repo-Man or Sds22 had no effect on FRET biosensor phosphorylation during early mitotic stages up to metaphase and on transfer of Aurora B to the central spindle at anaphase. The delayed substrate dephosphorylation kinetics on chromatin resulting from Repo-Man or Sds22 depletion, however, correlated with inefficient and frequently paused chromosome segregation during anaphase.
Conclusions: Our data suggest a model where after removal of Aurora B from chromosomes at anaphase onset, Repo-Man/Sds22-PP1 contributes to persistent force generation at the microtubule-kinetochore interface for faithful chromosome segregation.

B 034 The recruitment of BAF to chromatin during mitotic exit is regulated by dephosphorylation

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Background: Barrier-to-autointegration factor (BAF) is a conserved chromatin binding protein which links chromatin to the NE through interactions with inner nuclear membrane (INM) proteins. Hyperphosphorylated BAF is displaced from chromatin early during mitosis; this helps to sever the link between chromatin and the NE. It is however unclear how the recruitment of BAF to chromatin and to INM proteins is re-established upon mitotic exit.
Observations: We have used live-cell imaging and biochemical techniques to study the mechanism by which BAF is recruited to chromatin upon mitotic exit in human cells. We found that RNAi-mediated depletion of a previously uncharacterised protein (BAF-targeting, BAF-TA) prevents efficient recruitment of BAF to chromatin upon mitotic exit. We identified interaction partners of BAF-TA that are important for the dephosphorylation and chromatin recruitment of BAF. We propose a model in which the efficient recruitment of BAF to chromatin is dependent on dephosphorylation mediated by BAF-TA. We have also shown that this mechanism operates in the nematode worm C. elegans.
Conclusions: We have identified a conserved mechanism that regulates BAF chromatin recruitment during mitotic exit in human cells and C. elegans. We propose that this represents a novel feedback mechanism coupling NE reassembly to BAF chromatin recruitment.

B 035 Human papillomavirus oncoprotein E7 targets the cytoskeleton-nucleus interface to induce centrosome dysfunction

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Background: Centrosome aberrations are a typical feature of tumor cells. In order to test whether such defects can indeed cause oncogenesis, it is necessary to elucidate the centrosome-regulating mechanisms and how they are abrogated in oncogenesis. The aim of this study is to characterise the molecular machinery mediating centrosome reduction, i.e. the removal of

pericentriolar matrix (PCM) that occurs at the mitosis-interphase transition, and to assess its role in cellular transformation.

Observations: The comparative study of high- and low-risk HPV E7 variants revealed a previously uncharacterised activity of the oncoprotein that results in massive reduction of centrosomal PCM, loss of microtubule nucleation capacity and, consequently, disorganisation of the microtubule cytoskeleton. This activity was specific for high-risk HPV E7 and governed by the CR3 domain. Two homologous chromodomain helicase DNA binding proteins (CHD) were identified as E7 targets in an RNAi screen and the CHD domain required for centrosome reduction was identified. Additional components of the CHD-E7 complex were isolated by immunoprecipitation and identified by mass spectrometry. The identified proteins cluster into functional groups involved in protein folding, RNA binding/Splicing, nuclear matrix organisation and actin-microtubule cytoskeleton cross talk.

Conclusions: High- but not low-risk HPV E7 induces centrosome dysfunction,indicating a potential role in oncogenesis. This effect is independent of pRB inactivation since mediated solely by the CR3 domain. Proteins that link nuclear processes to the cytoskeleton are targeted in E7-induced centrosome dysfunction.

B 036 NONO couples the cell cycle to the circadian clock via p16INK4A

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Background: We have identified NONO as a member of a circadian clock protein complex that also contains the known clock proteins PERIOD and CRYPTOCHROME. Depletion of NONO in cells or mutation of its homolog in flies resulted in attenuation of circadian rhythmicity, possibly due to antagonism of PERIOD-mediated transcriptional repression. Previously, this protein has been implicated in diverse processes of transcriptional regulation as well as in RNA transport and metabolism.

Observations: To investigate NONO function both within and outside the circadian oscillator, we generated a Nono-genetrap mouse line. It possessed a shortened circadian period length when compared to wildtype mice in free-running (dark/dark) conditions. In fibroblast cells derived from Nono-genetrap mice, we also observed resistance to senescence as well as an accelerated cell doubling rate. We found that NONO directly regulates p16INK4A expression, a critical component of the mitogen-responsive retinoblastoma pathway, and is in fact responsible for gating of S phase to a specific time window during the day. Furthermore, mice with a defective clock or lacking NONO displayed impaired wound healing due to hyperproliferation of keratinocytes and fibroblasts, but reduced collagen deposition. While investigating NONO, we also discovered that it has two homologs, SFPQ and PSPC1. Misregulation of each of these transcript levels via RNAi or overexpression experiments led to perturbation of circadian rhythmicity, implying a crucial role for these homologs in the circadian clock.

Conclusions: We demonstrate that NONO couples the cell cycle to the circadian clock by regulating exit from the G1 phase. It acts as a transcriptional regulator of p16INK4A, a G1-S phase checkpoint gene. Furthermore, we show that homologs of NONO are involved in transcriptional regulation of the circadian clock.

B 037 Re-modelling the actin cortex through mitosis

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Background: Before an adherent cell divides it dramatically changes shape by detaching from the substrate and 'rounding up'. This process is driven by the re-modelling of actin filaments to form a cortical shell, which gives a mitotic cell its characteristic spherical shape and rigidity. Although actin re-modelling at mitosis is crucial for spindle positioning, chromosome capture and equal division into two daughter cells, it is not known how this process is controlled.

Observations: We carried out an siRNA screen in hela cells to find genes that affect mitotic cell shape, which identified Ect2 as a key regulator. Ect2 is a RhoGEF that plays a known role in cytokinesis by locally activating RhoA to generate the contractile actin-myosin ring that drives cell division. We show that Ect2 also acts early in mitosis to control rounding and re-modelling of the acto-myosin cortex. Ect2 RNAi cells, as well as those with reduced RhoA, Rho Kinase or Myosin II activity, have delayed mitotic rounding and a disorganized mitotic cortex. In addition, optical stretching experiments show that they are softer and more deformable than control cells. Thus, as well as positioning the cytokinetic ring at anaphase, Ect2, upstream of RhoA & myosin, also regulates actin in early mitosis. However unlike at cytokinesis, neither microtubules nor MgcRacGAP, which bring Ect2 to the spindle midzone at anaphase, are required for mitotic rounding.

Conclusions: These data lead us to propose a model whereby Ect2 activates RhoA diffusely in early mitosis to build a rigid acto-myosin cortex. Then, at anaphase, the contractile machinery is repositioned to the spindle midzone by MgcRacGAP where it controls formation of the acto-myosin ring.

B 038 Why should we line up?

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Background: As human cells get ready to enter anaphase, they align chromosomes on a metaphase plate. In the metaphase plate chromosomes oscillate along the spindle axis. After having traveled 2-3 micrometer for about 35-50s, chromosomes switch direction. The amplitudes of chromosome oscillations determine the metaphase plate width, which decreases on average to 2.4 micrometer just before the anaphase onset. One unresolved key question is why is it important to align chromosomes in such a narrow structure.

Observations: The potential purposes of a thin metaphase plate we aim to investigate are: faithful chromosome segregation, precise establishment of the cytokinetic machinery, and the conservation of nuclear architecture. To test these hypotheses

we established different perturbations to lengthen chromosome oscillations, and therefore give rise to wider metaphase plates. These perturbations include: a) the depletion of a small portion (20% of the total level) of the kinetochore protein CENP-H, where chromosomes are oscillating with a period that is twice as long as an unperturbed system, giving rise to a 40% wider metaphase plate, and b) decrease of the temperature to 32°C (instead of 37°C), which causes similar widening of the oscillation period and metaphase plate.Our results based on live-cell imaging indicate that a widening of the metaphase plate does not significantly perturb cytokinesis, based on the localization of critical proteins, such as the chromosome passenger proteins or the timing and localization of the cleavage furrow. Similarly, our preliminary results indicate that a wider metaphase plate does not lead to severe chromosome segregation defects.
Conclusions: However, we are currently establishing more rigorous assays to test for small increases in the rate of chromosome segregation errors, as well as the assays that will allow us to address whether the nuclear architecture is conserved in cells that lack narrow metaphase plates.

B 039 Polo-dependent phosphorylation of Centrosomin drives centrosome maturation in Drosophila

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Background: Centrosomes in mitosis mature through the addition of pericentriolar material (PCM) the site of MT-nucleation and anchoring. Mitotic centrosomes control the bipolar spindle formation by organising the MT-cytoskeleton and by the creation of spindle and astral MTs. Astral MTs contact the cell cortex and position the mitotic spindle according internal and external cues. How PCM recruitment is initiated and modulated in mitosis is not well understood.
Observations: Our findings show that Polo kinase phosphorylation of the major Drosophila PCM protein centrosomin (Cnn) is key for PCM nucleation on top of centrioles when cells enter mitosis. Constant Polo phosphorylation is needed to maintain Cnn at the centrosome. Cnn phosphorylation only takes place on the centriole scaffold. Cnn is phosphorylated at serine 567. Mutation abolishing phosphorylation results in a strong reduction of the PCM. In contrast, mutation of serine 567 mimicking constitutive phosphorylation is very toxic and induces PCM formation during all cell cycle stages. Ectopic expression of active polo to interphase centriole induces PCM recruitment and aster formation.
Conclusions: Thus, phosphorylation of Cnn by Polo in mitosis is both needed to initiate and maintain PCM in Drosophila.

B 040 Control of mitosis by microRNAs

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Background: Progression through the cell cycle depends on oscillating transcription and translation of many genes. Because microRNAs trigger mRNA degradation and inhibition of translation, they are interesting candidates to regulate cell cycle transitions. A growing body of evidence indeed shows that microRNAs play an important role in the control of cell cycle progression, particularly in the G1/S transition. Nevertheless, the role of microRNAs in the regulation of mitosis remains vastly unexplored.
Observations: To define the role of microRNAs in mitosis, we performed a live-cell imaging-based genome-wide gain-of-function screen using microRNA mimicking oligomers. This led to the identification of 30 candidate microRNAs that induced a statistically significant mitotic delay upon overexpression. To investigate the molecular basis of the observed phenotypes, we performed microarray based analysis to detect changes in the transcriptome under overexpression of the top-ranking candidates. We found several genes that bear seed matching sites, are down-regulated upon overexpression of the respective microRNA and have ontologies related to the observed phenotypes. In a complementary approach to identify cell cycle-relevant microRNAs, we deep sequenced small RNAs from samples of synchronized HeLa (a model human cancer cell line) and RPE1 (a model diploid non-cancer human cell line) cells. This revealed several microRNAs with cell-cycle-dependent expression, which was much less pronounced in Hela cells, as compared with RPE1.
Conclusions: This findings suggests that miRNAs play a role in regulation of mitosis and that deregulating cell cycle-dependent patterns of microRNA expression may contribute to cancer cell transformation.

B 041 Functional characterisation of a centrosome protein interaction network relating to centrosome biogenesis and aberration in cancer cells

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Background: The centrosome serves as the primary microtubule-organising centre in eukaryotic cells and is implicated in a range of fundamental cellular processes. Its function, replication, segregation and structure maintenance depend on regulatory proteins of the cell cycle and other cellular signalling pathways. Centrosomal aberrations and dysfunction of centrosomal proteins frequently occur in various diseases. Especially in cancer cells centrosome morphology and number are frequently deregulated.
Observations: The origin of most of these aberrations on a molecular level is unclear but there is a strong link between centrosome defects and deregulation of cell cycle regulatory kinases, tumor suppressors and oncogenes. In contrast,

mutations in genes coding for centrosome proteins that cause centrosome hypertrophy are rare. Hence, we investigated the protein-protein interaction network of some of the major centrosomal proteins in human cells to identify dependencies that functionally relate to centrosome abnormalities. Tandem affinity purification mass spectrometry (TAP-MS) identified three major hubs in the centrosome interaction network: TUBG1 (11 interactions), TUBGCP3 (11 interactions) and CEP250 (10 interactions) suggesting that interacting proteins are in close functional relationship or modulate interaction of these proteins as adaptor proteins. The network also identified a series of new centriolar and centrosome candidate proteins.

Conclusions: Functional analysis revealed a dual role for a subclass of the identified proteins for both centrosome hypertrophy and centriole biogenesis in human cancer cells and patients tissues. Based on our biochemical and functional data we are suggesting a pathway for centrosome hypertrophy in cancer cells.

B 042 Molecular architecture and mechanical properties of the kinetochore: a biophysical approach

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Background: Accurate chromosome segregation depends on proper attachment of kinetochores (KTs) to microtubules (MTs). Mounting evidence suggests that mechanical properties of the KT make fundamental contributions to faithful segregation of chromosomes. Merotelic KT orientation is an error in which a single KT binds MTs emanating from opposite spindle poles. In this project we want to establish merotelic KTs as a model for studying structural and mechanical properties of the KT.

Observations: 1) We will use fluorescently labeled kinetochore proteins representing major kinetochore complexes to determine their localization pattern at merotelic kinetochores. Our primary aim will be to establish which kinetochore complexes are laterally stretched upon merotelic attachment. 2) We argue that if we sever microtubules attached to a stretched merotelic kinetochore on one side, the change of the kinetochore shape should reflect its elasto-plastic properties. We expect that an elastic kinetochore should resume its normal globular shape shortly after the laser severing of the microtubules, whereas a plastic kinetochore will retain its stretched shape. 3) We will perform these experiments both in the fission yeast S. pombe and in mammalian tissue culture cells (PtK1). This will allow us to identify features that are either distinctive or evolutionarily conserved.

Conclusions: Studying mechanical properties of the kinetochore may reveal important kinetochore features relevant to many aspects of cell division and help to understand how the cell prevents and corrects merotelic attachments, a major mechanism of aneuploidy in mitotic cells.

B 043 Trypanosoma brucei Sec16 – Size Matters

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Background: The Trypanosome cell has a highly organized and polarized architecture, in which positions, sizes and shapes of its intracellular organelles are relevant for their cellular functions. However, how the cell regulates the size of the intracellular structures is poorly understood. Newly synthesized secretory proteins are exported from the ER at a specialized region known as the ER exit site (ERES) where COPII transport vesicles are produced.

Observations: We have identified a putative Sec16 in Trypanosoma brucei: a large peripheral endoplasmic reticulum (ER) membrane protein, which is required for the generation of COPII transport vesicles. Sec16 has also been proposed to be involved in the biogenesis of the ER exit site (ERES), acting as a key player in the early steps of the assembly process. Here, we show by epifluorescence microscopy that TbSec16 localizes to the ERES. We also demonstrate that TbSec16 is involved in the regulation of the size of the ERES. Overexpression leads to an enlarger ERES and this effect seems in turn to make a bigger Golgi. Conversely, knockdown of TbSec16 expression decreases the size of the ERES and the Golgi. Interestingly, the protein levels of Golgi stack proteins and other COPII proteins localized to the ERES do not appear to change by western blotting.

Conclusions: Based on these observations, we propose that TbSec16 might be involved in controlling the size of ERES and Golgi two intracellular structures positioned on the protein transport pathway.

B 044 Identification of a molecular link between the mitotic spindle and the plasma membrane during cytokinesis

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Background: Cytokinesis is the final step of cell division that leads to the birth of new daughter cells. It involves the ingression of a cleavage furrow and a subsequent membrane fusion reaction, called abscission. Although both cleavage furrow ingression and abscission occur at the plasma membrane, key conserved cytokinetic regulators are associated with the mitotic spindle. A key but unresolved question is how microtubule-associated protein complexes control cytokinetic events at the cell membrane.

Observations: The centralspindlin complex is a major component of the spindle midzone and midbody, two iconic microtubule-based structures that are required for cytokinesis in animal cells. Centralspindlin plays a key role in assembling these structures and in recruiting effector proteins that control cleavage furrow formation and abscission. To investigate how centralspindlin controls cytokinetic events at the plasma membrane, we developed a genetic complementation system for the centralspindlin subunit MgcRacGAP in human cells. We discovered a conserved C1 domain in MgcRacGAP that is essential for cell division. Deletion of this domain and point mutations therein

abrogate the execution of cytokinesis after furrow ingression. MgcRacGAP's C1 domain belongs to the atypical family and, unlike typical C1 domains, is not responsive to phorbol esters. We show that the atypical C1 domain of MgcRacGAP acts as a constitutive membrane targeting domain in vivo and binds to phospholipids in vitro. Mutations in the C1 domain that prevent cytokinesis invariably abolish membrane targeting activity. Finally, artificial membrane tethering of MgcRacGAP restores cell division in the absence of C1 domain function.
Conclusions: We propose that the atypical C1 domain of the centralspindlin subunit MgcRacGAP links the mitotic spindle to the plasma membrane during cytokinesis. Our work provides the molecular basis for a long-proposed function of the midbody: stabilizing the cleavage furrow to allow abscission to occur.

B 045 WD40 domain repeat protein 62 (WDR62) is a phosphorylated spindle pole protein required for centrosome cohesion during metaphase-anaphase transition

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Background: Centrosomes consist of a centriole pair surrounded by the pericentriolar matrix and are involved in regulating mitotic spindle polarity and fidelity in mammalian cells. The importance of these functions is highlighted by genetic studies linking abnormal neuroprogenitor cell division overwhelmingly with mutations in centrosome-associated proteins. Recently, WDR62 mutations were linked to human brain malformation although the precise roles of WDR62 in the dividing cell remain uncharacterized.
Observations: We show that WDR62 is cytosolic in interphase HeLa cells, but during mitosis, prior to nuclear envelope breakdown, accumulates around the centrosome in a microtubule-dependent manner. This localization of WDR62, which overlaps with NUMA and p150Glued, persists until the metaphase-anaphase transition. Using RNA-interference to probe WDR62 function, we demonstrated defects in centrosomal positioning and integrity. Specifically, metaphase centrosomes were displaced from the spindle poles and became fragmented in the absence of WDR62. Mitotic progression, specifically metaphase-anaphase transition, was delayed, but WDR62 depletion did not induce cell cycle arrest, prevent chromosome segregation or induce cytokinesis defects. We found that WDR62 was phosphorylated during mitosis and this correlated with its translocation to the centrosome. Surprisingly, Plk1 depletion or inhibition did not prevented WDR62 phosphorylation while JNK1/2 siRNA and JNK-specific inhibitors partially reversed WDR62 phosphorylation in mitosis. JNK-mediated WDR62 phosphorylation was not required for centrosome-associated localization but may be involved in WDR62 interaction with gamma-tubulin complexes.
Conclusions: Our results indicate that JNK signalling regulates WDR62 interaction with the PCM to maintain centrosome integrity, attachment to the spindle pole and normal mitotic progression. Notably, this is a novel mechanism underlying JNK regulation of centrosomes in early stages of mitosis.

B 046 The role of ORC1 in regulation of DSB repair in drosophila salivary gland cells

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Background: ORC1 is the key component of the origin recognition complex (ORC) and is essential for replication initiation and cell proliferation. However, it was previously shown, that ORC1 is not required for endoreplication in drosophila salivary gland cells. We induced RNAi-mediated Orc1 knock-out in salivary glands (AB1-GAL4 driver) and analyzed its cytological phenotype.
Observations: Normally, late-replicating regions that mostly contain silenced genes appear as breaks on polytene chromosomes. These regions fail to complete replication due to shortened S-phase in endocycles and become fragile because of the increased number of unrepaired DSBs, probably caused by stalled replication forks. We found that, when Orc1 is suppressed by RNAi in salivary glands, the frequency of chromosomal breaks is significantly lower. We hypothesized that in Orc1 knock-outs these regions might be polytenized above normal. To test this hypothesis we measured the levels of polyteny of four typical chromosomal break regions in Orc1 knock-outs compared to those in wild type. Surprisingly, despite the absence of breaks in Orc1 knock-outs, the level of polyteny in tested regions was not higher than in wild type. The most probable explanation for this paradox could be that ORC1 depletion triggers DSB repair via NHEJ. Consistent with this idea, Orc1 deficient chromosomes demonstrate increased signals when stained with antibodies against the phospho-H2Av histone, known to mark the initial stages of repairation. In this work we also analyzed the chromosomes of Ligase4/Orc1 double mutants.
Conclusions: Our data suggest that ORC1 may be involved in the regulation of DSB repair process in drosophila salivary gland cells. Notably, this new Orc1 function is independent from other ORC components: Orc2 down-regulation in salivary glands does not affect chromosomal breaks frequency.

B 047 An Eg5/Kif15-dependent kinetochore-coupling matrix is essential for metaphase plate organization and chromosome segregation

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Background: During human cell division, chromosomes are bound by dynamic spindle microtubules (MTs) through kinetochores and aligned one the metaphase plate. Kinetochore-tracking in HeLa cells indicates that kinetochores are not positioned in randomized manner, but that they form a well-defined doughnut-shaped metaphase plate. Sister-kinetochores display constrained diffusion within the plate and they never exchange position with neighboring sister-kinetochore pairs, implying a higher-order structure.

Observations: When analyzing the movements of non-sister kinetochores we demonstrate by correlation analysis that non-sister-kinetochores do not behave in an autonomous manner. Instead, they show a distance-dependent motion coupling. This suggests the existence of physical linkages that transmit forces between non-sister-kinetochores. Our perturbation-based analysis of the MT-cross-linking proteins PRC1, Eg5, Kif15 or HSET, chromokinesins or the spindle matrix protein TPR, indicate that this coupling activity is controlled in an antagonistic manner by Eg5 and Kif15. While Eg5 inhibition increases the motion coupling between non-sister kinetochores, Kif15 depletion decreases it. Further analysis shows that Eg5 inhibition in metaphase abrogates sister-kinetochore oscillations and causes a shrinking of the metaphase plate. Eg5 inhibtion does, however, not affect spindle length or inter-kinetochore distances, indicating a normal spindle morphology and a correct force generation within the spindle. Strikingly, when Eg5-inhibited cells enter anaphase, we observe a 3-fold increase in chromosome segregation errors, and in 15% of the cells, a complete arrest of chromosome segregation in early anaphase.
Conclusions: We postulate the existence of MT-dependent cross-linking matrix that transmits forces within the spindle via the kinesin-12 Kif15, and under the control of the kinesin-5 Eg5. This matrix is essential for correct kinetochore movements, metaphase plate organization and faithful chromosome segregation.

B 048 Misregulation of cell cycle, DNA repair and chromatin machinery during cellular senescence

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Background: Cellular senescence is a dynamic tumor suppression mechanism that limits the proliferation of impaired cells, by executing a stable cell cycle arrest. Understanding the molecular pathways and regulatory circuits that are involved in the process of senescence is presently incomplete. In this study, we determined the changes in gene expression during the establishment of replicative senescence in endothelial cells, by comparing the expression profiles of young and senescent HUVECs.
Observations: Exploration of array data using ingenuity pathway analysis showed that genes involved in cell cycle regulation, cellular assembly and organization, DNA replication, recombination and repair were significantly down regulated during replicative senescence. Dominant number of genes including cyclins, and CDK's that drive progression of cell cycle CCNA2, CCE2, CDC25A (G1/S transitions), CCB1, CCB2, CDK1, CDC25C, FOXM1 (regulating G2/M phase) and novel genes that drive G2/M transition including CDC2, CDC20, PLK1, and 5 genes CDC25C, AURKB, PRC1, SMC4 and MAD2L1 that regulate mitosis and mitotic chromosome structure were significantly down regulated. In addition, expression of genes associated to DNA damage check point regulation and DNA repair including EXO1, XRCC2, RAD51, RAD51AP1, RAD54L, TOP2A, BRAC2, FANCD2 and Replication Factor C variants 3, 4 were decreased. A significant number of genes involved in chromatin regulation, chromosome segregation and mitotic assembly, also showed altered gene expression. Interestingly, many of cell cycle regulatory genes previously identified as transcriptional repressional targets of p53 were down regulated during replicative senescence.
Conclusions: Our data suggest that activation p53 during cellular senescence may be important for repressing its specific gene targets that regulate cell cycle progression. Defective DNA repair mechanism and misregulated mitotic machinery are potentially involved in regulating the complex senescence phenotype.

B 049 Sgo1 is required for co-segregation of sister chromatids during achiasmate meiosis I

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Background: During meiosis single round of DNA replication is followed by a reductional division (meiosis I) and an equational division (meiosisII) resulting in 4 haploid gametes from original diploid cell. The three major features of meiosis I leading to reductional division are namely formation of chiasmata which physically link homologous chromosomes, monoorientation of sister kinetochores and protection of centromeric cohesion. This protection of cohesion is mediated by conserved Sgo1/MEI-S332 proteins.
Observations: We found that during haploid meiosis, sgo1Δ cells frequently segregated sister chromatids to opposite poles in anaphase I. However, previous experiments showed that sgo1Δ cells efficiently co-segregate sister chromatids to the same pole during diploid meiosis I. We therefore decided to test the possibility that Sgo1 is required for co-segregation of sister chromatids during meiosis I in the absence of chiasmata. Indeed, we found that fission yeast cells lacking both chiasmata (rec12Δ) and the protector of centromeric cohesion (sgo1Δ) frequently segregated sister chromatids to opposite poles during diploid meiosis I. This equational segregation is likely caused by the absence of chiasmata and not due to a potential role of Rec12 at centromeres, because we observed a similar phenotype in mde2Δ mutant cells, which are also defective in chiasma formation.
Conclusions: Sister kinetochores frequently bi-orient during achiasmate meiosis I but fail to segregate to opposite poles due to centromeric cohesion resisting pulling forces of the spindle. Absence of Sgo1 resolves this cohesion in anaphase I, allowing segregation of the sister chromatids to opposite poles.

B 050 Molecular mechanism of Ndc80 recruitment to kinetochores by a conserved CENP-T/W complex

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Background: Kinetochores are large proteinaceous complexes that physically link centromeric DNA to the plus-ends of spindle microtubules. Stable kinetochore-microtubule attachments are required for accurate distribution of the genetic material. At the core of kinetochore function in all eukaryotes lies the supramolecular KMN network. A critical question is how the

KMN network is anchored to centromeric chromatin, and which molecular mechanisms regulate this association over the cell cycle.
Observations: Here, we have used bioinformatic and proteomic analysis in combination with biochemical reconstitution experiments to elucidate the molecular mechanisms by which the KMN network is anchored to budding yeast kinetochores. Our analysis indicates that centromere-binding proteins are highly conserved between yeast and human cells and we identify a universal constitutive-centromere-associated network (CCAN) of proteins. Systematic tandem affinity purifications indicate distinct points of contact between CCAN and KMN. We characterize a novel budding yeast protein YDR374W-A as a CENP-W related protein and show that it is required for the localization of a CENP-T homolog to the kinetochore. Yeast CENP-T uses a conserved binding motif to directly anchor the microtubule-binding Ndc80 complex via an interaction with the Spc24-25 globular domain.
Conclusions: Our analysis indicates a universal building plan for kinetochores in which the KMN network is part of a conserved unit attachment fibre that combines microtubule-binding and DNA-binding elements.

B 051 Stepwise and additive multisite phosphorylation of Cdc25 regulates Cdc25 activity and controls mitotic timing

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Background: Computer models and computations from cell extracts predict that Cdk1 and Cdc25 activate one another in a positive feedback loop to drive mitotic entrance. How this loop contributes to the robustness of the mitotic entry switch has not been tested in live cells. In addition, Cdc14 dephosphorylation of Cdk1 substrates function in a positive feedback loop in initiating mitotic exit; however, contributions of Cdc14 interaction with Cdc25 to the mitotic exit switch have not been explored.
Observations: Here we use Schizosaccharomyces pombe to study in vivo interaction between Cdc25 with Cdk1 and S.pombe Cdc14 homologue, Clp1. We find that disrupting Cdc25 phosphorylation by Cdk1 radically elongates the time to mitotic entry within single cells and removes synchrony of the mitotic entry switch within the population. In addition, we define the mechanism of Cdc25 activation, showing that Cdk1 phosphorylation increases specific activity of Cdc25 and this activation is efficient when at least 6 out of 13 putative Cdk1 sites on Cdc25 are phosphorylated. Additional Cdk1 phosphorylation of Cdc25, after satisfying the initial 6 phosphorylations, further activates Cdc25 in an additive manner. Finally we show that Clp1 dephosphorylation of Cdc25 controls the time to mitotic exit, suggesting that the two proteins function as a double-negative feedback loop that contributes to the robustness of the mitotic exit switch.
Conclusions: Cdk1 phosphorylation increases Cdc25 specific activity in stepwise and additive manners. This activation is vital in controlling the switch-like timing of mitotic entrance. Conversely, Clp1 interaction with Cdc25 may be an important feedback loop that regulates timing in the mitotic exit switch.

B 052 Reconstructing ancestral enzymes reveals how gene duplication resolves drives evolutionary innovation

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Background: Gene duplication is widely recognized as a primary driving force of evolutionary innovation. Duplication can increase gene function (dosage effect); duplication can split the ancestral function over two genes (subfunctionalization), and –most excitingly- duplicates can develop novel functions (neofunctionalization). However, the precise molecular mechanisms and evolutionary forces by which duplication stimulates innovation are still fiercely debated, and many different models have been proposed.
Observations: To investigate the precise molecular mechanisms underlying evolutionary innovation by gene duplication, we studied a large family of fungal glucosidase genes in which multiple duplication and divergence events generated an arsenal of enzymes that allow growth on two broad classes of carbohydrates. We reconstructed the different pre-duplication ancestral enzymes and show that the very first pre-duplication enzyme was primarily active on maltose-like substrates, but also showed minor activity for isomaltose-like sugars. Subsequent gene duplications spawned novel genes in which the isomaltase activity was optimized through adaptive mutations at the expense of the ancestral maltase activity. By contrast, other paralogs show positive selection for the ancestral maltase function, which is further optimized at the expense of the minor isomaltase activity. Structural analysis of the enzymes reveals that a few key residues in the active site determine substrate preference and prevent optimization of both functions in one molecule.
Conclusions: Our results provide an unprecedented level of detail in the molecular mechanisms that occur during the evolution of duplicated genes in eukaryotes. More specifically, we show how gene duplication allows escape from an adaptive conflict and facilitates the development of a novel function.

B 053 Quantitative phenotyping of C. elegans reveals new genes affecting behaviour

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Background: Visible phenotypes have played a critical role in understanding the molecular basis of behaviour and development in the nematode worm C. elegans. However, many mutants have subtle phenotypes that are difficult or impossible to see by eye.
Observations: To broaden the applicability of this powerful approach to genetics, we are using an automated system to record high-resolution video of freely behaving worms and a computer vision system to identify and classify worms based on features of their movement, morphology, and posture yielding a rich phenotypic 'fingerprint'. Analysis of this fingerprint provides the most detailed view to date of the behavioural rep-

ertoire of C. elegans and defines a baseline for comparison to mutants. To this end, we have compiled a database comprising video data from several hundred mutant strains that reveal many previously undescribed phenotypes that can be clustered into related classes. Since mutants in the same class may have related functions, phenotypic clustering leads to new hypotheses for gene function.

Conclusions: Behavioural fingerprints provide a complementary view of genetic interactions to that provided by the high-throughput gene expression data from the modENCODE consortium.

B 054 Comparative genomics to explore evolutionary history of gonadotropin-releasing hormones and their receptors

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Background: Many GnRH and GnRHR genes have been identified using conventional biochemical tools in combination with bioinformatic tools. Phylogenetic approaches, based on amino acid sequence identity, classify these GnRHs and GnRHRs into several lineages. However, phylogenetic analyses cannot fully explain the evolutionary origins of each lineage and the relationships among the lineages. This study delineates the evolutionary mechanism of vertebrate GnRHs and GnRHRs based on extensive genome comparison.

Observations: Genome synteny, combined with phylogenetic analyses of teleost fish and tetrapods, revealed that two rounds of genome duplication events may have generated three vertebrate lineages of the GnRH peptides GnRH1, GnRH2, and GnRH3. The GnRH3 gene has been lost in the tetrapod. For GnRHRs, two rounds of duplication of an ancestral GnRHR generates four GnRHR lineages: GnRHRn1, GnRHRn2, GnRHRn3/m2, and GnRHRm1. GnRHRn1 and GnRHRn2 are likely generated by a local gene duplication. GnRHRn3/m2 arose by first or second round genome duplication from a common ancestor. GnRHRm1 likely translocated from either the third or fourth genome fragments to other chromosomes. Teleost-specific genome duplication has produced the fish-specific GnRHR lineages, GnRHRn1b and GnRHRn3b.

Conclusions: Together, GnRH and GnRHR provide an excellent model for understanding the molecular evolution of peptide ligands and their receptor pairs at the genome level.

B 055 Modeling Morphology Dynamics in Live Cell Microscopy by Unsupervised Learning

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Background: Analysis of cellular phenotypes in imaging-based screening conventionally involves supervised statistical methods, which require substantial amounts of user-annotated training data. The application of supervised methods thus remains confined to cases in which preexisting knowledge about the phenotypes is available.

Observations: Building on previously developed methods for cell segmentation, feature extraction, and tracking over time, we here introduce an annotation-free unsupervised method aimed at the identification of distinct classes of cellular morphologies. From image sequences of human cells expressing a fluorescent chromatin marker, morphologies are characterized by a Gaussian mixture model. The estimation is initialized by a combinatorial clustering algorithm that takes advantage of the strong temporal constraints of the system. The procedure constitutes the basis for the identification of Hidden Markov models, which describe cellular dynamics by incorporating both spatial and temporal information. The method is highly competitive compared to state-of-the-art supervised approaches for automated classification of cell cycle phases.

Conclusions: Our unsupervised method efficiently and accurately classifies cellular morphologies in movies of human cells. This enables new biological assays in image-based systems biology, particularly when ground truth of underlying morphology classes is unattainable.

B 056 Born this way: Insertions and deletions in non-coding DNA are equally deleterious

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Background: Elucidating the mechanisms of mutation accumulation and fixation is critical to understand the nature of genetic variation and genetic disease. To date, knowledge of processes shaping human genome evolution has primarily relied on analysis of single nucleotide changes, despite evidence that insertions and deletions (indels) lead to more differences between human and chimpanzee. Here we seek to determine the evolutionary forces shaping indel variation identified in the 1000 Genomes Pilot 1 data.

Observations: We analyze the ~2 million short (1-50 base pair) indels segregating in human populations by applying standard population genetic tests designed to detect changes in allele segregation frequencies that could reflect the signatures of either natural selection or biased gene conversion (BGC). We find that indel rates and derived allele frequencies (DAF) are highly dependent on the local DNA context. Interestingly, we discover a strong signature of purifying selection on indels in functional sequences, including coding exons and conserved non-coding sequences that likely harbor regulatory elements. However, we find no significant evidence for genome-wide selection against deletions as compared to insertions. Rates and DAF appear to be more dependent on context than on the local GC content and crossover rates, thus, the data do not provide conclusive support for biased gene conversion altering the fixation probabilities of either insertions or deletions.

Conclusions: The trends observed here thus allow us to infer the forces contributing to genome-wide variation in indel polymorphism and to the evolution of mammalian genome architecture.

B 057 An evolutionary model of myosin V-based transport in opisthokonts

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Background: In budding yeasts, class V myosin motors facilitate the bud-directed movement of most organelles, astral microtubules and some mRNA's. The recruitment of myosin V to each of its cargoes is mediated by cargo-specific adaptor complexes that bind to conserved patches on the surface of the carboxyl-terminal globular tail domain of myosin V. Efficient transport of cargoes carried by class V myosins requires tight control and regulation of its attachment to and detachment from its different cargoes.

Observations: To provide an evolutionary perspective on how regulation of the interaction between myosin V and its diverse cargoes is achieved, we have performed a comparative genomic and phylogenetic analysis on the class V myosins and their cargo-specific receptor complexes in opisthokonts. A common pattern that emerged from our comparative genomic survey is that the composition of myosin V receptor complexes typically consists of a conserved biogenic protein that is paired with an evolutionary novel adaptor protein. A phylogenetic analysis of the class V myosins demonstrated that all existing class V myosins in opisthokonts originate from a common ancestor. Combining these data, we were able to map the evolutionary conservation of receptors and their binding sites on the primary structure of the myosin V tail.

Conclusions: Our findings suggest an evolutionary mechanism for how diverse cargoes compete for access to myosin V. We propose the emergence of myosin V-based transport has utilized mechanisms of paralogy, the exploration of sequence space and the appearance of pliable, evolutionary novel adaptor proteins.

B 058 Changes in gene expression following segmental duplication in mammals

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Background: Gene duplications are powerful drivers of evolution that allow novel functions to emerge from a state of initial genetic redundancy. They were shown to be abundant in many organisms and to have contributed to their phenotypic diversity. Here, we focus on genes that emerged through DNA-based (segmental) duplication within mammals and study the evolution of their expression patterns in representatives of all major mammalian lineages; placental mammals, marsupials and the egg-laying monotremes.

Observations: We rely on a unique transcriptome dataset generated for six organs from eight mammalian species and a bird (the evolutionary outgroup) using RNA sequencing. Using a dedicated pipeline, we computed expression profiles for all protein-coding genes. We retrieved gene trees from the Ensembl database and identified duplication events and their respective ages using a phylogenetic approach. We observe that mammalian lineage-specific duplicated genes have on average lower expression levels and are more tissue-specific than their non-duplicated orthologous counterparts. Furthermore, the correlation of expression profiles among non-duplicated genes from different lineages is significantly higher than between non-duplicated outgroup genes and homologous lineage-specific duplicates. This uncoupling of expression profiles between paralogs and outgroups is particularly strong in the Old World primates. Thus, despite the large evolutionary time separating birds and mammals, non-duplicated genes seem to preserve their expression profile, while paralogs show divergent expression profiles, probably reflecting adaptive evolution of at least some duplicates.

Conclusions: Using newly generated transcriptome data, we provide initial insights into the expression profiles of segmentally duplicated genes in mammals. Our first results support the notion that segmental duplications promoted genomic innovations and contributed to mammalian phenotypic evolution.

B 059 Progenitors of mammalian X chromosome

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Background: Human X chromosome represents ancestral eutherian sex chromosome. Comparision of marsupial, chicken, platypus and human genome reveal X-conserved region (XCR) and X-added region (XAR). XAR is located in autosomes of non-eutherians. However, discrepancies in synteny between chicken and human XCR resulted in three X chromosome evolutionary layers hypothesis. Existence of ancestral XAR chromosome is inferred by some identical gene blocks in vicinity of XAR region in non-eutherian chromosomes.

Observations: Genome of the frog, Xenopus tropicalis, brings information necessary for elucidation of mammalian X chromosome evolution. We identified orthologs of human X-borne genes by screening of genome databases. Frog ortholog locations were determined using linkage map and in situ hybridization. The frog genome contains 456 orthologs of human X chromosome genes, 97.4% of these lie on chr. 2 and 8. Distribution of X borne genes between frog chromosomes agrees with ortholog positions in XAR or XCR regions. Map of the XAR- adjacent regions identifies homologies among opossum chr. 7 and 4, chicken chr. 1, frog chr. 2 and human chr. 1, 2, 3, 11, 13, 15, 19 and 21. Parts of human chr.2, 3 and 13 reveal mosaic of syntenic blocks with both opossum chromosomes, indicating existence of their predecessor, pre-XAR. Gene content of chicken chr. 1 and frog chr. 2 homologous to opossum chr. 4 and 7 are very similar, except short part of frog chr. 2 forming a third of chicken chr. 23. The size of opossum chr. 7 and 4, containing majority of XAR adjacent gene blocks, is considerably longer than human and opossum homologies with chicken and frog, suggesting fusion of chromosomes in pre-XAR genesis.

Conclusions: Frog genome brings no support to three X evolutionary layers hypothesis. Synteny blocks of opossum chr. 4 and 7 with human defines pre-XAR gene content. We infer the existence of a single proto-XAR chromosome, ancestral to the pre-XAR, in the progenitor of Synapsida and Sauropsida.

B 060 The mechanical properties of kinesin-1 change in connection to nucleotide binding and two-headed microtubule attachment

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Background: Kinesin-1 is a molecular motor that transports cellular cargo along microtubules by completing hundreds of consecutive steps of its two 'head' domains. This ATP-driven motility is not fully understood, and questions remain over the conformational changes that drive directionality and the method of coordination of the heads. Understanding this mechanism could provide a blue-print for future technologies and give insight into diseases involving kinesin malfunction.
Observations: For this study, in vitro bead assays were carried out using the Photonic Force Microscope, an optical trap with three-dimensional position detection. Using this method, the energy landscape of a bead, tethered to a microtubule by a single full-length Drosophila kinesin molecule, was analysed. The mechanical properties of kinesin molecules can be extracted from these energy landscapes and were found to alter measurably depending on whether no nucleotide, or AMP-PNP, was present in the experimental chamber. These variations included an increase in the effective axial stiffness away from the microtubule on AMP-PNP binding. They also included an additional increase in stiffness and a substantial decrease in the accessible volume available to the bead along the kinesin axis, attributed to the change from one- to two-headed microtubule binding. These transitions are explained by means of a model of kinesin as a system of rods and hinges. Simulations of this model structure undergoing thermal fluctuations, suggest that changes in angular stiffness and orientation of a hinge near the head domains are sufficient to explain the mechanical transitions observed.
Conclusions: Neck-linker docking on AMP-PNP binding and inter-head strain caused by two-headed microtubule binding could explain both the change in hinge orientation and angular stiffness thought to occur. Therefore this work supports the hypothesis that these phenomena are essential to kinesin's motility cycle.

B 061 Glutaric aciduria type 1: disease-related metabolites impair the succinate transport from astrocytes to neurons

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Background: The deficiency of the mitochondrial protein glutaryl-CoA dehydrogenase (GCDH) leads to the neurodegenerative disorder glutaric aciduria type 1, characterized by accumulation of glutaric (GA) and 3-hydroxyglutaric acid (3OHGA) especially in the brain. GA and 3OHGA are known to be substrates of sodium-coupled dicarboxylate transporters (NaCs). NaCs are crucial for the anaplerotic supply of citric acid cycle intermediates from astrocytes to neurons and may be blocked by accumulating GA and 3OHGA.
Observations: To examine the effects of GA and 3OHGA on the transport of citric acid cycle intermediates, primary cultured astrocytic and neuronal cells prepared from brains of wildtype and Gcdh-deficient mice were used for [14C]-succinate uptake and efflux experiments. In the presence of GA or 3OHGA, the sodium-dependent uptake of [14C]-succinate into neuronal cells was inhibited in a concentration-dependent manner. Quantitative real-time PCR analysis revealed highly upregulated mRNA transcripts of the sodium-coupled dicarboxylate transporter NaC3 in Gcdh-deficient neuronal cells. The efflux of radioactivity from astrocytes of Gcdh-deficient mice preloaded with [14C]-succinate was significantly reduced in comparison with cells from wildtype animals. HPLC analyses showed that radioactivity effused from these cells corresponds to [14C]-succinate and not to metabolized compounds.
Conclusions: Our results suggest that increased levels of GA and 3OHGA in the brain of Gcdh-deficient mice interfere with the anaplerotic supply of citric acid cycle intermediates from astrocytes to neurons, mediated by NaC3-dependent uptake. This may lead to neuronal injury via disruption of energy production.

B 062 PI(4,5)P2 and Tyrosine Phosphorylation Dependent Membrane Insertion of FGF2 Oligomers Implicated in Unconventional Secretion

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Background: Fibroblast growth factor 2 (FGF2) is a potent mitogen that is exported from cells by an ER/Golgi-independent mechanism. Unconventional secretion of FGF2 occurs by direct translocation across plasma membranes, a process that depends on the phosphoinositide PI(4,5)P2 at the inner leaflet as well as heparan sulfate proteoglycans at the outer leaflet of plasma membranes.
Observations: To explore the mechanism of FGF2 membrane translocation, we have established an in vitro system to study interactions between FGF2 and model membranes. We found that PI(4,5)P2-dependent recruitment on membrane surfaces causes FGF2 to oligomerize and to insert into membranes in a lipid composition-dependent manner. This process is strongly enhanced by phosphorylation of tyrosine 82 in FGF2, a modification that has previously been shown to be essential for unconventional secretion of FGF2.
Conclusions: Our data suggest that membrane inserted oligomers of FGF2 are intermediates of membrane translocation that is completed when cell surface heparan sulfate proteoglycans liberate FGF2 at the outer leaflet of plasma membranes.

B 063 The role of Penicillium chrysogenum Pex11, Pex11B, Pex11C and Pex16 in peroxisome development and function

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Background: Peroxisomes represent a class of important eukaryote organelles that are characterized by an unprecedented functional plasticity that varies with the organism in which they occur and environmental conditions. In general, two modes of peroxisome development have been documented namely 'de novo' synthesis from the endoplasmic reticulum (ER) and organelle multiplication by fission. Here we have analyzed the peroxisome proliferation machinery in the filamentous fungus P. chrysogenum.

Observations: P. chrysogenum contains three Pex11 protein family members: Pex11, Pex11B and Pex11C. CLSM analysis of strains producing GFP fusions of Pex11 proteins revealed that Pex11 and Pex11C are localized to the peroxisome membrane while Pex11B is a component of the ER. The analysis of all possible combinations of single, double and triple gene deletions revealed that the major factor required for peroxisome proliferation by fission is Pex11. Overproduction of both Pex11 and Pex11C resulted in massive proliferation of peroxisomes, while elevated levels of Pex11B caused formation of giant membrane structures. In contrast to yeast cells depleted of Pex11 family members, deletion of all Pex11 proteins in P. chrysogenum did not lead to a peroxisome deficient (pex) phenotype, but resulted in a drastic reduction of peroxisome numbers of increased size. In cells depleted of Pex11 proteins we also deleted PEX16, a peroxin involved in early stages of peroxisome assembly in higher eukaryotes and observed that, similarly to PEX16 single deletion cells, cells of the quadruple pex11.pex11b, pex11c.pex16 mutant contained normal but reduced numbers of peroxisomes with matrix protein import defect.

Conclusions: We show that Pex11 proteins control peroxisome proliferation by fission in P. chrysogenum. P. chrysogenum Pex11 and Pex11C are peroxisome borne in contrast to Pex11B protein which is confined to the ER. Interestingly, all Pex11 proteins and Pex16 are not crucial for de novo peroxisome biogenesis.

B 064 An investigation into the role of GRASP65 in epithelial cells

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Background: Polarized epithelial cells have distinct apical and basolateral plasma membrane domains that result from specific targeting of proteins and lipids. Essential to polarization is the correct deposition and trafficking of junctional proteins. Many junctional proteins are membrane spanning and should follow the classical secretory pathway. However, their individual transport routes are not well mapped, nor is the role of Golgi morphogenesis and function during epithelial cell polarisation.

Observations: We set about to analyse the trafficking dynamics of transmembrane proteins that target distinct plasma membrane domains, namely E-Cadherin for the adherens junction, Claudins for the tight junction and Pannexins for the gap junction, during establishment of epithelial cell polarity. Given our work on Drosophila follicular epithelial cells, which demonstrates certain transmembrane proteins bypass the Golgi in a GRASP65 dependent fashion, we set out to first determine whether the junctional proteins mentioned above follow the classical secretory route. We also depleted GRASP65 and its interacting partner GM130, both of which have strong effects on Golgi morphology.

Conclusions: Our work suggests that GRASP65 but not its Golgi receptor GM130 has specific roles in the transport of specific junctional cargos, which are only revealed in a polarised cell context. As such, we reveal GRASP65 to be an essential factor contributing to junction formation and epithelial polarisation.

B 065 Selective induction of autophagy throughout ER stress in glioma cells treated with 2-Hydroxyoleic acid

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Background: 2-Hydroxyoleic acid (HOA) is an oleic acid synthetic analogue that impairs lung cancer (A549) cell proliferation and induces apoptosis in human leukemia (JURKAT) cells. Recent evidence indicates that HOA induces a modulation of the plasma membrane lipid structure affecting membrane-bound protein function and a subsequent down-regulation of proliferative signals that cause down-regulation of DHFR expression.

Observations: In this work we have confirmed the down-regulation of DHFR expression in 1321N1 human glioma cells and we have further investigated the molecular mechanisms of HOA action by studying its effect on endoplasmic reticulum stress-Unfolded Protein Response (UPR), autophagy induction pathways and lipid composition in 1321N1 human astrocytoma cells and in MRC-5 human fetal fibroblast non-cancer cells. HOA up-regulated the levels of a number of important ER stress/UPR markers in 1321N1 human astrocytoma cells, such as phosphorylated eIF2alpha, IRE1alpha and CHOP proteins, ATF4, ATF6, IRE1alpha, CHOP mRNAs and the spliced form of XBP1 gene mRNA. Concomitantly, HOA arrested 1321N1 cells in the G2/M phase of the cell cycle as determined by DNA content analysis and the decrease on the expression of Cyclin B1 and Cdk1/Cdc2 proteins. HOA treatment also alters the phospholipid composition of 1321N1 cells, increasing sphingomyelin and decreasing phosphatidylcholine and phosphatidylethanolamine. Finally, HOA induced autophagy as determined by the formation of acidic vesicles and the induction of the autophagy-related proteins LC3BII and ATG7. In turn, HOA failed to induce the above changes in MRC-5 cells.

Conclusions: The present results show relevant data about the mechanism of action of this anticancer drug, and explain in part its specificity against cancer cells, with the concomitant lack of side effects observed after HOA treatments.

B 066 Dynamics of Rab7b-dependent transport of sorting receptors at the TGN

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Background: Rab proteins are small GTPases that tightly regulates the intracellular trafficking in the eukaryotic cells. Rab7b was originally thought to have the same function of Rab7 since it shares the highest homology with Rab7 and it is also localized on lysosomes. However we recently showed that Rab7b localizes also to Golgi and TGN and that its depletion inhibits the retrograde transport of the cholera toxin B subunit to the Golgi, demonstrating its main role in the transport from endosomes to TGN.
Observations: Sorting receptors transport lysosomal enzymes from the Golgi to late endosomes/lysosomes. After cargo dissociation, receptors recycle back to the TGN. In this work we aim to test whether Rab7b is directly involved in the recycling pathway of CI-MPR or other cargo receptors. In order to answer this question we used live-microscopy techniques and functional assays in cells depleted of or overexpressing Rab7b. In this way we found that endosomes-to-Golgi retrieval of sorting receptors is delayed in Rab7b depleted cells, suggesting that Rab7b controls the retrograde transport of these receptors. Sorting receptors normally leave the TGN generating tubular carriers, however we observed a reduced tubulation after the expression of Rab7b mutants or its silencing. Interestingly, we also demonstrated that the constitutively active mutant Rab7bQ67L, impairs the formation of CI-MPR positive carriers from TGN.
Conclusions: Our data demonstrate that Rab7b is required for the retrieval of sorting receptors from endosomes to TGN and that alterations of its functionality prevent the correct formation of carriers from TGN.

B 067 Structural and biochemical basis for an E2/co-activator complex

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Background: Ubiquitin-conjugating enzymes (E2s) coordinate distinct types of ubiquitination via specific E3 ligases, to many substrates. While most E2s need only the presence of an E3 ligase for substrate ubiquitination, a number require additional binding partners to specify their function. One such E2 is Pex4p, responsible for site-specific cysteine ubiquitination of the import receptor Pex5p. Pex4p requires the peroxisomal membrane protein Pex22p, yet its role in receptor ubiquitination remains elusive.
Observations: We have determined the crystal structure and function of the Pex4p:Pex22p E2/co-activator assembly. Pex4p binds the peroxisomal membrane protein Pex22p through an interface which does not overlap with any other known binding site in E2 enzymes. Pex22p association stimulates Pex4p's ubiquitin-conjugating activity in vitro in an E3 ligase-independent manner and Pex22p binding is essential for Pex4p to ubiquitinate its target, the Pex5p import receptor in vivo.
Conclusions: Our data demonstrate that the Pex4p:Pex22p assembly, and not Pex4p alone, functions as the E2 enzyme required for Pex5p ubiquitination, establishing a novel mechanism of E2 enzyme regulation.

B 068 Rab GTPases and phosphoinositides regulation for endosome fusion events in a double stranded virus endocytosis

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Background: Viruses have evolved to exploit the endocytic pathway for infection. African swine fever virus (ASFV) is a complex double stranded DNA enveloped virus. Analysis of major entry routes indicates that ASFV enters the cells by clathrin-mediated endocytosis. A viral protein binding dynein mediates virus transport to the perinuclear area where a characteristic replication site called viral factory is built. Nevertheless, the early events between entry and viral factory formation are not yet clear.
Observations: In this work we have identified the relevance of the endosomal pathway on ASFV entry by endocytosis. Incoming virus particles should traverse the early endosome at very early time points to enter the cytosol showing a clear colocalization. Viral uncoating was evaluated to occur in a later step, within the first 60 min after infection. Although, the dependence of the late endosome was not obvious in basal conditions, viral particles could be found retained in late endosomes under acidification inhibition conditions. Acidification inhibition stopped endosomal membrane maturation process and virus egress. Hence, virus cytoplasmic transport was impaired and late endosomes containing virions remained at the cell periphery. Moreover, viral production was severely affected in Rab7 dominant negative mutant, transfected and sorted cells. Also, endosome fusion events were found to be crucial for infection and the inhibition of kinases PI3K and PIKfyve resulted in infection inhibition at early infection steps due to an alteration of the normal cycle of phosphoinositides conversion.
Conclusions: Our results show the strict requirement of endosomal passage at the first steps of the viral cycle. The acidic pH of late endosomes, as well as the integrity of the endocytic compartment, Rab GTPases and endosomal membrane phosphoinositides were found necessary for a successful viral infection.

B 069 Study of Type III Secretion System ATPase from the opportunist pathogen Pseudomonas aeruginosa

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Background: Pseudomonas aeruginosa, a gram negative bacterium, is a common infectious agent in hospitals that exhibits multiple antibiotic resistances. It possesses many virulence factors including the Type III Secretion System (T3SS). This system, used by several bacteria, mediates the injection of effectors directly into the target cell cytoplasm, and leads to the hijacking of host cell main functions. T3SS ATPase is crucial for the activity of this system but its exact function remains unclear.

Observations: To get new insights in function of P. aeruginosa ATPase, PscN, we undertook a multi-approach study. First, the PscN protein was expressed in E. coli. Purifying this protein is challenging as it is hydrophobic and has a poor solubility. However, it was purified in complex with its regulator PscL, from a co-expression in E. coli, which confirms the interaction between these two proteins. The ATPase was also expressed directly in P. aeruginosa and a Strep-tag affinity chromatography led to the obtention of a semi-purified sample, with ATPase activity. The interacting proteins were analysed by mass spectrometry. Another approach consisted in the phenotype analysis after a site directed mutagenesis, based on sequence homology with other ATPases. P. aeruginosa strains that have a mutation on the catalytic site or on the amino acid supposed to be located in the catalytic site of adjacent subunit were shown to be non cytotoxic. Interestingly, these mutations have dominant negative effect.

Conclusions: In conclusion, T3SS ATPase PscN from P. aeruginosa was studied by in vivo and in vitro approaches. PscN was obtained in complex with PscL from expression in E. coli. The purification from P. aeruginosa gave a semi-purified preparation with ATPase activity.

B 070 A Role for the PLD1 pathway in Autophagosomal Membrane Dynamics

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Background: Macroautophagy is known to be an essential degradative process. While autophagosome (AP) formation involves massive changes in membrane architecture, the lipid changes underlying autophagosomal membrane dynamics are largely undetermined. We have recently reported that phospholipase D1 (PLD1), an enzyme producing bioactive lipid phosphatidic acid (PA), is a modulator of autophagosomal membrane dynamics and maturation during starvation-induced autophagy, downstream of PI 3-kinase Vps34.

Observations: Our current efforts are focused on providing mechanistic insights into how PLD1 and its product PA regulate the maturation step involving the fusion of autophagosomes (AP) with endosomes (End) and, ultimately, lysosomes (Lys). We use a combination of lipidomic analysis (LC-MS), cell free fusion assays, Western blotting and light microscopy techniques to characterize organelles (AP, End and Lys) purified from liver/brain tissues derived from Pld1-deficient mice. In particular, we are assessing their ability to fuse with each other heterotypically. Our results show decreases fusion efficacy between AP and both End and Lys, when these organelles are derived from Pld1 KO mouse livers. Moreover, we have obtained the full lipid profiles of WT and KO AP and Lys and we found differences in the lipid composition, which we hypothesize may contribute, along with PA changes, in their differential fusogenicity. We are currently attempting to modulate the relevant lipids changes in order to rescue the fusion phenotypes.

Conclusions: Our results demonstrate the involvement of PLD1 in the maturation of APs. We anticipate our studies will provide a better understanding of the lipid-based mechanisms controlling autophagosomal membrane dynamics and a molecular basis for novel therapeutic avenues.

B 071 Mgr3p and Mgr1p Are Adaptors for the Mitochondrial i-AAA Protease Complex

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Background: While most mitochondrial proteins are encoded by nuclear DNA, a handful of mitochondrial proteins are encoded by a small genome located in the mitochondrial matrix. Damage to mitochondrial DNA (mtDNA) can lead to severe illness and may be a cause of aging, and it is therefore important to fully understand the cellular consequences of mtDNA loss.

Observations: To ascertain the requirements for cell viability after mtDNA damage and to potentially find new mutants affecting mitochondrial assembly and function, we performed genetic screens to search for mutants that are inviable after loss of the mitochondrial genome. We found that cells lacking the prohibitin complex, a likely inner membrane chaperone, are inviable after mtDNA loss. Furthermore, we identified two previously uncharacterized proteins that are required for the survival of cells lacking mtDNA: Mgr1p and Mgr3p. Both proteins biochemically associate with the catalytic subunit of the i-AAA protease, Yme1p, which was previously shown to degrade misfolded proteins at the mitochondrial inner membrane. Mgr1p and Mgr3p are required for maximal binding of substrate proteins to Yme1p, and both Mgr1p and Mgr3p can bind to misfolded

proteins in the absence of other known proteins involved in i-AAA protease function.
Conclusions: These data indicate that Mgr1p and Mgr3p are adaptor proteins that recruit misfolded polypeptides to the catalytic core of the i-AAA protease. Moreover, our work highlights the importance of mitochondrial protein quality control by the i-AAA protease and prohibitin complexes in mtDNA-minus cells.

B 072 Characterisation of the Hereditary Spastic Paraplegia Protein Strumpellin and the WASH complex in Neuronal and Non-Neuronal Cells

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Background: The WASH complex acts at the interface of cytoskeletal regulation and membrane dynamics, and is recruited to endosomal compartments by retromer. Knockdown of any of several complex members results in aberrant endosomal tubulation. The WASH complex member strumpellin is mutated in a rare form of hereditary spastic paraplegia, a group of disorders characterised by progressive axonopathy leading to paralysis of the legs. We aim to investigate the effects of strumpellin mutation on the WASH complex.
Observations: We transfected HeLa cells with either wild-type strumpellin or strumpellin containing one of the three point mutations causative of disease, V626F, L619F and N471D, and showed by co-immuniprecipitation that these mutations do not ablate binding of other WASH and retromer complex members. We have further shown that, in contrast to strumpellin knockdown, transfection of these disease mutants does not result in aberrant endosomal tubulation. By the use of co-immunofluorescence techniques in rat primary cortical neurons, we have found that WASH and retromer complex members localise to the endosomes, as previously reported for non-neuronal cell types. After transfecting these neurons with either wild-type or mutant strumpellin we found that all these forms of strumpellin co-localise with other WASH complex members. We found that WASH and retromer complex members displayed a widespread distribution in neuronal cells, being present in the cell body, dendrites and axons. Finally, by performing co-immunoprecipitation in rodent brain tissue, we have confirmed that strumpellin, WASH1 and retromer interact in this cell type.
Conclusions: We suggest that mutation of strumpellin affects complex function rather than complex assembly and, as the WASH and retromer complexes maintain their interaction in neuronal cells, that disruption of their role in vesicle trafficking is highly likely to be causative of progressive axonopathy.

B 073 RT qPCR analysis of the expression of a novel ABC transporter split system for the acquisition of haem and the xenosiderophores ferrichrome and ferroxamine B in Sinorhizobium meliloti 2011

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Background: Iron is essential for the survival of nearly all organisms. It plays crucial roles as an electron donor/accepter and cofactor in metabolic processes due to its ability to interchange between the $Fe2+$ and $Fe3+$ states. However, iron in the oxidised $Fe3+$ state forms insoluble compounds in physiological conditions, limiting its availability. To counter this, microorganisms produce iron chelating molecules known as siderophores that display very high affinity for $Fe3+$ and facilitate its acquisition.
Observations: Sinorhizobium meliloti 2011 produces rhizobactin 1021 as its endogenous siderophore. In addition to native siderophore utilisation many microorganisms have attained the ability to utilise xenosiderophores. S. meliloti can utilise haem along with the xenosiderophores ferrichrome and ferroxamine B as iron sources. The transport systems for haem and the above xenosiderophores have been characterised and were found to be members of the ABC superfamily of transporters. The transport systems each comprise a periplasmic binding protein, HmuT for haem and FhuP for the siderophores and, interestingly, they share the ATPase/Permease components, HmuUV. The regulation of this novel split system is of interest as the hmuT gene is co-transcribed with hmuUV while fhuP is located distally. We have used RT qPCR to measure the effect of each ligand on the expression pattern of the system. Our results have shown that the expression of fhuP is upregulated in the presence of a cognate siderophore. Although hmuTUV are members of the same operon they are differentially expressed in a manner dependent on the presence of haem or ferroxamine B.
Conclusions: These results imply that specific siderophores or haem influence the expression of specific components of their transport systems. It remains to be determined whether the cognate iron ligands regulate the system through transcriptional regulators or riboswitch like interactions with RNA.

B 074 Characterisation of novel transport systems for the utilisation of hydroxamate type siderophores by Sinorhizobium meliloti 2011 and Pseudomonas aeruginosa PAO1

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Background: Many bacteria biosynthetically produce low molecular weight ferric specific chelators termed siderophores. The FhuACDB transport system of Escherichia coli is established as the model for hydroxamate type siderophore utilisation. The inner membrane components are an ABC transport system consisting of a periplasmic binding protein, an integral membrane permease and an ATPase. The Fhu system has been extensively characterised and is functionally conserved across many bacterial species.
Observations: S. meliloti 2011 produces an asymmetric citrate hydroxamate siderophore termed rhizobactin 1021. The genetic determinants required for rhizobactin 1021 production and utilisation have been identified and characterised. In contrast to the model FhuCDB system, transport of rhizobactin 1021 is facilitated by a single unit siderophore permease termed RhtX. The RhtX permease can functionally substitute for FhuCDB transport of rhizobactin 1021 in E. coli. Additional single unit permease transporters have been identified and characterised in P. aeruginosa. The FptX transporter of P. aeruginosa, displaying significant homology to RhtX, is the inner membrane component for the utilisation of the endogenous siderophore termed pyochelin. The identification of RhtX and FtpX as the first members of a novel family of single unit siderophore transporters lead to the identification of another P. aeruginosa single unit inner membrane transporter termed FoxB. Heterologous expression of FoxB in S. meliloti transport mutants enabled identification of its cognate xenosiderophores. These inner membrane transporters and their cognate target siderophores are the subject of this investigation.
Conclusions: The transporters RhtX, FptX and FoxB are the first members of novel families of inner membrane hydroxamate siderophore single unit transporters. Furthermore, FptX and FoxB are the only inner membrane siderophore transporter components identified thus far in P. aeruginosa.

B 075 Epsin1 bends membranes by molecular crowding

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Background: Dynamic remodeling of membrane curvature is essential to many cellular processes. Two primary mechanisms have been proposed to explain how specific membrane binding proteins can induce membrane curvature: (1) membrane scaffolding and (2) wedge-like insertion of protein domains or amphipathic helices. Based on studies of Epsin1, a protein involved in clathrin-mediated endocytosis, we propose a third general mechanism – membrane bending by molecular crowding.
Observations: Using fluorescence lifetime imaging of labeled Epsin1 (ENTH domain), which binds to the surfaces of giant unilamellar vesicles by recognizing the lipid phosphatidylinositol-4,5-bisphosphate and inserting an amphipathic peptide helix (helix0), we demonstrate that a protein surface coverage (percentage of surface area occupied by proteins) of greater than 25% is required to bend membranes. A simple mechanical model suggests that this degree of protein coverage could lead to membrane bending simply through steric interactions between tightly bound diffusing proteins. To test this molecular crowding concept we replaced ENTH's helix0 with a hexa-histidine tag, which strongly binds metal chelating lipids and measured that a similar coverage threshold was required to induce bending. Further, we found that a structurally similar domain (AP180 ANTH), which binds the membrane with lower affinity than ENTH, could not reach the 25% coverage threshold and did not bend the membrane. Full-length Epsin1, which has three times the molecular weight of ENTH, reached the surface coverage threshold and bent the membrane with a smaller number of molecules per membrane area than the ENTH domain alone.
Conclusions: Our results demonstrate that Epsin1 can bend membranes through molecular crowding and suggest that amphipathic anchoring motifs found often in membrane bending proteins may function to enable crowding by increasing protein-membrane affinity, rather than by directly bending membranes via insertion.

B 076 Import oligomers induce positive feedback to promote peroxisome differentiation and control organelle abundance

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Background: A fundamental question in cell biology is how cells control organelle composition and abundance. Woronin bodies are fungal peroxisomes centered on a crystalline core of the self-assembled HEX protein. Despite using the canonical peroxisome import machinery for biogenesis, Woronin bodies are scarce compared to the overall peroxisome population.
Observations: Here, we show that HEX oligomers promote the differentiation of a subpopulation of peroxisomes, which become enlarged and highly active in matrix protein import. HEX physically associates with the essential matrix import peroxin, PEX26, and promotes its enrichment in the membrane of differentiated peroxisomes. In addition, a PEX26 mutant that disrupts differentiation produces increased numbers of aberrantly small Woronin bodies.
Conclusions: Our data suggest a mechanism where HEX oligomers recruit a key component of the import machinery, which promotes the import of additional HEX. This type of positive feedback provides a basic mechanism for the production of an organelle subpopulation of distinct composition and abundance.

B 077 Regulation of endoplasmic reticulum and mitochondria morphology by spatacsin and spastizin, two proteins associated with hereditary spastic paraplegias

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Background: The hereditary spastic paraplegias (HSP) are genetic neurological disorders mainly characterized by progressive spasticity. The symptoms are caused by axonal degeneration of the cortical motoneurons, especially in their terminal portions. So far 21 causative genes have been cloned. The putative roles of the encoded proteins suggest that mitochondrial function, protein folding and intracellular trafficking are implicated in the dying back of pyramidal tract axons in these disorders.

Observations: A common form of autosomal recessive HSP associates spastic paraplegia with thin corpus callosum. Most families (~60-80%) are linked to SPG11, while ~11% of the families are linked to SPG15. SPG11 and SPG15 encode spatacsin and spastizin, respectively, two proteins of unknown function. In most cases, the pathology is due to loss of protein function. Furthermore, SPG11 and SPG15 have similar patterns of expression, as shown by in situ hybridization in adult rat brain. Spatacsin and spastizin are members of a protein complex with KIAA0415. Interestingly, mutations in the latter gene have been found in patients with HSP (SPG48), suggesting that this protein complex is strongly linked to the physiopathology of HSP. To get an insight in the molecular function of this protein complex, we have analyzed the consequences of the absence of spatacsin, spastizin or KIAA0415 on organelle morphology and dynamics. This has been done by RNA interference in cultured cell lines and by examining lymphoblasts and fibroblasts derived from patients. It appears that endoplasmic reticulum and mitochondria are the main organelles affected in the absence of these proteins.

Conclusions: These data suggest that spatacsin, spastizin and KIAA0415 affect the same organelles, and are consistent with a role for these proteins in intracellular trafficking and/or mitochondrial function. Whether the three proteins are acting together on these organelles remains to be determined.

B 078 Multivariate clathrin-coated vesicle profiling

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Background: Clathrin-coated vesicles (CCVs) are major endocytic and intracellular transport intermediates. Here we report a multivariate comparative proteomics approach to mapping the machinery and cargo of CCVs in HeLa cells. We establish multiple objective criteria that allow us to distinguish CCV proteins from co-purifying contaminants, and also to differentiate between different types of CCVs.

Observations: We used SILAC-based quantitative mass spectrometry to compare the composition of CCV fractions prepared under different experimental conditions. Over 2,500 proteins were identified in the CCV fraction. Cluster delineation based on Principal Component Analysis predicts that 133 of these proteins are CCV associated, including 36 novel proteins. Furthermore, the clustering also predicts if a CCV protein functions in endocytosis or intracellular trafficking. Our analysis identified >91% of known CCV proteins, and assigned >93% of them correctly as either intracellular or endocytic. Therefore, the novel predicted CCV proteins can be functionally assigned with high confidence. Although the experimental design is optimized to identify CCV proteins, a by-product of the multivariate clustering is a comprehensive analysis of the >2300 'contaminant' proteins present in the CCV fraction. Numerous known and novel associations among non-CCV proteins were detected. These included for example 16 Retromer-associated proteins, five of which had not been reported previously. Six of the novel proteins were analysed by immunofluorescence microscopy, and all had the predicted sub-cellular localization.

Conclusions: This study is the most complete characterization of the CCV proteome to date. Although we focused on clathrin-mediated trafficking, we also uncovered many associations among non-CCV proteins. This powerful approach can be adapted to address related cell- and systems biological questions.

B 079 Involvement of the vacuolar membrane in autophagosome formation

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Background: Autophagy is one of the cellular defense responses taken place under the starvation condition. Cells conquer such situation by forming double lipid bilayer organelle called autophagosome that sequesters cytosolic proteins and free ribosomes to be degraded. Many Atg proteins were found and studied how autophagosome formation takes place; however, many questions are still addressed regarding the autophagosome membrane formation, especially the origin of the membrane.

Observations: In this study, we studied the relationship between the vacuolar membrane and the autophagosome since the formation site, pre-autophagosome structure (PAS), is found always in vicinity to the vacuole membrane. The lipophilic dye, FM4-64, was used to detect the vacuolar membrane. Vacuolar membrane was stained prior to induction of autophagy; the autophagosome and autophagic bodies become stained in wild-type cells. Such staining was significantly reduced in mutant cells that has defect in retrograde trafficking from the vacuole. So far, blocking the vps pathway does not show defect in autophagosome formation thus flow from endosome is not required. Furthermore, similar results were seen using GFP tagged vacuolar membrane protein. FYVE-GFP also stained accumulated autophagic bodies. The purpose of using the vacuolar membrane in autophagosome formation could be to provide SNARE complex. We found a fusion defect in autophagosome formation in one of the SNARE complex mutant cells.

Conclusions: These results suggest that the vacuolar membrane is one of the membrane sources for autophagosome. It is; however; not a requirement since autophagy still occurs in such mutant cells. We are now trying to see the link between vacuolar membrane flow and SNARE complex in autophagosome formation.

B 080 Peroxisomal membrane protein recognition by Pex19p

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Background: Pex19p is a 299-residue protein with a conserved C-terminal farnesylation site, which functions as a receptor and chaperon for peroxisomal membrane proteins (PMPs). According to the current model, Pex19p recognizes newly translated PMPs in the cytosol, thus functioning as a soluble PMP receptor. In a second step, the PMP cargo is directed to the peroxisomal membrane where it docks to the membrane-bound Pex3p, assigning Pex3p the function of a Pex19p co-receptor.
Observations: To unravel the molecular basis of Pex19p function as PMP receptor, we performed quantitative peptide-binding experiments, using a number of Pex19p deletion and point mutants. The basis of the experimental design was the available crystal structure of the C-terminal domain of Pex19p. To gain further structural information on the full-length Pex19p, we used a combined approach, including thermal denaturation experiments, small angle X-ray scattering and modeling. Low-resolution structural data suggest that full-length Pex19p is an elongated molecule, of which the N-terminal part appears to be loosely folded, as mirrored by its continuous unfolding. Apparent melting temperature values reveal a two-step unfolding for the tested constructs.
Conclusions: We showed that binding of mPTS by the C-terminal bundle domain is sufficient for complete PMP recognition. Three-dimensional model of the Pex19p molecule suggests that it consists of three domains connected by flexible loops that may enable large conformational changes during the transport process.

B 081 A fifth adaptor protein complex involved in endosomal sorting

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Background: For many years it has been assumed that there are only four adaptor protein (AP) complexes; AP-1, AP-2, AP-3 and AP-4. All four are heterotetramers, consisting of two large subunits, a medium-sized subunit, and a small subunit that share significant homology between corresponding subunits. Each of the AP complexes, which are present in most eukaryotes, has a distinct localisation and function to sort cargo into vesicles for transport between membrane compartments.
Observations: We have identified four subunits which share structural features and sequence homology with the known AP subunits, and we propose to call this complex AP-5. Intriguingly, AP-5 subunits can be found in all five eukaryotic supergroups, but they have been co-ordinately lost in many organisms. Concatenated phylogenetic analysis provides the first robust resolution into the evolutionary order of emergence of the AP complex family. Using a combination of subunit tagging and siRNA knockdown we have shown that AP-5 functions in endosomal trafficking, and like AP-4 does not associate with clathrin.
Conclusions: We report the existence of a fifth AP complex (AP-5) that is an evolutionarily ancient complex involved in endosomal sorting.

B 082 Mechanistic aspects of membrane elongation: Pex11 proteins and peroxisome proliferation

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Background: Compartmentalization of eukaryotic cells into organelles increases metabolic efficiency. Yet, these organelles must be maintained throughout cellular life. Especially, organelle proliferation relies on factors that remodel membranes to ensure proper execution of the elongation and fission steps. Studying peroxisomes, versatile organelles whose function is associated with lipid metabolism and ROS detoxification, we established the Pex11 proteins as membrane elongation factors.
Observations: Through structure/ function analysis of the membrane protein PEX11C in human cells, we demonstrate the importance of the interaction network between Pex11 proteins and the peroxisomal fission machinery. Mutation analyses revealed domains essential for the function of PEX11C including an amphipathic alpha-helix, motif previously shown to generate positive membrane curvature. Furthermore, to find out how PEX11C acts on the peroxisomal membrane we performed detailed topology studies. We also show the ability of Pex11 proteins to elongate peroxisomal remnants (devoid of matrix content) proving that metabolic signalling from inside the organelle is not absolutely required for peroxisomes to engage in proliferation. Finally, in dynamic studies exploiting the properties of photoactivatable GFP, we show that matrix proteins are sequestered during membrane elongation illustrating the asymmetric proliferation of peroxisomes under the influence of Pex11 proteins.
Conclusions: We propose peroxisome proliferation to be non-stochastic and initiated by polarized outgrowth of their membrane, a process driven by Pex11 proteins. Our detailed analysis of PEX11C allows for mechanistic explanations on how the proliferation of the peroxisomal compartment might be regulated.

B 083 The Class C VPS complex is required for autophagosome maturation in Drosophila

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Background: During autophagy, portions of the cytoplasm are engulfed in double-membrane autophagsosomes, which then fuse with lysosomes to deliver their contents for degradation. Understanding the molecular mechanism of autophagosome formation and maturation is important, as autophagy plays a role in a number of human pathologies, including aging, cancer, cardiovascular and neurodegeneration diseases, infection.
Observations: We identified a late larval lethal Drosophila mutant line that showed a characteristic accumulation of autophagosomes in larval fat body cells, suggesting impaired fusion of these

vesicles with lysosomes. Recombination, deletion and complementation mapping suggested that the mutation affects Drosophila Vps16a, a subunit of the Class C VPS complex required for vacuolar fusion events in yeast. The mutation caused a new splice variant, resulting in a truncated protein unable to function properly. Transgenic RNAi silencing of Vps16a or other members of the Class C VPS complex including deep orange, carnation and Vps11 showed similar phenotypes. Importantly, expression of wild-type Vps16a in our mutants completely rescued the autophagy phenotype as a final proof that the mutant phenotype is due to loss of Vps16a function. Analysis of our mutants and RNAi lines revealed that the Class C Vps complex is also required for the uptake and clearance of Texas Red-avidin, and for the proper trafficking of LAMP1. Detailed analysis of homotypic and heterotypic vesicle fusion events will reveal the exact function of this conserved protein complex in Drosophila.
Conclusions: We have discovered that loss of Vps16a function is responsible for the accumulation of autophagosomes in our Drosophila mutant. Vps16a is required for the proper trafficking of multiple lysosomal targeting pathways, including autophagy, endocytosis, and sorting of resident lysosomal proteins.

B 084 An in vivo hierarchy analysis of Drosophila Atg proteins required for autophagosome formation

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Background: Evolutionarily conservedAtg proteins cooperate during autophagy induction to promote the assembly of autophagosomes, double-membrane vesicles that deliver cytoplasmic components for lysosomal degradation and recycling. Given the demonstrated role of this process in development and disease, a more complete understanding of this core protein network is necessary.
Observations: Yeast Atg proteins are recruited to pre-autophagosomal structures in a hierarchical manner, which has also been documented for a subset of mammalian homologs in cell culture studies. Promoted by these scenarios, we carried out an in vivo epistasis analysis in Drosophila larvae. We are using a combination of transgenic GFP- or mCherry-fusion reporters and antibodies to follow the localisation of 12 selected proteins during autophagy induction in wild-type larval fat body and midgut cells, and in mutants or RNAi knockdown cells for 30 genes involved in this process. These genetic interventions enable us to selectively block the process of autophagy at distinct stages, such as various steps of pre-autophagosomal structure formation or isolation membrane closure.We can also analyze the localization of these structures relative to other intracellular organelles, such as the ER, Golgi, mitochondria etc. Remarkably, Drosophila melanogaster lacks a DFCP1 homolog, although this gene is present in other Drosophila species. We find that ER-derived omegasomes are formed in cells of transgenic flies expressing human DFCP1, suggesting a conserved role for ER in the earliest steps of autophagy.
Conclusions: We established the hierarchy of Drosophila Atg proteins during autophagosome formation. This analysis found important differences compared to yeast studies, and also included metazoan-specific genes to further understand the mechanisms of autophagosome formation in higher eukaryotes.

B 085 The Emerging Role of VHS Domain-Containing Tom1, Tom1L1 and Tom1L2 in Membrane Trafficking

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Background: It is estimated that over 30% of the proteins encoded by the human genome, projected to encode about 25 000 proteins and other macromolecules, are delivered to the secretory and endocytic pathways where movement of proteins between various compartments is primarily mediated by vesicles/carriers budding from one compartment for delivery to another. Sorting of cargo proteins into budding vesicles/carriers is mediated by adaptors that link the cargo proteins to the coat proteins.
Observations: EGF stimulates transient tyrosine phosphorylation of Tom1L1 by the Src family kinases, resulting in transient interaction of Tom1L1 with the activated EGFR bridged by Grb2 and Shc. Cytosolic Tom1L1 is recruited onto the plasma membrane and subsequently redistributes into the early endosome. Mutant forms of Tom1L1 defective in Tyr-phosphorylation or interaction with Grb2 are incapable of interaction with EGFR. These mutants behave as dominant-negative mutants to inhibit endocytosis of EGFR. RNAi-mediated knockdown of Tom1L1 inhibits endocytosis of EGFR. The C-terminal tail of Tom1L1 contains a novel clathrin-interacting motif responsible for interaction with the C-terminal region of clathrin heavy chain, which is important for exogenous Tom1L1 to rescue endocytosis of EGFR in Tom1L1 knocked-down cells.
Conclusions: Recent studies suggest that Tom1, Tom1L1 and Tom1L2 subfamily of VHS domain proteins, which do not exist in yeast, are emerging as novel regulators for post-Golgi trafficking and signaling.

B 086 Dynamics of anthrax toxins delivery into the host cell cytosol

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Background: Anthrax toxins are the major virulence factors secreted by Bacillus anthracis during anthrax infection. They enter target cells hijacking the host endocytic route, but instead of crossing the limiting membrane of endosomes, the anthrax toxin-receptor complex is sorted into the intraluminal vesicles (ILVs) where translocation occurs. Once in the lumen of ILVs, the toxins await transport to late endosomes (LEs) and back fusion of the ILVs with the limiting membrane to reach the cell cytoplasm.
Observations: In this study we observe that a brief exposure to anthrax lethal toxin (LTx) results in its sustained protease activity towards cytosolic targets. The continuous activity observed is not due to the high persistence of the toxin in the cell cytosol, as LTx is rapidly degraded by the proteasome. This was confirmed by directly expressing the lethal factor (the enzymatic subunit of LTx) in the cell cytosol. We therefore hypothesize that the persistence of the toxin in the cell is due to its sheltered presence in ILVs, from where delivery to the cytosol would be slow. Little is known about the mechanisms that govern back fusion events. Using drugs and RNA interference, we are currently identify-

ing factors that would control ILVs fusion and thereby the time window during which LTx is effective on cells.
Conclusions: The cytosolic release by gradual ILVs back fusion may be a strategy for anthrax toxins to be stored in LEs, not exposed to protein degradation. The slow delivery to their targets would result in a sustained cytotoxic activity. This finding has important implications for anthrax infections treatment.

B 087 Identifying novel proteins involved in Multivesicular Body formation

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Background: Multivesicular bodies (MVBs) are late endosomes formed by limiting membrane invaginations that give rise to many small internal vesicles. ESCRT complexes are essential for MVBs biogenesis, but MVBs can be also formed without participation of known ESCRT proteins (Hrs, Tsg101, Vps22 and Vps24, components of ESCRT-0, I, II and III, respectively). This suggests the involvement of other proteins in MVB formation.
Observations: To identify novel proteins involved in MVBs formation, we silenced by siRNA more than 21 thousand proteins in human melanoma cell line (MJS) and identified seven proteins that could control MVB formation. One candidate is Tollip that interacts with members of the Tom1 family (Tom1, Tom1L1, and Tom1L2), but Toms do not interact with each other. The Tollip-Tom1 complex binds ubiquitylated proteins and clathrin, suggestive for involvement in the canonical or alternative ESCRT-0 complexe(s). We identified their interactions. Tom1 family members redistribute from cytosol to endosomal membranes when co-expressed with Hrs, Tom1L1 and Tom1L2, but not Tom1. When co-expressed with Tollip, Tom1 and Tom1L1, but not Tom1L2, becomes associated to the vesicles. Tollip and Hrs do not interact and localize to the distinct populations of endosomes. This suggests the existence of two different multi-enzyme complexes, one formed by Tollip, another by Hrs, and each containing one of Tom1 family members. To assess the role of Tom1 family members in targeting ubiquitylated proteins for degradation, we used EGFR as a model cargo. We found that Tom proteins affect EGFR phosphorylation and/or endocytosis.
Conclusions: Our data suggest the existence of two different ESCRT-0 complexes; one controlled by Tollip that interacts with Tom1 or Tom1L1; the other by Hrs interacting with Tom1L1 or Tom1L2. How the two different ESCRT-0 complexes affect endosomal transport and substrate selectivity, is currently assessed.

B 088 Drosophila FIP200 is a master regulator of autophagy

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Background: Autophagy degrades and recycles old or dispensable cell components through lysosomal digestion. Atg genes were first identified in yeast, and most Atg gene homologs exist in all eukaryotes including Drosophila and mammals. Mammalian FIP200 (also known as RB1CC1) is possibly analogous to yeast Atg17, a scaffold protein in the Atg1 kinase complex in yeast. In addition, FIP200 is an important tumor suppressor that interacts with other tumor suppressor proteins such as RB1 and TSC1.
Observations: We started our functional analysis of Drosophila FIP200 (CG1347) by generating a genetic null mutant. FIP200 knockout animals die in the late pupal stages. Both starvation-induced and developmental autophagy is inhibited in mutant larval fat body cells. Similar phenotypes were observed in transgenic RNAi knockdown experiments. Loss of FIP200 results in persisting larval salivary glands and midguts, organs that normally undergo histolysis during metamorphosis. In addition, ubiquitinated protein aggregates accumulate in FIP200 mutant neurons. Transgenic mCherry- and GFP-fusion reporters of FIP200 colocalize with early pre-autophagosomal markers, indicating the evolutionarily conserved role of FIP200 in autophagy induction. Interestingly, overexpression of FIP200 promotes autophagy even without starvation, similar to our previous findings regarding Atg1. Finally, knockdown of FIP200 had no obvious effect on cell size, unlike in the case of TSC1. We are further testing the role of Drosophila FIP200 in cell growth and proliferation.
Conclusions: Drosophila FIP200 is necessary for autophagy induction as a subunit of the Atg1 kinase complex, and its overexpression is sufficient to promote autophagy. Mutant phenotypes further verify the importance of autophagy in tissue histolysis during Drosophila metamorphosis, and in neuronal homeostasis.

B 089 Pex5p- and Pex14p-induced pore formation in the peroxisomal membrane

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Background: To date, the molecular mechanisms of protein translocation across the peroxisomal membrane are largely unknown. Recently, Erdmann and colleagues demonstrated that the cycling import receptor Pex5p, together with its docking factor Pex14p, can form a gated ion-conducting channel after reconstitution in a planar lipid bilayer. The aim of this study was to investigate whether or not Pex5p and Pex14p also have channel- or pore-forming properties at the peroxisomal membrane in living cells.
Observations: To monitor pore formation, we transfected mammalian cells with constructs coding for a peroxisomal redox sensor or peroxisomal HaloTag-fusion proteins of different sizes. To study the pore-forming properties of Pex5p and Pex14p at the peroxisomal membrane, we interfered with the function of the wild-type proteins by (i) cultivating the cells under conditions depleting the cellular pool of free ubiquitin, (ii) overexpression of Pex5p(C11S), a mutant of Pex5p accumulating at the peroxisomal membrane and blocking the docking-translocation machinery, and (iii) overexpression of Pex14p-KillerRed, a variant of Pex14p which can be inactivated by chromophore-assisted light inactivation. We found that interfering with Pex5p or Pex14p function resulted in the formation of a pore at the peroxisomal membrane. We also obtained evidence that this pore displayed a size-based selectivity, which depends on the concentration of Pex5p.
Conclusions: Our data suggest that Pex5p and Pex14p may display pore-forming properties in their natural membrane. A

further characterization of the composition of this pore and how it is regulated will further our understanding of how matrix proteins are translocated across the peroxisomal membrane.

B 090 Structural requirements for the interaction of peroxisomal targeting signal 2 and its receptor PEX7

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Background: The recognition of peroxisomal targeting signals (PTS) by their receptors is a key step in the import of peroxisomal matrix proteins. Whereas the PTS1 is well characterized and its binding to the receptor has been resolved the PTS2 is more loosely defined and attempts to clarify the interaction of PTS2 with its receptor PEX7 have failed until now.
Observations: We combined a mutational analysis of the least conserved residues of a canonical PTS2 with a computational investigations of naturally occuring PTS2 signals. Thereby we demonstrate that PTS2 signals are amphipathic helical structures orienting all important residues of the consensus sequence towards one side. 3D structural modelling of the PTS2-receptor PEX7 reveals a groove with an evolutionary conserved charge distribution complementary to PTS2 signals. Mammalian two-hybrid assays and cross-complementation of a mutation in PTS2 by a compensatory mutation in PEX7 confirm the interaction site. The PTS2-helix is connected to the core protein by a flexible linker domain.The relevance of the identified parameters is confirmed by the demonstration that their implementation into a prediction algorithm for PTS2 signals enables the identification of novel PTS2 signals in naturally occurring human proteins.
Conclusions: PTS2 signals consist of an amphipathic helix connected to the core protein by a flexible linker. The helix fits spatially into an evolutionary conserved groove on the surface of the PTS2 receptor PEX7 and this interaction is experimentally verified.

B 091 The superoxide dismutase SodA is targeted to the periplasm by a novel mechanism

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Background: Most periplasmic proteins cross the cytoplasmic membrane in an unfolded state via the Sec-dependent (Sec) pathway. Additionally some proteins are exported fully folded by the twin-arginine translocation (TAT) machinery. A feature of these pathways is the presence of a signal sequence on the N-terminus of substrate proteins, which is typically cleaved upon export and consists of a positively charged extreme N-terminus followed by a hydrophobic region and a polar region containing a cleavage site.
Observations: The manganese/iron-type superoxide dismutase (SodA) of Rhizobium leguminosarum bv. viciae 3841 does not possess the hydrophobic cleaved N-terminal signal peptide typically present in soluble proteins exported by the Sec pathway or the TAT pathway, but is nevertheless exported to the periplasm of R. l. bv viciae 3841, Escherichia coli and other proteobacteria. A tatC mutant of R. l. bv viciae still exported SodA to the periplasm, ruling out export of SodA as a complex with a TAT substrate protein as a chaperone. The export of SodA was unaffected in a secB mutant, but export was inhibited by azide, an inhibitor of SecA ATPase activity. A temperature-sensitive SecA mutant of E. coli also exhibited severely reduced SodA export. Sequence analysis revealed that a 10-amino acid sequence within SodA was sufficient to target a reporter protein to the periplasm, and mutational analysis of this sequence determined the conserved residues involved in efficient periplasmic targeting. Our results demonstrate the export of a protein lacking a signal peptide to the periplasm by a novel SecB- and signal peptide-independent Sec targeting mechanism.
Conclusions: Our results demonstrate a novel SecB- and (classical) signal peptide-independent pathway for targeting proteins to the periplasm. The targeting mechanism may be widespread, as export of SodA to the periplasm was also observed in other proteobacteria.

B 092 Tracking the endocytic pathways of internalized platelet-derived growth factor (PDGF) and their impact on signaling

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Background: Platelet-derived growth factors (PDGFs) belong to a family of disulphide-linked dimer proteins which act via transmembrane receptors with tyrosine kinase activity (PDGFRs). By activation of the receptor PDGF regulates major cellular physiological processes like proliferation, migration and differentiation. Upon PDGF binding to PDGFR, ligand-receptor complexes are internalized and their endocytic trafficking may affect PDGF-dependent cellular signaling.
Observations: We undertook studies to characterize the endocytic routes of the PDGF-PDGFR complexes and to elucidate the role of endocytosis in PDGF signaling. We established methods to label PDGF molecules for microscopy detection and to reduce strong extracellular background resulting from unspecific adhesion of PDGF to glass or plastic surfaces. Quantitative microscopy assays indicated that PDGF can be internalized by dynamin-dependent and -independent pathways. After internalization, PDGF exten-

sively colocalizes with markers of early endosomes EEA1 or APPL2, and with endocytosed epidermal growth factor (EGF). It is subsequently sorted into CD63-positive multivesicular bodies followed by lysosomal degradation. No significant recycling of a PDGF-PDGFR complex was detected. Importantly, the uptake route of PDGF can affect its signaling. In particular, the full activation of certain signaling effectors requires dynamin-dependent internalization of PDGF.

Conclusions: These studies indicate that changes in endocytic trafficking of PDGF have impact on its signaling machinery and that certain signaling events take place intracellularly on endosomal compartments.

B 093 Characterisation of novel machinery involved in clathrin-mediated endocytosis

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Background: This project aims to identify novel components of clathrin-mediated endocytosis through the validation and characterization of hits that emerged from a recent genome-wide siRNA screen. This screen involved siRNA transfection of HeLa cells expressing CD8 chimeras containing either YXX or FXNPXY sorting motifs which are well-established recognition sequences in cargo sorted by AP-2 or 'alternative adaptors' such as Dab-2 respectively.

Observations: Hits from the genome-wide screen were indicated by an increase in CD8 on the plasma membrane and these included well-characterised machinery of clathrin-mediated endocytosis such as dynamin 2 and AP-2, a number of unknown proteins and interestingly, multiple subunits of the vacuolar-type H+-ATPase (V-ATPase). So far the project has involved validation of hits using an endocytosis efficiency assay to evaluate whether accumulation of CD8 on the surface is attributable to reduced endocytosis. Currently this assay is using smartpools of 4 siRNAs (as in the original screen) but these will soon be deconvoluted in order to test each siRNA individually. Ultimately a list of validated hits will be established which can be subjected to further high-throughput assays to investigate their involvement at different stages of clathrin-mediated trafficking such as the recruitment of membrane proteins and organelle acidity.

Conclusions: Ultimately hits of interest will be studied in greater detail to establish their exact contribution to clathrin-mediated endocytosis. This will involve a range of techniques including comparative proteomics, immunofluorescence, live cell imaging and electron microscopy.

B 094 Regulation of Endoplasmic Reticulum Morphology by ERMO1/GNOM-LIKE1 and ERMO2/SEC24a in Arabidopsis thaliana

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Background: The endoplasmic reticulum (ER) forms diverse complex structures with the largest surface area among organelles in eukaryotic cells. They are composed of tubules, sheets, and three-way junctions, resulting in a highly conserved polygonal network. These morphology supports various cellular functions of the ER, although the molecular mechanisms underlying the formation and maintenance of these structures remain poorly understood.

Observations: In this study, we used a transgenic Arabidopsis thaliana plant (GFP-h) in which the ER was fluorescently labeled. We isolated two mutants so-termed ermo1 and ermo2, for 'endoplasmic reticulum morphology', that showed defects in ER morphology (Nakano, R.T. et al., Plant Cell, 2009). The cells of both mutants developed a number of ER-derived spherical bodies, ~1 micrometer in diameter, in addition to the typical polygonal network of ER. The spherical bodies were distributed throughout the ermo1 cells, while they formed a large aggregate in ermo2 cells. The formation of these abnormal structures were independent of cytoskeletons. We identified the responsible gene for ermo1 to be GNOM-LIKE1 (GNL1) and the gene for ermo2 to be SEC24a. GNL1/ERMO1, SEC24a/ERMO2 and their homologs were thought to be involved in ER-Golgi protein trafficking in various eukaryotes. However, we found that the defects in ER-Golgi transport was not the direct trigger of the defects in ER morphology.

Conclusions: Our results demonstrate that ERMO1 and ERMO2 are involved in novel mechanisms that are unique to higher plant, rather than typical pathways such as interaction with cytoskeletons. ERMO1 and ERMO2 are transporting some specific key proteins that are crucial for organizing ER morphology.

B 095 Desmosome assembly and cell-cell adhesion are membrane raft-dependent

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Background: Desmosomes are the strongest intercellular junctions and are especially abundant in epithelia. Membrane rafts, cholesterol enriched membrane domains, are implicated in many cellular processes including cell adhesion. Our aim was

to determine if desmosome assembly in MDCK cells depends on the level of cell cholesterol, particularly on membrane rafts.

Observations: We demonstrated that desmosomal proteins associate with membrane rafts; as well as that desmosome assembly depends on cholesterol content. Biochemical analysis proved the association of desmosomal cadherin desmocollin 2 (Dsc2) with isolated rafts. Confocal microscopy and immunoelectron microscopy have proved significant colocalization of desmosomes with protein ostreolysin, a novel protein which binds specifically to cholesterol-sphingomyelin enriched domains. FRAP experiments have shown that depletion of plasma membrane cholesterol decreases lateral mobility of Dsc2. Live-cell imaging with controlled light exposure microscopy (CLEM) has revealed that the rate and the extent of desmosomal assembly were decreased in cells with reduced cholesterol concentration. Moreover, depletion of membrane cholesterol significantly reduces the strength of well-established cell-cell junctions.

Conclusions: Our data indicate that desmosomes associate with membrane rafts; particularly with the ostreolysin type of membrane rafts and that intact membrane rafts are necessary for desmosome assembly. These data suggests that cholesterol is a potential regulator of desmosome assembly.

B 096 Reconstitution of insulin secretory granules transport in vitro

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Background: Impaired insulin secretion in response to elevated blood glucose level is the hallmark of type 2 diabetes. Insulin is produced by pancreatic beta cells, stored in the insulin secretory granules and secreted upon glucose stimulation. Prerequisite of insulin release is the transport of secretory granules to the plasma membrane. Our aim is to establish the in vitro assay to study the transport of insulin secretory granules in the minimal system.

Observations: We use the motor protein-dependent affinity of the insulin secretory granules (ISG) to the in vitro polymerized microtubules (MT) to isolate ISG-motor protein complexes from the post nuclear supernatant. Separation of MT-ISG complexes from free ISGs is based on the high speed centrifugation through glycerol cushion. Addition of MTs alone or with AMPPNP (the nonhydrolyzable analog of ATP) results in the enrichment of the ISG markers and motor proteins in the pellet compared to the addition of MTs with ATP or to the control without MTs. ISG-motor protein complexes are then released from the MTs by addition of ATP and this material is used in the in vitro stepping assay. Fluorescently labeled ISGs and MTs are observed by TIRF microscopy and evaluated for the movement of ISGs on the MTs. Currently we observe mostly the localization of immobile ISG to MTs but few motility events are being evaluated and the assay is being further optimized.

Conclusions: The effect of AMPPNP in contrast to ATP, as well as correlation of secretory granules marker with motor protein levels implies the involvement of endogenous moloecular motors in MT-ISG preparation. The motility assay is still under the development and first events are being analysed.

B 097 Mechanism of transport through the Golgi complex

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Background: The Golgi apparatus arranged as a stack of cisternae is a central sorting station in the secretory pathway. A main question in the transport field is how proteins are transported through the Golgi: whether anterograde transport of cargo mediated by carriers or by maturation of the entire cisternae? To address this, we are studying the dynamics of the Golgi residents to determine how these are coupled to the dynamics of the cargoes that are transported through the Golgi.

Observations: First, to investigate the dynamics of the Golgi enzymes, we have engineered a Golgi-resident glycosylation enzyme that can be polymerised in a regulated way, to obtain a polymer that is large enough not to enter retrograde carriers. We are also studying if and how this polymerization affects the localisation of the Golgi-resident proteins and the functioning of the Golgi apparatus. First, we find that the polymerisation of a cis Golgi resident enzymes (ManI-FM) induces its shift to the trans-Golgi, a displacement that is not apparently mediated by vesicles or tubules. Second, the depolymerisation of the enzyme, once this has reached the trans side of the Golgi, induces its recycling back to its steady state position (cis/medial) in a few minutes.

Conclusions: The polymerised ManI traverses the Golgi without entering the carriers. The depolymerisation of the enzyme at the trans level induces its recycling to the steady state position. Our results strongly fit with cisternal maturation model.

B 098 Urothelial plaques are not endocytosed in differentiated urothelial cells in vivo

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Background: Differentiation of normal superficial urothelial cells is characterized by the formation of specialized membrane domains, called urothelial plaques. Plaques are thought to accommodate bladder's luminal surface area by exocytosis and endocytosis of unique membrane carriers, i.e. fusiform vesicles. Controversial results about these two processes were published. Our aim was to follow endocytosis of urothelial plaques in vivo by obtaining physiologic conditions of the experiment.

Observations: Gold nanoparticles (AuroVist, NanoProbes, USA) were injected into tail vein of normal mouse. Ninety minutes after injection, bladders were fixed, silver enhanced and processed for electron microscopy. Results showed that 1.9 nm gold particles were filtered in kidneys and reached the urinary bladder. However, gold particles were not detected in the cytoplasm of majority of superficial cells. In those cells that contained gold particles, they were seen in endosomes, but never in the fusiform vesicles. To test the ability of urothelial cells to endocytose gold nanoparticles, we added AuroVist also to cultured normal swine urothelial cells and to transformed urothelial cell line (RT4). Results showed that these cells did not have any fusiform vesicles, which demonstrated that these

urothelial cells in vitro are less differentiated than normal superficial urothelial cells in vivo. Gold nanoparticles were commonly seen in endosomes of cultured cells, which further proved the suitability of gold nanoparticles for endocytotic experiments.

Conclusions: Gold nanoparticles are appropriate markers to follow endocytosis of urothelial cell under physiologic conditions in vivo. However, endocytosis in differentiated superficial urothelial cells is very limited and does not commonly involve urothelial plaques.

B 099 Internalization of the Clostridium botulinum C2 toxin is mediated by clathrin- and Rho-dependent mechanisms

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Background: The bacterial C2 toxin, produced by Clostridium botulinum, is an actin-ADP-ribosyltransferase, causing depolymerization of the actin cytoskeleton in eukaryotic cells. The C2 toxin belongs to the family of binary toxins and consists of the enzymatic subunit C2I and the binding subunit C2II. C2I is able to bind to activated C2IIa oligomers, and it has been suggested that the whole complex is internalized by a raft-dependent mechanism.

Observations: Here we analyzed by which mechanisms C2 toxin is endocytosed. In HeLa cells expressing a dominant negative dynamin mutant, cytotoxicity and C2 toxin uptake were blocked. Furthermore, siRNA mediated knockdown of flotillins or inhibition of Arf6 function, proteins suggested to be involved in dynamin-independent endocytosis, did not affect C2 toxicity. Likewise, knockdown of caveolin did not inhibit endocytosis of C2 toxin. In contrast, inhibition of clathrin function reduced the uptake of C2 toxin and delayed the cytotoxic effect. Finally, we demonstrated that internalization of C2 toxin depends on the small GTPase Rho.

Conclusions: In summary, C2 toxin is endocytosed by dynamin-dependent mechanisms and we provide evidence for involvement of clathrin and Rho.

B 100 Understanding the molecular mechanism of Golgi biogenesis in Trypanosoma brucei

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Background: The Golgi apparatus is the central organelle in the secretory system which modifies and sorts secretory cargo. Despite its crucial role in secretion, the biogenesis of this organelle is still poorly understood. One question remains unanswered concerns the source of Golgi components during biogenesis; specifically whether the newly forming Golgi uses material provided by the existing Golgi. Initial studies have provided indirect evidence to suggest this; however direct proof is not yet available.

Observations: In this project, we aim to provide direct evidence for the involvement of existing Golgi in the formation of new Golgi. The large number of Golgi stacks in mammalian cells makes it difficult to address mechanistic questions about Golgi biogenesis. To circumvent this problem, we have chosen the simple parasite Trypanosoma brucei to study Golgi biogenesis in detail. T. brucei cells have a single Golgi apparatus which is duplicated and partitioned precisely during cell division. We established stable cell lines in T. brucei that express Golgi components fused to optical highlighter fluorescent proteins such as photoactivatible green fluorescent protein (PAGFP). These cell lines will allow us to track Golgi components during Golgi biogenesis by live-cell microscopy. We will then determine the potential contribution of the existing Golgi to new Golgi formation and investigate whether the kinetics of the contribution of this material correlate with the kinetics of new Golgi formation.

Conclusions: Tracking a subpopulation of proteins in the cell using photoactivatible fluorescent proteins is an excellent tool to study the order of events and kinetics of organelle biogenesis. Using this approach we hope to address key questions on the formation of Golgi apparatus.

B 101 Lipid droplets are functionally connected to the endoplasmic reticulum in Saccharomyces cerevisiae

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Background: Cells store metabolic energy in the form of neutral lipids that are deposited within lipid droplets (LDs). In this study, we examine the biogenesis of LDs and the transport of integral membrane proteins from the endoplasmic reticulum (ER) to newly formed LDs.

Observations: In cells that lack LDs, otherwise LD-localized membrane proteins are homogenously distributed in the ER membrane. Under these conditions, transcriptional induction of a diacylglycerol acyltransferase that catalyzes the formation of the storage lipid triacylglycerol (TAG), Lro1, is sufficient to drive LD formation. Newly formed LDs originate from the ER membrane where they become decorated by marker proteins. Induction of LDs by expression of the second TAG-synthesizing integral membrane protein, Dga1, reveals that Dga1 itself moves from the ER membrane to concentrate on LDs. Photobleaching experiments (FRAP) indicate that relocation of membrane proteins from the ER to LDs is independent of temperature and energy, and thus not mediated by classical vesicular transport routes. LD-localized membrane proteins are homogenously distributed at the perimeter of LDs, they are free to move over the LD surface and can even relocate back into the ER, indicating that they are not restricted to specialized sites on LDs.

Conclusions: These observations indicate that LDs are functionally connected to the ER membrane and that this connection allows the efficient partitioning of membrane proteins between the two compartments.

B 102 Function of Amphiphysin in clathrin/AP-1 coated vesicle formation

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Background: Transport of cargo within the endocytic and secretory pathway is generally mediated by coated vesicles. Clathrin and the adaptor complex AP-2 are the major coat components of endocytic vesicles originating from the plasma membrane. They are at the centre of a complex interactome of proteins accessory to vesicle formation. Less is known about the formation of clathrin coats at the trans-Golgi network and endosomes, which involves the adaptor complex AP-1.
Observations: In vitro studies showed the minimal requirements to recruit AP-1 to liposome membranes to be activated Arf1, phosphoinositides, and either sorting signals or an unknown cytosolic factor. We have purified this unknown factor from calf brain cytosol by several chromatographic methods, testing the fractions for in vitro activity to recruit AP-1 with Arf1•GMPPNP to cargo-free liposomes. The final fractions contained three major proteins that were identified by mass spectrometry to be amphiphysin 1, amphiphysin 2, and endophilin A1. All three proteins are known components of the AP-2/clathrin interactome at presynaptic terminals. They consist of an N-terminal N-BAR domain for membrane binding and dimerization and a C-terminal SH3 domain for interaction with dynamin. Amphiphysins 1 and 2 in addition contain a middle domain with binding sites for AP-2 and clathrin. The wild-type proteins as well as mutant forms were bacterially expressed, purified, and tested for in vitro AP-1 recruitment activity. Only amphiphysin 2 showed activity, both as a homodimer and as a heterodimer with amphiphysin 1. Activity depended on a PWDLW motif that is also important for interaction with clathrin.
Conclusions: Our results indicate that the amphiphysin 1 and 2 and endophilin function not only in endocytosis, but are also part of AP-1/clathrin coats at the trans-Golgi network and/or endosomes in intracellular membrane traffic.

B 103 Micropatterning for lipid-raft localization analysis of proteins in living cells

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Background: We have developed an assay for quantitative analysis of the interaction between a fluorescently marked protein (prey) and a membrane protein (bait) using microstructured surfaces, produced by micro-contact printing, covered with ligands (antibodies) targeted against the bait. The proof-of-concept was demonstrated for the interaction between CD4, a major co-receptor in T cell signalling, and Lck, a protein tyrosine kinase essential for early T cell signalling (Schwarzenbacher Nat.Met.2008).
Observations: Here we present the applicability of the assay for the analysis of protein interactions within lipid rafts in the inner and outer leaflet of the plasma membrane. Therefore we stably expressed several fluorescently labelled raft and non-raft proteins in the human T24 cell line as prey proteins and determined the degree of interaction with the antibody targeted bait proteins CD59 (GPI-anchored protein, raft marker) and CD71 (Transferrin-receptor, non-raft marker). Our results display a colocalization of CD59 and Actin-GFP as well as an interaction between CD59 and the GPI-anchor of the decay acceleration factor fused to a GFP, suggesting that these proteins are present within the same lipid domains (Weghuber Anal.Bioanal.Chem. 2010). Surprisingly the GPI anchors of other proteins fused to GFP showed different affinities. The distribution of folate-receptors GPI-anchor fused to GFP remained unchanged when patterning CD59, while this patterning displayed repulsive forces on the GPI-GFP of the Trail-receptor 3: The Trail-R3-GPI-GFP was excluded from the CD59 positive sites. Importantly all analyzed proteins didn't show any redistribution upon CD71 patterning.
Conclusions: While the detected absence of CD71 from and the presence of CD59 in lipid rafts confirms current knowledge, our results suggest that there is not only one sort of lipid rafts. However, there are still numerous open questions concerning rafts and our assay will be of great interest to address them.

B 104 Heterotrimeric kinesin-II is involved in odor receptor localization in the ciliary outer segments after their assembly in Drosophila

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Background: Kinesin-2 family motors play distinct roles in the assembly and maintenance of the structurally and functionally diverse sensory cilia in C. elegans and vertebrates. However, we showed that the bipartite cilia on olfactory neurons assemble before the expression of the olfactory receptors (ORs) and heterotrimeric kinesin-II is necessary and sufficient for the process. Nevertheless, the mechanism of activating and maintaining the olfactory reception in the adult antenna remain unclear.
Observations: The olfactory neurons innervating the sensilla basiconica in the adult Drosophila antennae expressed elaborate bipartite cilia. A range of ORs, involved in specific odor reception, are displayed on the membrane of the ciliary outer segments. The receptor localization is the key step in activation of olfactory reception. We found that the odor reception in the basiconic sensilla in adult Drosophila antennae progressively increases after eclosion. The kinesin-II motor subunits (KLP64D and KLP68D) and the accessory protein (DmKAP) are enriched in the ciliary outer segment in the adult antenna, and actively move into the compartment throughout the adult life. In addition, specific mutations in the Klp64D gene selectively affect the maturation of ordor reception and olfactory receptor localization to the ciliary outer segment in the adult antenna.

Conclusions: These observations suggest that a second phase of kinesin-II dependent transports of ORs and other associated proteins help to activate the sensory function in Drosophila antenna, and established a quantitative paradigm to explore the mechanism of sensory maturation and plasticity in future.

B 105 An in vivo, whole-genome RNAi screen for genes involved in autophagy in Drosophila

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Background: Autophagy is a conserved pathway for the degradation of cytoplasmic material in lysosomes for subsequent reuse. This process is involved in various physiological and pathological conditions such as aging, cancer, immunity, neurodegeneration diseases etc, and various drugs affecting autophagic activity are already used in clinical trials. In spite of the tremendous progress made in recent years, we are still far from complete understanding of the genetic networks involved in autophagy.
Observations: To gain further insight into the molecular mechanisms and regulation of autophagy, we carried out an RNAi screen in mosaic fat bodies of starved Drosophila larvae using an mCherry-Atg8 reporter assay. We tested the effect of knocking down 7,118 individual genes, corresponding to 90% of the conserved genome. Importantly, we identified almost all known regulators of autophagy including Atg (autophagy-related) gene homologs in a blind fashion. In this primary assay, a total of 488 lines suppressed and 559 lines enhanced mCherry-Atg8 puncta formation, compared to surrounding wild-type tissue. We next carried out secondary tests, such as analyzing the effect of these gene knockdowns in well-fed larvae, and independent reporter assays that include multiple transgenic mCherry- or GFP-tagged Atg proteins, reporters for autophagic flux and lysosomes, and LysoTracker Red staining to detect the proper formation of acidic autolysosomes. With a combination of these in vivo assays, we were able to classify our hits into distinct phenotype categories, establishing their roles in the hierarchy of Atg proteins, autophagosome formation or maturation, and autolysosomal degradation.
Conclusions: We found most known core components and regulators of autophagy in our in vivo functional genomics approach. We also identified hundreds of additional genes potentially involved in this process, and established the stages when these gene products are required for proper progression of autophagy.

B 106 ClC-7 mediates slowly voltage-gated chloride/proton-exchange and requires Ostm1 for transport activity

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Background: The CLC family of anion transporters comprises both plasma membrane chloride channels and intracellular chloride/proton exchangers. ClC-7 localizes to lysosomes and to the ruffled border of osteoclasts. It forms homodimers and it needs Ostm1 for protein stability. Loss of either ClC-7 or Ostm1 leads to osteopetrosis and lysosomal pathology. The intracellular localization of ClC-7/Ostm1 has so far precluded a detailed biophysical characterization.
Observations: We have identified dileucine motifs in the N-terminus of ClC-7 that mediate endosomal/lysosomal sorting through binding to AP adaptor proteins. Mutation of these motifs resulted in cell surface expression of ClC-7. When expressed with Ostm1, this mutant gave robust, strongly outwardly rectifying plasma membrane currents. The presence of both the N-terminus and the transmembrane part of Ostm1 were required for transport activity, while the transmembrane part was sufficient to mediate ClC-7/Ostm1 interaction. ClC-7/Ostm1 currents displayed slow activation and relaxation kinetics, allowing for tail current analysis. This showed that the open transporter has an intrinsically almost linear voltage dependence. Reversal potentials of tail currents revealed a 2Cl/1H-exchange stoichiometry. Some disease-causing CLCN7 mutations reduced currents while others accelerated gating. The latter mutations cluster to either side of the potential contact zone between the second CBS domain of the cytosolic C-terminus and the transmembrane segment.
Conclusions: Slow gating of ClC-7/Ostm1 may be physiologically important and may involve interaction between the cytosolic and the transmembrane parts. Our work suggests that gating underlies the rectification of all intracellular CLCs and extends the concept of voltage-gating beyond channels to ion exchangers.

B 107 Cargo sorting by the exomer complex requires a tetratricopeptide repeat backbone and a composite cargo recognition domain

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Background: In yeast, a specific subset of proteins is exported from the trans-Golgi network to the plasma membrane via the exomer complex. Exomer comprises Chs5p and the ChAPs family (Chs5p-Arf1-binding proteins: Bud7p, Chs6p, Bch1p, Bch2p) and is essential for the sorting of cargoes into secretory vesicles. It is thought that the complex may act as a coat, although the evidence for this hypothesis remains inconclusive. Also, little is known about the assembly of exomer and its mode of cargo recognition.
Observations: We found that each of the ChAPs contains five conserved tetratricopeptide repeats (TPRs), indicating that they might play a role in exomer-mediated cargo export. Indeed, we could show that mutant ChAP constructs lacking either the first four or the fifth TPR fail to export cargo, demonstrating that the TPRs are required for the function of the ChAPs. Interestingly, the individual repeats perform different functions: Chs6p lacking TPR1-4 could not interact with Chs5p and thus lost proper TGN localisation, while Chs6p lacking TPR5 still associated with Chs5p and Arf1p but showed inefficient cargo binding. Chimeric mutants of the ChAPs revealed that their specificity for particular cargoes does not depend on any of the TPRs, but on the rest of the protein including the N- and C-terminus, as well as a region interspersed between the repeats. Accordingly, we

found that most of the TPRs are highly conserved in evolution, while the sites of lowest sequence conservation are located in the regions determining cargo specificity.
Conclusions: These results show that the exomer uses TPRs to assemble its subunits into a higher-order structure, similar to the COPI coat or the anaphase promoting complex. Moreover, unlike for most other cargo receptors, the ChAPs' cargo recognition domain appears to be of non-linear, purely structral nature.

B 108 Recombinant Mpv17 is a channel-forming protein

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Background: Mpv17 is a mammalian inner mitochondrial membrane protein with unknown function. Knock-out of mouse Mpv17 results in glomerulosclerosis and in ultrastructural and physiological defects in the cochlea. Human Mpv17 is mutated in infantile hepatic mitochondrial DNA depletion syndrome.
Observations: Mpv17 belongs to a small family of proteins that includes the peroxisomal membrane protein Pxmp2. Recently we described function of Pxmp2 as a channel-forming protein involved in transfer of small solutes across peroxisomal membrane. This led us to suggest that other members of the family including Mpv17 protein are in fact membrane channels. Mouse His-tagged Mpv17 was expressed in the yeast Pichia pastoris and isolated using conventional chromatography. Circular dichroism analysis confirmed preservation of the secondary structure of purified protein. Size-exclusion chromatography showed mainly monomeric form of isolated Mpv17 while homodimes and homotrimers have also been detected. Characterization of the channel-forming activity of Mpv17 was conducted using planar lipid bilayer technique. The measurements revealed the pore-forming activities mainly at three conductance levels: 1.6, 3.2, and 5.2 nS in 3.0 M KCl. Voltage ramp protocol showed linear dependence between an applied voltage and a channel current in the diapason of ± 100 mV. The Mpv17 channel showed weak anion selectivity and no voltage dependence.
Conclusions: The relatively high conductance and long-lasting open states of the channel indicate that recombinant Mpv17 protein is able to form a general diffusion pore in the membrane. The potential functional role of Mpv17 channel in the inner mitochondrial membrane is discussed.

B 109 Cholesteryl hemiesters in LDL cause endolysosomal lipid accumulation, foam cell formation and apoptotic cell death of macrophages

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Background: The 'oxidation of Low Density Lipoproteins (LDL)' is one of the oldest and most tested hypothesis for the etiology of atherogenesis. Oxidized LDL (Ox-LDL) exist in vivo in the artery wall and stimulate endothelial cells to produce pro-inflammatory molecules that recruit monocytes and promote their differentiation to macrophages. These are known to internalize the ox-LDL and form 'foam cells'.
Observations: Ox-LDL have been used to study 'foam cell' formation from macrophages and elucidate the origin of atherogenesis. Since Ox-LDL contain a large number of potentially bioactive products of lipid peroxidation, it is impossible to establish etiological hierarchies among the Ox-LDL constituents in foam cell formation. Therefore, we developed a new LDL model in which native (unoxidized) LDL are loaded with a single chemical species that represents a family of compounds that may be formed upon oxidation of LDL. Specifically, we demonstrate this principle using cholesteryl hemissuccinate to model the cholesteryl hemiesters that are the chemically expected stable end-products of oxidation of cholesteryl linoleate and arachidonate, the major cholesteryl esters of LDL. Exposure of macrophages to native LDL enriched in cholesteryl hemisuccinate induced intracellular lipid accumulation in a time- and concentration-dependent manner. Large lipid structures decorated with LAMP-2, a lysosomal marker, were observed. Lipidomic analysis also show that lysosomes are not able to digest the LDL enriched in cholesteryl hemisuccinate. Massive apoptotic cell death is also observed.
Conclusions: In conclusion, this study describes a new approach to study in vitro foam cell formation, identify chemical components of Ox-LDL that may be responsible for irreversible lipid accumulation in endolysosomes, macrophage death and atherogenesis.

B 110 Endocytic regulator NDRG1 is required for LDL-receptor plasma membrane localization and LDL-cholesterol uptake

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Background: Mutations in NDRG1 (N-myc downstream regulated gene 1) cause a recessively inherited demyelinating neuropathy, Charcot-Marie Tooth Disease type 4D (CMT4D) but the cellular function of NDRG1 and the disease pathogenesis are poorly understood. We found NDRG1 as a hit when searching for proteins involved in the cellular processing of low-density lipoprotein (LDL)-cholesterol. NDRG1 was upregulated in cells treated with the compound U18666A that induces late endosomal cholesterol deposition.
Observations: Here we show that upon NDRG1 knockdown (k/d) in A431 cells, free cholesterol was redistributed from the plasma membrane to modified early endosomes (EEs) and cholesterol ester stores were depleted. NDRG1 silencing resulted in diminished LDL-cholesterol binding to cells due to the accumulation of LDL-receptors (LDLR) intracellularly in EEs. In NDRG1 depleted cells, the maturation of EEs into multivesicular bodies was disturbed and the dynamic trafficking of tubules, required

for cargo recycling to the plasma membrane, was reduced. Overexpression of wild-type NDRG1 rescued the endosomal LDLR accumulation while the major CMT4D -disease mutant form of NDRG1 (R148X) produced an unstable protein that failed to rescue the phenotype. In a murine oligodendroglial cell line (Olineu), NDRG1 k/d also reduced LDL uptake and induced a cholesterol imbalance. Moreover, NDRG1 k/d caused a significant reduction in the amount of the oligodendrocyte transcription factor, Olig2.
Conclusions: Together, our findings indicate that NDRG1 deficiency leads to a lipid imbalance due to endosomal LDLR trafficking defects. The dysfunction of LDLR family members in oligodendrocytes may contribute to the demyelination in the absence of functional NDRG1 in CMT4D.

B 111 Structure of the chaperone of the Type III Secretion System translocators of Pseudomonas aeruginosa

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Background: The type III Secretion System (T3SS) is a multimeric complex present on the surface of many human pathogens (eg Shigella, Samonella, Yersinia) that allows injection of toxins directly into the cytoplasm of eukaryotic cells. This system can be divided into 3 parts: a basal structure that anchors it to the bacterial membranes, a fibrous-like needle, and a translocation pore that is inserted into the host membrane. The integrality of each part is an absolute requirement for a functional system.
Observations: Our group is particularly interested in the translocation pore formed by membrane proteins PopB and PopD (in Pseudomonas aeruginosa). These proteins share a common chaperone, PcrH, whose role is to stabilize them within the cytoplasm prior to secretion. The site(s) of recognition of Pops within the PcrH have been a matter of controversy. We purified the 1:1 PopD:PcrH and PopB:PcrH complexes in E. coli, and solved the high-resolution structure of apo-PcrH as well as of PcrH bound to a short region of PopD (residues 47 to 56). The PcrH in apo form is highly similar to that of its Yersinia homolog SycD. Notably, the PopD:PcrH complex structure reveals that the PopD peptide binds within the concave region of PcrH, a binding pattern which is analogous to that of the PopB:PcrH homolog in Shigella, IpaB:IpgC. Thus, in different T3SS, both translocation proteins occupy the same pocket in their common chaperone. Moreover, by employing site-specific mutagenesis combined with co-purifications in E.coli and in vivo tests in Pseudomonas, we demonstrated that 4 hydrophobic residues within the PopD interaction region (47-56) are responsible for PcrH complex formation and PopD stabilization.
Conclusions: This structural and functional data provides tools for the screening of drug-like molecules that could potentially disrupt chaperone:translocator interactions, preventing formation of the translocation pore and thus blocking toxin injection through the T3SS.

B 112 Quality control mechanisms inside peroxisomes: the role of a Lon protease

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Background: The removal of damaged or misfolded proteins by proteolytic digestion helps to avoid potentially catastrophic aggregation in the densely packed intracellular environment. The ATP fueled proteolysis performed by members of the family of AAA proteases constitutes vital quality control mechanism in all cells. The Lon protease family, which is the most widespread family of ATP-dependent proteases, is conserved in prokaryotes and in eukaryotic organelles such as mitochondria and peroxisomes.
Observations: The contribution of Lon protease in protein quality control surveillance in mitochondria has been well established, but the role of the peroxisomal Lon protease is still poorly understood. We have studied the function of the putative peroxisomal Lon protease (pln) from the filamentous fungus Penicillium chrysogenum. We confirmed that the PLN gene indeed encodes a peroxisomal matrix protein with proteolytic activity. Disruption of the PLN gene caused accumulation of protein aggregates in the peroxisomal matrix. The major constituents of these aggregates have been identified as heme-containing catalase-peroxidase and FAD dependent oxidoreductase from the GMC family. The PLN deficient cells were characterized by decreased catalase and peroxidase activities and increased oxidative stress.
Conclusions: Our data suggest that peroxisomal catalase-peroxidase is an important substrate of the peroxisomal lon protease in P. chrysogenum. In cells lacking Pln, the activity of catalase-peroxidase is reduced, which most likely causes the observed enhanced oxidative stress.

B 113 A novel approach for the detection of short-lived enzyme-substrate interactions – Identification of protein phosphatase 2A (PP2A) substrates

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Background: PP2A(CDC55) is indicated to be a master regulator of mitosis. Even though genetic assays have suggested many substrates for PP2A, the verification of these substrates proves to be difficult, because most PP2A-substrate-interactions are very short-lived and therefore hard to detect with current methods.
Observations: Thus, our lab adopted and developed a new two-hybrid approach termed M-TRACK, in which a protein-protein

interaction is detected by a second, simultaneously occuring enzyme-substrate interaction between a histone lysine methyl transferase (HKMT) and the histone H3 N-terminus, which results in a specific and persistent methylation of the prey. Our bait CDC55-fusion-protein rescued a cdc55Δ defect in the spindle assembly checkpoint and was incorporated into an active holoenzyme complex, indicating its functionality in vivo. Consistent with this, we detected in an M-TRACK assay a direct interaction between PP2A(CDC55) and NET1, a protein involved in the regulation of mitosis. The detection depended on the presence of CDC55 and the Cdk-targeted phosphosites in NET1. The transcription factor YAP1, however, a substrate of the phosphatase PP2B was not detected by the bait HKMT-CDC55.

Conclusions: Together, these results provide strong evidence for NET1 as a true in vivo-substrate of PP2A as well as for M-TRACK's ability to detect in vivo the short-lived interaction between an enzyme and its specific substrate.

B 114 Chaperones suppress protein oligomer toxicity: insight into the molecular mechanism of action

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Background: Deficiencies in the homeostasis of the proteome lead to many human diseases. Indeed cells have evolved the molecular chaperone machinery as a key control mechanism for regulation of proteostasis. Chaperones facilitate folding, inhibit aggregation and promote clearance of misfolded aggregates. Little is known, however, about their ability to suppress the toxicity of aberrant protein aggregates, that are considered the major deleterious species in protein misfolding diseases.

Observations: The effect of five human chaperones, namely alphaB-crystallin, Hsp70, clusterin, alpha2-macroglobulin and haptoglobin, was tested on the toxicity of misfolded oligomers formed by the N-terminal domain of HypF from E. coli (HypF-N), the 42-residue form of the amyloid beta peptide (Abeta42) and the islet amyloid polypeptide (IAPP). Measures of the cell viability showed all five chaperones are effective in suppressing the toxicity of these protein oligomers. An investigation at the molecular level showed that the ThT-binding of the oligomers was not affected by chaperones, indicating that they do not dissolve the oligomers. In addition, the chaperones do not change the N-(1-pyrene)maleimide fluorescence spectra of the oligomers labelled with the dye, suggesting that they do not remodel the structure of the oligomers. Experiments carried out using intrinsic fluorescence, SDS-PAGE and immuno-dot blot show that the chaperones bind to the oligomers. Atomic force microscopy images reveal that, following binding, the chaperones induce the assembly of the oligomers into larger species.

Conclusions: These data suggest that the chaperones promote the clustering of the oligomers by binding them. The cluster are harmless because they are unable to interact and cause damage to the cells, due to the shielding of the reactive patches on the oligomers and the decrease of their diffusional mobility.

B 115 TRIM50 forms a complex with HDAC6 and p62/SQSTM1 that localizes to aggresomes

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Background: TRIM50 is hemizygous in patients affected by Williams Beuren syndrome, a neurodevelopmental genomic disorder, caused by a 1.5-1.8 Mb deletion at 7q11.23 including 28 genes. We recently demonstrated that TRIM50 encodes a cytoplasmic E3-ubiquitin ligase that catalyzes the binding of specific substrates to the ubiquitin leading them to the degradation by activating the Proteasome or autophagy system.

Observations: Further characterization of this protein was able to show that TRIM50 forms highly labile and dynamic cytoplasmic bodies that are aggresome precursors. Indeed in response to proteasome inhibition, TRIM50 localizes to the aggresome in a microtubule dependent manner. In addition, we identified HDAC6 as a new binding partner of TRIM50, an association that is strengthen under conditions of protein impairment. Using HDCA6-deficient fibroblasts we demonstrated that HDAC6 is required for the proper localization of TRIM50 within the aggresome. Furthermore, we provide experimental compelling evidences that TRIM50 localizes and interacts with p62, a multifunctional adaptor protein implicated in various cellular processes including autophagic clearance of aggregation-prone polyubiquitinated proteins. Of note TRIM50 modulates the protein level of HDAC6 and p62, mainly increasing their insoluble fractions.

Conclusions: TRIM50 protein is an aggresome precursor. When its E3-Ub-ligase activity fails to drive its substrates to proteasomal degradation, an alternative route is taken to ensure their sequestration to the aggresome via the association with HDAC6 and their subsequent removal by p62-mediated autophagy.

B 116 The dodecameric megasynthase AccD1-AccA1 of Mycobacterium tuberculosis is a functional acyl-CoA carboxylase

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Background: Mycobacterium tuberculosis is an obligate human pathogen, whose survival in the host is directly linked to its unique ability to synthesize novel lipids. Among the enzymes required for the synthesis of these membrane lipids are the acyl-CoA carboxylases (ACCase), large multi-functional nano-machines, that synthesize the extender units used by fatty acid synthases for the synthesis of fatty acids. We aim to determine the first high-resolution native structure of an ACCase holo-enzyme.
Observations: We report the discovery and characterization of a third novel ACCase complex encoded by Rv2501 and Rv2502, as well as the first low-resolution structure of a complete M. tuberculosis ACCase holo-enzyme. The complex is a functional carboxylase. It carboxylates both acetyl-CoA and propionyl-CoA, the extender units used in the synthesis of the mycolic acids and the multimethyl-branched trehalose-base lipids of the mycobacterial outer membrane, respectively. The alpha- and beta-subunit protomers encoded by Rv2501 and Rv2502 physically interact to form a large symmetrical dodecamer composed of a hexamerix beta-subunit flanked on either side by three alpha-subunit protomers. Precision knockouts of orthologouse genes in Mycobacterium smegmatis imply that these genes are non-essential for survival, but may be required for effective growth and have a distinct colony morphology.
Conclusions: These data demonstrate significant redundancy build into the mycobacterial ACCase family, highlighting the biological importance of this family of genes. We have produced a functional, homogenous and stable dodecameric ACCase, which can be expressed in quantities suitable for crystallography.

B 117 Mechanisms of amyloid fibril formation: focus on domain-swapping

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Background: Common to conformational diseases (among them amyloidoses) are changes in protein conformation, leading to proteins intracellular aggregation and toxicity or/and, in some cases, to extracellular deposition, burdening physically the organs in question or the brain vessels in diseases such as Alzheimer's. Models for the mechanism of the ordered aggregation into amyloid fibrils can be classified as: 1. Templating and nucleation; 2. Colloid-like assembly of spherical oligomers; 3D Domain-swapping.
Observations: It has been demonstrated that protein aggregation to amyloid fibrils and even the aggregates toxicity is in common to all proteins, folded and natively unfolded, pathological and those which are not connected to any disease. Thus, studying cystatins can be seen of general importance but they also represent a special case of proteins which swap domains. We are studying predominantly human stefin B (cystatin B), a globular protein not involved in amyloid pathology. However, this gene is mutated in EPM1, a progressive myoclonus epilepsy of type 1. One characteristic feature in common to stefins and cystatins is domain-swapping. In addition to solution structure of the domain-swapped dimer of stefin A, we recently have determined 3D structure of stefin B tetramer, which proved to be composed from two domain swapped dimers. Interaction between the dimeric units occurs by a proline switch in the loop surrounding Pro 74. It has been shown that the nucleation and fibril elongation reactions indeed have energies of activation (Ea's) in the range of proline isomerisation.
Conclusions: Our aim remains to understand the molecular mechanisms of protein aggregation to amyloid fibrils and the source of amyloid induced toxicity. Understanding the molecular details of these processes is of major importance and could contribute to more effective therapies.

B 118 Amyloid promoting properties of an RNA chaperone

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Background: Proteinaceous amyloid fiber deposits in cells are linked to a variety of devastating neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease. The molecular determinants of amyloidogenesis are the focus of intensive investigations. The small basic protein MOAG-4/SERF has been recently described as an ageing-related endogenous modifier of amyloid fiber assembly and toxicity when over-expressed in cells. The molecular details of this relationship, however, are obscure.
Observations: We observed a direct influence of MOAG-4/SERF on amyloid fiber maturation. In an isolated in vitro system, the human isoform SERF1b was able to significantly accelerate the assembly of disease-linked amyloidogenic proteins into fibers by interfering with the intermediate steps of the amyloid pathway. Intriguingly, SERF1b also exhibited pronounced in vitro RNA chaperone activity by accelerating the annealing of complementary RNA strands. This led us to speculate that SERF1b had an additional function in RNA-related cellular processes. The inherent structural disorder of SERF1b, together with its low binding affinity for single stranded nucleic acids (Kd approx. 5 microM), argues for a protein engaged in promiscuous binding processes, as expected for a chaperone. The amyloid-promoting and RNA chaperone activities had different structural requirements. The intact protein was necessary for RNA binding and chaperone activity, while the C-terminal region of SERF1b was sufficient to accelerate amyloidogenesis.
Conclusions: We identified SERF1b as a bifunctional protein with both amyloid-promoting and RNA chaperone properties. We suppose that this is mediated by the polypeptide's intrinsic structural disorder, which would allow it to conformationally rearrange into either an amyloid-promoting or an RNA binding fold.

B 119 FKBP12 regulates the localization and processing of amyloid precursor protein in vitro

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Background: One of the pathological hallmarks of Alzheimer's disease is the presence of insoluble extracellular amyloid plaques, which are mainly constituted of amyloid beta peptide, a proteolytic product of amyloid precursor protein (APP). APP processing also generates the APP intracellular domain (AICD). We have previously demonstrated that AICD interacts with FKBP12, a peptidyl-prolyl cis-trans isomerase (PPIase) ubiquitous in nerve systems. This interaction was interfered by FK506 dose-dependently.
Observations: To elucidate the roles of FKBP12 in the pathogenesis of Alzheimer's disease, the effect of FKBP12 overexpression on APP processing in cultured cells (HEK293T and SH-SY5Y) was evaluated. Our results revealed that APP processing was shifted toward the amyloidogenic pathway, accompanied by a change in the subcellular localization of APP, upon FKBP12 overexpression. This FKBP12-overexpression-induced effect was reverted by FK506. These findings in conjunction with the observation that FKBP12 is preferentially associated with Thr668 phosphorylated APP support our hypothesis that FKBP12 may participate in the regulation of APP processing in a fashion similar to Pin1, a peptidyl-prolyl isomerase belonging to another PPIase family.
Conclusions: FKBP12 overexpression may lead to the stabilization of a certain isomer of the Thr668-Pro669 peptide bond in AICD, therefore change its affinity to flotillin-1 or other raft-associated proteins, and eventually change the localization pattern and cause a shift in the proteolytic processing of APP.

B 120 Antioxidant systems in cord blood erythrocytes versus mother venous blood erythrocytes

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Background: Pregnancy related oxidative stress is well documented by now. Literature reports suggest causes of oxidative stress belonging to metabolic patways perturbations and immune system response to pregnancy. Maternal oxidative stress may influence foetus antioxidant systems. Blood systems fighting with oxidative stress include antioxidant enzymes present in erythrocytes. We have considered of interest to study antioxidant systems performance in venous and cord blood erythrocytes.
Observations: The study included 200 pregnant women with normal pregnancies :gestation longer then 37 weeks and fetal weight more then 2700 g – also no pathologies associated to the mother or to the new born child. Venous and cord blood have been collected at birth. On erythrocites lysates catalase, superoxide dismutase, glutathione transferase, glutathione peroxidase activities and total and non proteic thiols level(mainly glutathione) have been determined.Statistical analysis performed revealed a linear correlation of superoxide dismutase activity in cord blood erythrocites with the enzyme activity in mother venous blood. No other correlations have been identified. Superoxide dismutase, catalase, glutathione peroxidase activities were significantly higher in mother venous erythrocites while glutathione transferase was higher in cord blood. No statistical significant differences have been found between thiol levels,both proteic and non proteic.
Conclusions: Increased level of glutathione transferase activity in cord blood erythrocites suggest it as one of the principal enzymes implicated in foetus antioxidant defence. Further studies are needed to elucidate how fetus antioxidant systems are fighting with mother increased oxidative stress.

B 121 Stress-regulation of PML nuclear bodies

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Background: Promyelocytic leukemia (PML) nuclear bodies (NBs) are multiprotein complexes involved in tumor suppression, DNA repair, transcription, apoptosis, and antiviral response. Treatment with arsenic trioxide (ATO) induces the SUMO-dependent ubiquitylation and degradation of the PML-RAR fusion protein in acute promyelocytic leukemia (APL) cells and remission of the disease in APL patients.
Observations: PML-NB assembly is mediated by attachment of the small ubiquitin-like modifier (SUMO) to PML and other proteins and their interaction with proteins containing SUMO interaction motifs (SIMs). However, we found that polysumoylated proteins are subject to a ubiquitin-dependent proteolytic control mediated by specific SIM containing ubiquitin-ligases for SUMO conjugates (ULS). In mammals, we identified the RING finger protein RNF4 as ULS involved in the ATO induced degradation of PML. The analysis of the interaction of SUMO chains with RNF4 by titration calorimetry revealed that a combination of two of the four putative SIMs in RNF4 is sufficient for a high affinity interaction with SUMO chains. ATO and other stress factors increase SUMO-2/3 modification and can thereby contribute to proteolytic targeting of PML. The cellular response to osmotic stress, however, differed from ATO treatment and indicates that additional factors regulate the stability of PML. Furthermore, different isoforms of PML displayed distinct changes of their modification pattern and stability upon stress conditions.
Conclusions: We conclude that the numerous functions of PML-NBs are regulated by carefully balanced posttranslational modifications including phosphorylation, mono- and polysumoylation as well as ubiquitylation and proteasomal degradation.

B 122 The beta-sheet-breaking T72P-hexapeptide protects neuronal cells from alpha-synuclein aggregate-induced neurotoxicity

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Background: The aim of this study is to suppress the fibrillation of human wild-type or Parkinson's disease (PD)-linked alpha-

synuclein variants, which is implicated in the pathogenesis of PD. We screened for alpha-synuclein mutant proteins that block the aggregation of alpha-synuclein. Short synthetic peptides derived from these mutant sequences were designed to block alpha-synuclein fibrillation, and the effects are evaluated. The peptides may serve as a lead compound for the development of therapeutics for PD.

Observations: We have identified more than 20 single-amino-acid substitutions that block self-aggregation, even during prolonged incubation. Among those self-fibrillation-defective alpha-synuclein mutants, six mutants (V66S, V66P, T72P, V74E, V74G, and T75P) also completely suppressed aggregation of wild-type and PD-linked alpha-synuclein molecules. beta-Sheet-breaking peptides not only prevented alpha-synuclein fibrillation but also dissolved preformed alpha-synuclein-aggregates. The beta-sheet breaking peptides protected neuronal cells from alpha-synuclein aggregate-induced cell death, when monitored using conventional methods. Furthermore, the peptide was resistant to proteolytic degradation in human plasma.

Conclusions: The hydrophobicity of the central hydrophobic region and the total negative charge of alpha-synuclein are important factors in fibrillation regulation. The hexapeptide was the shortest peptide that blocked alpha-synuclein fibrillation and protected neuronal cells from alpha-synuclein-induced neurotoxicity.

B 123 An improved evaluation system for the unfolding activity of chaperone unit ClpA using Fe-S protein Ferredoxin as a model substrate

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Background: ATP-dependent proteases unfold substrates and then refold or degrade them. They choose between these two activities by selecting substrates; however, little is known about the substrate selection. To address this, a reaction system that can measure both activities simultaneously must be constructed. As a first step of constructing such a system, we attempted to establish the new method to evaluate the unfolding activity of Escherichia coli ClpA, a chaperone unit of ATP-dependent protease ClpAP.

Observations: By using a protein whose physicochemical property change according to the unfolding reaction as a model substrate, the unfolding activity of ClpA could be evaluated from the change of the property. Such a model substrate, we decided to use Escherichia coli Ferredoxin (Fd). Fd has 2Fe-2S cluster in its native structure. The cluster is disrupted according to the unfolding of Fd and could not be remodeled quickly once disrupted. Therefore, we hypothesized that the unfolding activity of ClpA could be evaluated from the disruption of Fe-S cluster by using Fd as a model substrate. To prove this, we examined whether the Fe-S cluster of Fd could be disrupted according to the unfolding by ClpA. We evaluated the disruption of Fe-S cluster from the change of two specific absorption peaks (414 and 460 nm). As a result, by the reaction with ClpA, the absorption peaks derived from the cluster of Fd decreased according to the reaction time. Moreover, the decrease of the peaks correlated with the increase of free $Fe2+$ in the reaction mixture. These results show that the unfolding activity of ClpA could be quantified by using Fd as a substrate.

Conclusions: In this study, we show that by using Fd as a model substrate, the ClpA unfolding activity could be evaluated from the amount of Fe–S cluster disruption. The reaction system could be established to evaluate ClpAP substrate selection, if the method is used even in the presence of ClpP.

B 124 Quality control of disulfide bond formation in E. coli type 1 pilus biogenesis by the periplasmic chaperone FimC

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Background: Type 1 pili from Escherichia coli are proteinaceous surface organelles responsible for bacterial adhesion to the host tissue. All structural pilus subunits are homologous proteins sharing an immunoglobulin-like fold with a single, invariant disulfide bridge. In this study we focus on the initial steps in the assembly of type 1 pili, namely disulfide bond formation, binding of unfolded pilus subunits to the assembly chaperone FimC and chaperone-assisted catalysis of folding.

Observations: We have used the major structural subunit from type 1 pili, FimA, to investigate the role of the disulfide bond on chaperone-subunit complex formation and subsequent pilus subunit folding. Our in vitro results show that unfolded FimA is unable to bind the chaperone in its reduced state. Only when the disulfide bond is introduced by disulfide exchange with the periplasmic dithiol oxidase DsbA, unfolded FimA has the ability to bind to the chaperone. Thus, disulfide bond formation in the pilus subunits is required for recognition by FimC and thus essential for the biogenesis of these organelles. Stopped-flow fluorescence measurements and kinetic simulations performed with the measured in vivo concentrations of DsbA and FimC provide strong evidence that the oxidation and subsequent assembly of FimA with the periplasmic chaperone proceed rapidly in a sequential manner. The deduced in vivo half-life of oxidative FimA folding is about two seconds. Further we report that FimC accelerates the folding of FimA by at least four orders of magnitude compared to spontaneous FimA folding, and that binding of unfolded, oxidized FimA to FimC is rate-limiting for FimC-catalyzed folding of FimA.

Conclusions: FimC neither catalyzes folding of reduced FimA, nor forms complexes with reduced, unfolded FimA. FimC acts as a quality control system ensuring that i) only disulfide-intact subunits become assembly competent and ii) each of the subunits in the assembled pilus contain its structural disulfide bond.

B 125 Progressive loss of constitutive autophagy in Cln6-defective brain

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Background: Neuronal ceroid lipofuscinoses (NCL) are a group of neurodegenerative lysosomal storage disorders with clinical features like seizures, blindness, progressive mental and motor deterioration. NCL can be caused by mutations in the CLN6 gene that encodes a polytopic ER membrane protein of unknown function. To investigate mechanisms contributing to neurodegeneration in CLN6 disease we examined the nclf mouse, a natural model of the disease developing clinical symptoms closely resembling human CLN6.
Observations: In symptomatic nclf mice at 54 weeks of age, an accumulation of the autofluorescent storage material lipofuscin was found throughout the brain in both neurons and microglia. Activation of microglial and astrocytic cells was present in all brain regions but not consistent with autofluorescent storage material. Although mutant Cln6 is rapidly degraded by the proteasome, no significant differences were detected in several ER stress markers such as BiP, Hsp70 and eIF2alpha phosphorylation. In contrast, with progression of the disease, a 3.5-fold increase of the amount of autophagosomes was observed in the nclf brain as well as in the heart, liver, lung and spleen as assessed by LC3-II levels. This was accompagnied by a progressive formation of p62-positive inclusion bodies beginning at 20 weeks of age in nclf brain. These p62-positive aggregates associated with ubiqitin-containing cargo and were neither detected in astroglial and microglial cell nor colocalized with lysosomal marker proteins.
Conclusions: The progressive loss of constitutive autophagy in the brain causes accumulation of p62- and ubiquitin-positive inclusions. These neuronal inclusions seem not to be part of lysosomal structures and could contribute to the neurodegeneration observed in this fatal disease.

B 126 Identification of specific amino acid sequences of type I collagen accelerating fibril formation

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Background: Type I collagen is the most abundant protein in connective tissue of all vertebrates. The N- and C-telopeptide domains of collagen play an important role in the fibril formation involving an azimuthal and a lateral growth. We have reported that collagen hydrolyzed by actinidain proteinase, purified from kiwifruits, forms self-assembly indicating a novel meshwork. In this study, our aim is to determine the N- and C-terminal sequences of chicken type I collagen cleaved with pepsin or actinidain.
Observations: Crude type I collagen was hydrolyzed by pepsin or actinidain according to our previous procedures. The time-dependent appearances of collagen self-assembly were observed by scanning electron microscopy. The N-terminal amino acids of the alpha1 and the alpha2 chains, cleaved with pepsin or actinidain, were determined by Edman degradation. Moreover, each alpha chain was digested with trypsin for MS measurements, and the C-terminal amino acid sequence determined by MALDI-TOF/TOF mass spectrometry. To investigate whether the C-terminal telopeptide domain affects the fibril formation, we synthesized five nonapeptides mimicking the C-terminal sequence and measured the turbidity of AHCol in the presence of each nonapeptide. The SEM observations demonstrated that the rate of self-assemblies from AHCol was markedly different from that of PHCol. The N-terminal amino acid sequence of AHCol showed no cross-linking between alpha chains. Each C-terminal sequence of all alpha chains was determined by identifying the specific peak originating from the cleaved peptide. Only one synthetic nonapeptide meaningly accelerated the formation of self-assembly from AHCol.
Conclusions: Our experiments suggested that the specific sequence of the C-telopeptide plays a crucial role in stipulating collagen suprastructure and in subsequent fibril formation.

B 127 Rapid Protein Oligomer Formation Induced by Heparan Sulfate Studied by a Stopped-Flow Device

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Background: Many human diseases are caused by the conversion of proteins from their native state into amyloid fibrils that deposit in the extracellular space. Heparan sulfate (HS), a component of the extracellular matrix, has been found to be universally associated with amyloid deposits and promote fibrillization. The formation of cytotoxic prefibrillar oligomers is challenging to elucidate due to its rapidity and the heterogeneity of the species generated, and is even more complex with agents such as HS.
Observations: We applied a new strategy using a stopped-flow device coupled to optical absorption to monitor rapid aggregation kinetics. Using the model protein human muscle acylphosphatase (mAcP) we acquired multiple time courses of HS-induced aggregation as a function of a number of parameters, such as mAcP or HS concentration as well as amino acid substitutions, in a remarkably reproducible manner. (i) Monitoring aggregation kinetics of wt mAcP at varying concentrations of either mAcP or HS, aggregation occurred independent of mAcP but linearly dependent on HS concentration. wt aggregation was also assessed in the presence of a fluorescent derivative of heparin by fluorimetry. Here, heparin fluorescence was quenched in the presence of aggregating mAcP. Finally, aggregates that formed in the presence of HS were detected by AFM using an HS specific antibody. The antibody detected free HS only, not when in complex with aggregating mAcP. (ii) Creating a battery of 24 mAcP mutants that varied in the distribution and density of positive charges, we assessed rapid aggregation kinetics of these variants. HS-induced aggregation was found to be significantly decelerated for 5 of the variants tested.
Conclusions: Establishing a novel strategy, we show that (i) HS provides the nuclei of aggregation and is incorporated into oligomers after it pre-adsorbs mAcP molecules and (ii) identify 5 residues located in the flexible regions of mAcP that are the key players in HS-induced aggregation.

B 128 Palmitoylation within the RING finger motif of the endoplasmic reticulum-localized gp78 E3 ubiquitin ligase

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Background: Palmitoylation is a dynamic post-translational modification that is important in receptor stability and intracellular trafficking, and commonly occurs when a lipid palmitate binds to one or more cysteine residues. Gp78, an E3 ubiquitin ligase in the endoplasmic reticulum-associated degradation (ERAD) pathway, contains 10 cysteines in the N-terminal transmembrane domains and 6 cysteines in the C-terminal RING finger motif responsible for its ubiquitin ligase activity.
Observations: Using two methods, [3H]-palmitate metabolic labeling and the Biotin-BMCC assay, we found that immunoprecipitated full-length Flag-gp78 is palmitoylated in COS7 cells. To identify the palmitoylation site, we used site-directed mutagenesis to sequentially substitute all cysteines to alanines. The Biotin-BMCC assay showed that Flag-gp78 undergoes sulfhydryl cysteine palmitoylation (S-palmitoylation) on multiple cysteines within the RING finger motif. Next, we screened 19 palmitoyltransferases (PATs), enzymes that add protein palmitate, and identified an ER-localized PAT, DHHC6, that both increases gp78 palmitoylation and enhances its peripheral ER distribution. Reduced gp78 palmitoylation due to mutations of RING finger cysteines or by using the general inhibitor 2-bromopalmitate, restricts gp78 to the central ER labeled by GFP-Sec61. Preliminary data suggest that palmitoylation of the RING finger motif may also target gp78 for proteasome degradation.
Conclusions: RING finger motif palmitoylation may be a novel post-translational modification that modulates gp78 expression and distribution, thus controlling substrate degradation in the ERAD pathway. Whether palmitoylation of E3 ubiquitin ligases is a general mechanism to control their activity remains unclear.

B 129 Expression and characterization of cytochrome c6 from Chlamydomonas reinhardtii using a designer gene

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Background: Cytochrome c6 is a lumenal redox carrier in oxygenic photosynthesis. The cytochrome is a class I cytochrome with histidine and methionine serving as axial ligands. Its midpoint potential ranges from +340mV to +390mV and is found to be pH dependent in all species examined. We have constructed a synthetic gene, expressed, purified, and conducted an initial characterization for cytochrome c6 from Chlamydomonas reinhardtii.
Observations: The synthetic gene, constructed by the removal introns and the substitution to E. coli biased codons, was incorporated into a pUCF2 plasmid downstream of the lac operon and a pelA leader sequence. The protein is expressed by cotransformation in E. coli of the pUCF2 plasmid and the PEC86 plasmid, which contains genes for the covalent attachment of the heme to the protein. The spectral characteristics were determined by UV-Vis spectrophotometry and include a reduced alpha peak at 553nm, beta peak at 523nm, Soret band at 417nm, the oxidized peak at 423nm and one at 693nm indicative of the His-Met ligation of the heme. Mutants K29I and K57I were constructed using site-directed mutagenesis and show a shift in the alpha peak to 552nm. The midpoint potentials at pH 7 as determined by redox titrations are 365 ± 5mV for the wild type and +322 ± 5mV and +335 ± 5mV respectively for the K29I and K57I mutants. The redox potentials of the wild type and mutants do not show a dependence on pH. Differential scanning calorimetry experiments reveal the folding of the wild-type protein and mutants to be irreversible. The Tm for the wild type is 78°C and 70°C and 71°C for the K29I and K57I mutants respectively.
Conclusions: Cytochrome c6 from C. reinhardtii shows typical spectrum, but the midpoint potential is not pH dependent as far as pH 10 differing from any previously described c6. Also, the conserved K29 and K57 residues contribute significantly to the midpoint potential and stability of the protein.

B 130 The Investigation of a recombinant GalNAc binding protein from Bacillus thuringiensis as a tool for glycan analysis and detection

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Background: Pathogen-Host interactions have been known to involve many carbohydrate-protein interactions. Bacterial toxins may interact with host cell receptors or with a specific tissue due to lectin like properties. Carbohydrates are considered as one of the most important classes of biomarkers for disease states, protein functions, and developmental states. New methods for the analysis and detection of carbohydrates by lectin screening would have applications in the diagnosis of disease states.
Observations: It has been observed that the Cry toxins from Bacillus thuringiensis have carbohydrate binding abilities. In particular GalNAc has been shown to inhibit the toxicity of Cry1Ac on insect BBMV's. The lectin like properties of this protein can be exploited as these toxins have evolved to specifically target a host cell or tissue. Therefore Cry1Ac could potentially be used for the analysis and detection of GalNAc, especially in disease states, where aberrant glycosylation can be observed. A truncated Cry1Ac was cloned, expressed and purified on immobilised metal affinity chromatography. The purified recombinant Cry1Ac binding specificities were analysed by Enzyme Linked Lectin Assay (ELLA). The ELLA showed very strong binding of recombinant Cry1Ac to GalNAc in comparison with commercial plant lectins. Further ELLA's are being carried out to examine the specificity and stability of Cry1Ac. The recombinant Cry1Ac could be immobilised onto novel platforms for analysis and detection of GalNAc. Therefore future work will include investigating the applications of Cry1Ac for the differentiation of glycan variants in glycoprotein production and the study of disease states.
Conclusions: It has been shown that a truncated form of Cry1Ac has a strong specificity for GalNAc. This would prove useful in

the analysis and detection of many disease states where aberrant O-glycosylation is observed.

B 131 Performance of Peptide array versus Protein array

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Background: When using only a few microlitres of patient serum, protein microarray serves as an efficient means for serum auto-antibody -based detection. On the other hand, peptide microarrays have proven to be a very useful means to study protein-protein interaction. This property can be exploited in the field of cancer diagnostics. However, the performance of peptide microarrays in comparison to protein microarrays is something which needs to be examined.
Observations: A panel of 642 clone-proteins were used to generate protein microarrays. A set of 1150 antigenic peptides were identified upon in silico analysis of 642 proteins. Using SPOT technology these peptides were synthesized and peptide microarrays were generated. For both protein and peptide microarray generation ARChip Epoxy surface was used.The peptide microarrays along with the protein microarrays will be processed with breast cancer serum samples (benign n = 16 and malignant n = 16) and health control serum samples (n = 16). The reactivity of the protein will be compared to the reactivity of the peptides with regards to the serum sample.
Conclusions: This experiment will give us an insight on the role of peptide microarray has to play in the field of cancer diagnosis. We will identify a panel of clone-proteins and peptides which would differentiate not only tumour class from non-tumour class but also within tumour class (benign and malignant).

B 132 Structure-based drug design of selective 5'-nucleotidases inhibitors

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Background: The 5'(3')-deoxyribonucleotidases, catalyze the dephosphorylation of nucleoside monophosphates. Substrate cycles between nucleotidases and kinases may affect the therapeutic action of nucleoside analogs used as anticancer and antiviral agents. Such compounds require the nucleoside kinases activity for phosphorylation to their active forms. Results of clinical and in vitro studies propose that an increase in nucleotidase activity can interfere with nucleoside analogue activation.
Observations: The main goal of this project is the search for potent and selective inhibitors of mammalian 5'-nucleotidases based on nucleoside phosphonic acids and their derivatives and comparison of sensitivity of 5'-nucleotidases isolated from various sources toward individual inhibitors. This is done using specific activity assay based on separation of dUMP and dU on reverse phase column. Kinetic parameters are than calculated using non-linear regression. Two strategies for finding potential inhibitors are used. First, a random series of nucleoside phosphonic acids derivatives are tested. Second, testing of rationally designed compounds based on a published structure of known inhibitor-enzyme complex. Best inhibitors are used for co-crystallization and crystal soaking experiments.
Conclusions: We have prepared 2 types of human 5'-nucleotidase: cytosolic and mitochondrial by recombinant expression in E. coli. Using structure-based design we have already found 100x better inhibitor of mitochondrial nucleotidase and some other promising compounds.

B 133 New strategies for directed evolution of fluorescent proteins in yeast

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Background: Fluorescent Proteins (FPs) are widely used in modern cell biology as in vivo highlighters of specific molecules and processes. Advanced light microscopy methods and single molecule detection techniques require bright, photostable and fast-folding FPs.
Observations: However, current methods for directed evolution of FPs use E.coli as a host selecting for a prokaryotic folding environment. Furthermore, single cell scoring using fluorescence activation cell sorting (FACS) is unreliable due to cell-to-cell variability. Moreover, in vitro DNA shuffling protocols and the subsequent library construction are tedious and time-consuming processes. Here we describe a new strategy that involves 'in vivo DNA shuffling' for efficient directed evolution of FPs in the eukaryotic environment using the budding yeast S. cerevisiae as host. The strategy is based on in vitro random mutagenesis followed by recombination of mutations, expression of the FPs in a eukaryotic folding environment – yeast – and ratiometric fluorescence activated cell-sorting (FACS) methods for selection of optimized FPs. We applied this strategy to two RFPs – mKeima and mCherry – to produce brighter and faster maturing variants. The evolved variants of both FPs exhibited increased molecular brightness, as demonstrated with endogenous tagging of various yeast proteins.
Conclusions: Our results establish a new strategy for directed evolution and demonstrate its application by evolving brighter variants of mKeima and mCherry.

B 134 Thermal Stabilization of Baeyer-Villiger Monooxygenases via a Structure Guided Consensus Approach

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Background: Baeyer-Villiger monooxygenases (BVMOs) were recognized as highly versatile biocatalysts for oxygenation of ketones to esters or lactones. A prominent transformation of such enzymes is the stereoselective oxidation of cyclic and/or aliphatic ketones to chiral lactones/esters which are interesting building blocks for the synthesis of bioactive- and natural

compounds. However due to number of reasons,mainly stability, large-scale application was not enforced on a satisfactory level so far.

Observations: Cyclohexanone monooxygenase (CHMO) originating from Acinetobacter NCIB 9871 was chosen for this study. The reason behind being its ability to act on wide range of substrates and its low stability. Mutations to stabilize helical structure motifs in particular were predicted by sequence comparison with the structure of phenylacetone monooxygenase (PAMO) from Thermobifida which was recently established by X-ray diffraction. Certain combinatorial and rational protein design approaches were applied for the development of several generations of mutants. Parallel screening methods both on whole-cells as well as crude cell extracts were utilized to assess biocatalytic performance. Herein we report the development of CHMO mutants with increased thermal and kinetic stability without compromising the substrate specificity and selectivity. Shelf life of mutant enzymes at 30°C can be improved from 80 hours (WT) to 722 hours (mutant). An increase of more than 1400% in the yield during the life time (TTN) of enzyme, over the wild type is achieved.

Conclusions: In summary, the generality of the consensus based approach for the improvement of thermal stability of biocatalysts could be successfully applied to a flavin monooxygenase for the first time.

B 135 O-linked GlcNAcylation regulates limited proteolysis

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Background: Defects in cell cycle regulation are major determinants in cancer development. The Host Cell Factor-1 (HCF-1) plays critical roles in regulating gene expression, and is involved in promoting mitosis. Following synthesis, HCF-1 protein undergoes a proteolytic cleavage that is important for cell cycle progression. The signaling enzyme O-linked-N-acetylglucosaminyl transferase (OGT) was shown to interact with and glycosylate HCF-1. However, the role of HCF-1 O-GlcNAcylation remained enigmatic.

Observations: We wanted to investigate the functional significance and mechanism of HCF-1 O-GlcNAcylation. We found that a large proportion of OGT is stably associated with HCF-1. We further demonstrated that HCF-1 is essential for maintaining proper OGT stability in the nucleus. Moreover, O-GlcNAcylation is, in turn, required for proteolytic maturation of HCF-1. Although, we showed that OGT interacts with and glycosylates the basic domain in HCF-1. Neither the interaction nor the O-GlcNAcylation of this region are required for HCF-1 proteolysis. To provide further insight into the mechanism of HCF-1 O-GlcNAcylation, we found that OGT regulates HCF-1 cleavage via direct protein interaction with and O-GlcNAcylation of the HCF-1 central domain, termed the proteolytic processing domain. Furthermore we showed that OGT-mediated modulation of HCF-1 proteolysis impacts the expression of HCF-1 target genes. These data indicate that O-GlcNAcylation of HCF-1 is a signal for its proteolytic processing and revealed for the first time a novel crosstalk between O-GlcNAcylation and proteolytic cleavage.

Conclusions: Our results shed light on the HCF-1 proteolytic maturation pathway. Since HCF-1 is a major regulator of cell cycle, defects in this cellular pathway might cause chromosomal instability and promote cancer development.

B 136 TFEB Links Autophagy to Lysosomal Biogenesis

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Background: Autophagy is a cellular catabolic process that relies on the cooperation of autophagosomes and lysosomes. During starvation the cell expands both compartments to enhance degradation processes.

Observations: We found that starvation activates a transcriptional program that controls major steps of the autophagic pathway, including autophagosome formation, autophagosome-lysosome fusion and substrate degradation. The transcription factor EB (TFEB), a master gene for lysosomal biogenesis, coordinated this program by driving expression of autophagy and lysosomal genes. Nuclear localization and activity of TFEB were regulated by serine phosphorylation mediated by the Extracellular Regulated Kinase 2 (ERK2), whose activity was tuned by the levels of extracellular nutrients.

Conclusions: Thus, we identified a novel mechanism that regulates autophagy by controlling the biogenesis and partnership of two distinct cellular organelles.

B 137 A novel mannose-6-phosphate receptor homology (MRH) domain containing protein is involved in plant ER-associated degradation of proteins

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Background: To avoid aggregation and cell damage, unfolded and terminally misfolded proteins in the endoplasmic reticulum must be quickly degraded via the ER-associated degradation (ERAD) pathway, in which the substrate is ubiquitinated and degraded by the cytosolic proteasome. N-glycans and mannose trimming reactions play an important role in glycoprotein ERAD.

Observations: Although the ERAD pathway is known to operate in plant cells, there is very little knowledge about the glycan-dependent degradation process. A specific alpha,1-6-linked mannose residue present on N-glycans of misfolded glycoproteins was recently reported to act as a 'degradation signal' in yeast and mammalian cells. This glycan structure is recognized by lectins containing a mannose-6-phosphate receptor homology (MRH) domain. By searching the Arabidopsis thaliana genome we discovered a yet uncharacterized MRH-domain containing

protein (AtMRH1). AtMRH1 is ubiquitously expressed in different plant organs, and confocal laser scanning microscopy and Endo H sensitivity showed that it resides in the endoplasmic reticulum of plants. A putative null allele of AtMRH1 (mrh1) was able to prevent the degradation of the mutant brassinosteroid receptor BRI1-5, which is a well established plant ERAD substrate, and suppressed the severe growth defect of the bri1-5 mutant. Co-immunoprecipitation experiments indicate that AtMRH1 specifically interacts with the ERAD substrate BRI1-5, but not with the wild type BRI1 protein.

Conclusions: These results suggests an important role of AtMRH1 in the plant glycoprotein ERAD pathway. Additionally, knockout mutants showed increased salt sensitivity compared to wild type plants, indicating the involvement of AtMRH1 in ER stress response reactions.

B 138 Visfatin regulates endoplasmic reticulum stress through the activation of sirtuin-1 in HepG2 cells

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Background: Endoplasmic reticulum (ER) stress is the leading cause of insulin resistance in insulin-sensitive tissues such as liver. Visfatin (also called Nampt/PBEF) is an intracellular NAD-cycle enzyme that has been shown to improve insulin sensitivity. The aim of this study was to investigate a possible relationship between ER stress and the expression of visfatin in HepG2 human hepatocellular carcinoma cell line. The molecular mechanism of visfatin action was also studied.

Observations: Tunicamycin and palmitic acid were used to induce ER stress in HepG2 cells. Expression of ER stress markers GRP78 (HSPA5) and GADD153 (DDIT3) were assessed by real-time PCR. Expression of visfatin was evaluated by both real-time PCR and immunoblotting. Sirtuin-1 activity was measured by a fluorometric method. Both tunicamycin and palmitic acid increased the expessions of GRP78 and GADD153, indicating ER stress. Visfatin expression was significantly increased during ER stress induced by either tunicamycin or palmitic acid. Increasing intracellular visfatin by transfection showed a protective effect indicated by a decrease in the expression of ER stress markers. Both ER stress and visfatin transfection increased the activity of NAD-dependent enzyme sirtuin-1. The protective effect of visfatin against ER stress was abolished by silencing sirtuin-1 expression through RNA interference.

Conclusions: Visfatin expression is increased in response to ER stress in liver cells and increased visfatin controls ER stress in return. Sirtuin-1 is involved in the molecular mechanism of the protective effect of visfatin. We suggest that PPARs might also be involved in the mechanism downstream sirtuin-1.

B 139 Ubiquitin: Not just a tag?

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Background: The ubiquitin-proteasome system is responsible for degradation of numerous proteins. Many proteins are targeted for destruction by modification of a ubiquitin chain which serves as a tag for their degradation. It is unclear whether ubiquitination can additionally affect degradation by altering the physical stability of the substrate. Here, we examine the effects of ubiquitination on substrates' thermal stability, and study the structural characteristics and evolution of ubiquitination sites.

Observations: We analyzed the structural characteristics of a large dataset of in vivo ubiquitinated substrates. We observed that the disordered regions that are required to support degradation are missing in ~25% of the substrates, and that many proteins are ubiquitinated on structured regions. Using molecular dynamics simulations we showed that ubiquitination may significantly alter the thermal stability of the modified substrates. Ubiquitination of the substrate Ubc7 at the residues that are modified in vivo prior to proteasomal degradation uniquely results in significant thermal destabilization and a local unwinding near the modification site. These effects are specific to these sites, while other lysine residues which are not used in vivo, display diverse behaviours upon ubiquitination. This indicates that ubiquitination possibly facilitates the unfolding process and improves substrate degradation efficiency. With respect to substrate p19, which is phosphorylated prior to its ubiquitination, our results support a synergetic effect of ubiquitination and phosphorylation on the degradation process via enhanced thermal destabilization.

Conclusions: We find that in addition to its signaling role, ubiquitination may change the thermal stability of modified proteins in a manner that could assist degradation. These effects are important in proteins that lack intrinsically disordered regions, and may affect the evolution of ubiquitination sites.

B 140 'Noodle'-like filaments of bovine insulin and beta2 microglobulin were non-cytotoxic due to abbreviated cell binding

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Background: Amyloid fibrils are associated with more than 20 diseases including Alzheimer's disease and type II diabetes. Recent studies have shown that a number of non-disease-associated proteins can also form toxic amyloid-like fibers under appropriate conditions in vitro. However, the origin and underlying mechanism of amyloid toxicity are still elusive.

Observations: Previously, we have shown that in the presence of a reducing agent, Tris (2-carboxyethyl) phosphine, bovine insulin forms flexible 'noodle'-like filamentous amyloid, whose toxicity was remarkably lower than that of the intact insulin 'needle'-like fibrillar amyloid (Zako et al. Biophys J. (2009)). In this study, we report our finding that cytotoxicity of beta2-microglobulin (beta2m) filaments was also very low against different cell lines, which supports the notion that filamentous amyloids are generally non-toxic. More importantly, cell binding of these fibrillar and filamentous amyloids from insulin and beta2m was examined in an aim to understand the difference in cytotoxicity. For this purpose, a luminescent conjugated polythiophene (LCP) was used to stain both filamentous and fibrillar amyloids since

insulin filaments could not be efficiently stained by classical amyloid probes such as Thioflavin T and anti-insulin antibody. We have demonstrated for the first time that cell binding of both filamentous amyloids from insulin and beta2m was less efficient than that of fibrillar amyloids, clearly suggesting that amyloid toxicity could be correlated with prolonged binding of amyloids to cells.
Conclusions: We demonstrated that both two known filamentous amyloids of insulin and beta2m are non-toxic to various cells, supporting the notion that filamentous amyloids are generally non-toxic. More importantly, the toxicity of amyloids might be correlated with their sustained cellular binding properties.

B 141 SwissNS: A database of non-natural sidechains to computationally screen new ligands targeting protein-protein interactions

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Background: Protein-protein interactions encode the wiring diagram of cellular signaling pathways and deregulations of these interactions underlie a variety of diseases, such as cancer. Recently, protein-protein interactions have witnessed a renewed interest as targets for the development of novel therapeutics. Towards this goal, peptido-mimetics provides one of the most promising approaches to design efficient inhibitors.
Observations: Here, we develop a structural and molecular mechanics database of hundreds of commercially available non-natural amino-acid sidechains to probe in silico their insertion into naturally occurring peptides or proteins. For any solved 3D structure, all desired mutants can be readily generated. The results can be first used for visual inspection of the structural environment of the new sidechains. For these new residues, we further provide all topologies and parameters for standard molecular mechanics software (CHARMM and Gromacs) to perform more detailed and quantitative analyses, such as molecular dynamics and free energy simulations. Our results on non-natural mutants of a BCL9-derived peptide targeting beta-catenin show very good correlation between predicted and experimental binding free-energies, illustrating the relevance of the method.
Conclusions: Our database provides access to a large amount of new sidechains that can be tested in silico before selecting the most promising candidates for synthesis. This enables the design of peptide inhibitors taking full advantage of the information contained in naturally occurring protein interactions.

B 142 Structural analysis of alpha-macroglobulin from Escherichia coli in native and methylamine reacted states

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Background: Alpha-macroglobulins (AMGs) are large, ubiquitous protease inhibitors present in all metazoans, and recently identified in bacterial genomes. Upon protease recognition, almost all AMGs suffer conformational change due to recognition of a bait region and cleavage of a thioesther bond, leading to physical entrapment of the target protease. Here we present SAXS and Electron microscopy data that reveal that AMG from E. coli (EcAMG) displays a comparable conformational change upon activation.
Observations: We have over-expressed and purified EcAMG, which elutes as a monomer of 180 kDa in gel filtration. In order to induce the conformational change, the thioesther bond was cleaved by incubating an aliquot of EcAMG with methylamine, generating the activated form. Activated EcAMG displayed increased mobility in a native PAGE gel as compared to the native form. To better characterize this modification, we analyzed both EcAMG forms by electron microscopy, and digitized images were subjected to 3D reconstructions. This resulted in final models of resolutions of 17 and 22A, respectively. In addition, we also performed SAXS experiments with both EcAMG forms and generated models of these experiments performed in solution. Both techniques produced similar results, in that the fold of EcAMG resembles that of complement factor C3, a monomeric molecule which belongs to the AMG family. Native EcAMG displays an empty central cavity, which becomes shallower after activation, in addition to enlargement of the base of the structure. It is possible that these modifications are required to entrap the protease blocking it from reaching its target substrate.
Conclusions: AMG-encoding genes in bacteria imply the presence of an ancient form of immune system. Our work suggests that EcAMG displays similar activation and structural characteristics to eukaryotic proteins of the AMG/complement family, suggesting a functional mechanism that emulate those found in metazoans.

B 143 Negatively charged membranes induce lysozyme aggregation under physiological conditions: a structural and photophysical study

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Background: Hen egg-white lysozyme (HEWL) is used as a model to investigate protein folding and adsorption at interfaces and membrane fusion. HEWL aggregates into amyloid-like prefibrillae and fibrillae when incubated at pH 2 and 60 °C for some days. This transition from native to amyloid-like structure is also triggered by lipid membranes under physiological conditions. The current study aims at characterizing the aggregation process of HEWL in presence of vesicles with different surface charge density.

Observations: We used liposomes with different surface charge density resulting from various molar ratios of zwitterionic POPC and negatively charged POPS and POPG. The fusion of negatively charged liposomes by interaction with the protein was observed trought Dynamic Light Scattering and as the negative surface charge of liposomes increased, an alteration of native HEWL secondary structure was detected by Circular Dichroism analysis. Besides, an accelerated aggregation kinetics was observed by ThT binding experiments. The effect of HEWL on liposomal membrane fluidity and on vesicle fusion was investigated by fluorescence polarization of 1,6-diphenyl-1,3,5-exatriene, a decrease of liposome membrane fluidity occourred upon binding to HEWL. We also observed a reduced HEWL enzymatic activity in presence of liposomes. Electron Microscopy revealed amyloid-like fibrils structures that showed to be cytotoxic as revealed by MTT assays. Eventually, Confocal Microscopy experiments performed on Giant Unilamellar Vesicles incubated with HEWL further confirmed protein/ liposome interaction and induced vesicle fusion.

Conclusions: POPG or POPS membrane models induce the formation of amyloid-like structures of HEWL. Elucidating the role of cell membranes in the amyloid fibrillogenesis should be taken into account for the development of aggregation inhibitors as potential drugs to cure amyloid-related diseases.

B 144 Surprising substrate protonation states in Aspergillus flavus urate oxidase – combining information from X-ray and neutron crystallography with computational chemistry

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Background: Urate oxidase is an enzyme that oxidises uric acid to 5-hydroxyisourate without any cofactors. It is used as a protein drug to treat tumour lysis syndrome, but the catalytic mechanism has remained enigmatic, as the protonation state of the substrate could not be reliably deduced. We have therefore determined the neutron structure of A. flavus urate oxidase in the presence of the substrate and the inhibitor chloride, as well as a neutron structure with the inhibitor 8-azaxanthine.

Observations: In addition to the neutron data we collected X-ray data at room temperature for the purpose of joint X-ray/neutron refinement and atomic resolution X-ray data at a low temperature from both complexes. The neutron maps show that the substrate is not uric acid, but its enol tautomer 8-hydroxyxanthine. The proposed catalytic triad T57-K10-H256 shows an interesting pattern of alternate protonation states with H256 50% protonated on each nitrogen. This catalytic triad is connected to substrate deprotonation site N9 through a hydrogen bond network where the protons are partly disordered and that can only be observed at high resolution. The enzyme was postulated to form a triplet biradical intermediate even in the absence of molecular oxygen, but our quantum mechanics/molecular mechanics (QM/MM) calculations suggest that the structure is a singlet. Based on these calculations we also detected a catalytically important water molecule that is disordered in the X-ray and neutron structures.

Conclusions: The unexpected tautomeric state of the substrate changes the current ideas about the first step of catalysis – the deprotonation of the substrate. The disordered water molecule connects the substrate to the postulated general base.

B 145 Structural analysis of vesicular stomatitis virus glycoprotein: implications for membrane fusion study

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Background: Vesicular stomatitis virus (VSV) infection is mediated by virus spike glycoprotein (G), which induces membrane fusion at the acidic environment of the endosomal compartment. Crystal structure of G protein revealed the presence of four domains in which domain IV was claimed as a fusogenic domain by structure similarities with other viruses. In our previous work, we identified a specific sequence, comprising residues 145-164, part of domain IV, directly involved in membrane interaction and fusion.

Observations: In the present work we compared the fusogenic activity of peptide 145-164 and domain IV of VSV G protein by fluorescence methods. We also constructed a domain IV that lacks part of the peptide 145-164. Our results showed that domain IV is able to mediate fusion with the same efficiency as the virus, but not the truncated domain IV. SAXS data with IV domain in the presence of OG showed the native trimeric conformation of the protein.

Conclusions: Our results suggest that peptide 145-164 participates in the fusion process of VSV. More experiments exploring mutation on domain IV and others peptides need to be done to allow a better understanding of the fusion mechanism of this virus.

B 146 The 3D Architecture of Radial Spokes in Cilia and Flagella

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Background: Eukaryotic cilia and flagella, due to their motility and sensory functions, are crucial for human physiology and

development. Radial spokes (RS) are important components of flagella/cilia axonemes and are involved in cell motility. Assembly failures of RS give rise to flagella paralysis and several human diseases. Experimental evidence indicates that RS are mechanochemical transducers that transmit signals in the axoneme for local control of dynein-driven microtubule sliding.
Observations: We reconstructed the 3D structure of RS in situ and revealed details of their architecture in Chlamydomonas flagella and Tetrahymera cilia using Cryo-Electron Tomographic. By comparative analysis of Chlamydomonas RS pairs (RS1, RS2) and Tetrahymena RS triplets (RS1, RS2, RS3) in situ, we clarified similarities and differences between RS1, RS2 and RS3. We also identified new, additional components of the Chlamydomonas RS pair architecture, showing that part of the RS3 structure is conserved in Chlamydomonas flagella. RS are known to be composed of more than 23 different polypeptides, whose interactions and functions are not yet understood well. Analyzing Chlamydomonas RS-protein mutants we clarified the specific location of RS polypeptides and their subsets. Knowing how these proteins interact is crucial for understanding how RSs perform mechanochemical signal transduction to regulate dynein activity. Our 3d reconstructions also reveal a 2-fold symmetrical structure of RS, suggesting that the fully assembled axonemal RSs are produced by dimerization of big cytoplasmic RS precursors.
Conclusions: Based on our Cryo-ET data we propose models for RS assembly, interactions between the 23 RS proteins, and the interaction of RSs with other axonemal components. Our results are a fundamental step towards understanding the machinery responsible for cilia and flagella motility.

B 147 The EF loop in the LG3 domain of endorepellin is involved in antiangiogenic activity

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Background: The C-terminal region of perlecan, referred to as endorepellin, inhibits angiogenesis by disrupting actin cytoskeleton and focal adhesions. Among the three laminin-like globular (LG1-LG3) domains of endorepellin, the LG3 domain possesses most of this antiangiogenic activity, and interacts with integrin alpha2beta1 receptor. We intended to determine the crystal structures of LG3 domain and identify important functional regions.
Observations: Crystal structures of the LG3 domain of endorepellin were determined in apo and Ca2+-bound forms, and revealed that Ca2+-induced structural changes were very restricted. But, the substitution of the conserved Ca2+ binding residue showed that the coordination of Ca2+ was essential for the antiangiogenic function. Separately, the structural alignment with LG4 of laminin alpha1 implied that the EF loop may be also involved in the function of LG3. From the actin disassembly assay and surface plasmon resonance (SPR) analysis, the H4268 located on the EF loop appeared to be another important element for integrin binding and antiangiogenesis.
Conclusions: It was proposed that the antiangiogenic function of LG3 depends on the bound Ca2+ rather than the Ca2+-induced structural rearrangements, and the EF loop which was newly identified as an distinctive functional element in this study.

B 148 Dual Chaperone Role of the C-Terminal Propeptide in Folding and Oligomerization of the Pore-Forming Toxin Aerolysin

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Background: The bistable nature of PFTs raises two interesting questions. The first is: since PFTs can adopt two quite different conformations, how is the folding reaction during biogenesis directed towards obtaining the soluble fold? The second question is: what mechanisms prevent pore-formation from occurring in the producing cell? To address these related questions, we have chosen the PFT aerolysin, which is produced by the human pathogen Aeromonas hydrophila as an inactive precursor called proaerolysin.
Observations: Conversion of proaerolysin to aerolysin involves proteolytic cleavage of a flexible 43-residue loop near the C-terminus. To characterize the molecular interactions between the CTP and Domain 4, we performed classical molecular dynamics (MD) simulations. The MD suggested that the CTP remains bound to aerolysin upon proteolytic activation of the protoxin. In order to identify key residues responsible for binding of the CTP to Domain 4, we performed in silico alanine scanning on most of the CTP and generated constructs to express the mutants in the E. coli periplasm. We identified several mutantions in the CTP which induced aggregation of proareolysin in the bacterial periplasm, either due to the exposure of a hydrophobic patch or improper folding of part of the protein. In vitro refolding experiments showed that the folding was impaired for several aerolysin mutants and the extent correlated with the ability of the mutants to fold into a soluble state in vivo.
Conclusions: We here show that the CTP is essential for the folding of aerolysin into a soluble toxin. Due to the fact that it promotes folding but is not part of the final active conformation of the protein, i.e. the transmembrane heptameric pore, the CTP qualifies as a chain-linked molecular chaperone.

B 149 Quantitative analysis of HIV-1 Gag clusters and the impact of fluorescently labelled Gag using super-resolution imaging

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Background: A successful viral infection is ultimately marked by the assembly and release of progeny viral particles. In the case of HIV-1 this process is driven by the Gag polyprotein. Gag assembles at the plasma membrane into virus-like particles (VLPs) when expressed in mammalian cells. VLP assembly has been studied using fluorescently labelled Gag (GagFP), but expressing GagFP alone leads to aberrant VLP morphology, which

can be rescued by additional expression of unlabelled Gag (Gagwt).

Observations: However, the impact of unlabelled to labelled Gag stoichiometry on the morphology of VLPs has not been quantified. This is due to two main limitations: the size of VLPs, which is too small to resolve using conventional fluorescence imaging and the lack in quantification of the Gag protein stoichiometry in single cells. To address these limitations we perform photoactivated localization microscopy (PALM) of mammalian cells expressing Gag labelled with mEos2 or the larger tandem dimeric tdEos to parametrize Gag cluster shapes at the plasma membrane. Gag protein stoichiometry in single cells is quantified using fluorescence correlation spectroscopy and a fluorescent reporter protein for Gagwt. We show that for mEos2 the label has no impact on VLP morphology or size, whereas the bigger label tdEos leads to an increase in VLP size, which can be rescued by additional expression of Gagwt. This increase in size is due either to an increase in Gag to Gag lattice distance in forming VLPs, to a decrease in relative Gag to Gag angle or a combination of both. These changes in Gag lattice lead to the decrease in local membrane curvature and thus an increase in measured VLP radius.

Conclusions: The use of biological model systems behaving in a native fashion is crucial to gain meaningful insights into biological processes. We show that the assembly of VLPs can be captured using Gag labelled with monomeric fluorescent proteins as demonstrated by super-resolution imaging.

B 150 Crystal structure of the autochaperone region of the Shigella flexneri autotransporter IcsA

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Background: The IcsA autotransporter is a key virulence factor from Shigella flexneri that is essential for the actin based motility of the pathogen inside infected cells. IcsA also plays a role in the escape of the bacterium from destruction by autophagy. Autotransporters consist of a N-terminal signal sequence, a passenger domain and a C-terminal transmembrane domain.

Observations: We identified a stable IcsA fragment corresponding to the autochaperone region of the passenger domain. The crystal structure of the autochaperone region (IcsA-AC) comprising residues 591-758 was determined at 2.0 Å resolution. Using far-UV CD measurements we could show that thermal unfolding of the autochaperone region is reversible, demonstrating that IcsA-AC is self-sufficient in adopting its native conformation.

Conclusions: IcsA-AC adopts a beta-helical structure. Self-contained folding of IcsA-AC is a prerequisite for its suggested function as an autochaperone promoting the folding of the passenger domain when it reaches the surface of the bacterium.

B 151 GTPgammaS microtubules mimic the growing microtubule end structure recognized by end-binding proteins (EBs)

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Background: Plus end tracking proteins (+TIPs) are essential for proper cytoskeleton function and most +TIPs are recruited to microtubule ends by proteins of the end binding protein (EB) family. EBs have the unique property of autonomously recognizing an extended region at the growing ends of microtubules. It was, however, unknown which feature of tubulin is recognized by EBs in the microtubule end region. This study aimed to reveal the feature recognized by EBs at the microtubule end region.

Observations: Using a combination of in vitro reconstitution, quantitative time-lapse Total Internal Reflection Microscopy (TIRF-M) and Cryo-Electron Microscopy (Cryo-EM), we find here that microtubules grown in vitro in the presence of the slowly hydrolysable GTP analogue GTPgammaS mimic the dynamic end structure of growing microtubules. By TIRF-M, we comparatively quantified EB binding to the microtubule end region and GTPgammaS microtubules by measuring the stoichiometry of binding sites, the affinity of EBs and single molecule binding/unbinding turnover. We then employed cryo-EM to show that GTPgammaS microtubules are closed B-lattice microtubules. We furthermore found that the addition of the inorganic phosphate analogue beryllium fluoride to microtubules grown in GTP also increases the affinity of EBs to the microtubule lattice.

Conclusions: Our findings indicate that the guanine nucleotide gamma–phosphate binding site is crucial for determining the affinity of EBs for lattice-incorporated tubulin. This finding is signficant as it defines the molecular mechanism by which EBs recognize growing microtubule ends.

B 152 One beta Hairpin after the Other: Folding Mechanism of a Transmembrane beta-Barrel Protein

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Background: Despite their importance, the folding mechanisms of only a few transmembrane proteins have been studied. Current models describe that transmembrane beta barrels fold into the lipid membrane in two major steps. Firstly, the unfolded polypeptide interacts with the lipid surface where it folds, tilts, and then inserts into the membrane. Consequently, it is thought that beta-barrel proteins (pre-)fold prior to their insertion into the cellular membrane.

Observations: To date, the refolding of beta-barrel membrane proteins into a lipid membrane has never been addressed by SMFS. Herein we report the application of SMFS to unfold and refold Outer Membrane Protein G (OmpG) from E coli. Previously, we found that the beta barrel of OmpG unfolds in single

beta hairpins. In our refolding experiments, OmpG that had been reconstituted in native E. coli lipid membranes was first imaged by AFM. Then, the AFM tip was pushed onto the OmpG surface to facilitate the nonspecific attachment of the N terminus. Withdrawal of the AFM tip stretched the terminus and induced the unfolding of OmpG. Force–distance (F–D) curves recorded the force peaks that reflect the unfolding steps of a single OmpG. Each unfolding step represents that of a beta hairpin of the transmembrane beta barrelTo refold the partially unfolded OmpG, we stopped withdrawal before unfolding the last beta hairpin VII. Then, we relaxed the unfolded polypeptide by approaching the AFM tip close to the membrane (ca. 5 nm). After a given time to allow the polypeptide to refold, the protein was unfolded again to probe which structural regions refolded into the lipid membrane.
Conclusions: Our results suggest a fundamentally different folding mechanism for transmembrane beta-barrel proteins. In contrast to the two-stage folding and insertion model of a transmembrane beta barrel, we observe distinct folding steps for beta hairpins.

B 153 Thermodynamic instability of collagen fibril formed without telopeptide domains

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Background: The triple-helical structure of type I collagen forms by hydrogen bonding among alpha chains. The N- and C-terminal telopeptide domains were not thought to contribute to the stability of the triple helix. Therefore, no work has focused on the importance of amino acid sequences in telopeptide domains. In this study, we report the thermodynamic characterization of the collagen self-assembly lacking telopeptide domains by differential scanning calorimetry.
Observations: Acid soluble type I collagen was prepared from chicken skin, and was treated by pepsin or actinidain proteinase, namely PHCol and AHCol. The thermal denaturation curve was measured from 20°C to 60°C by using a differential scanning calorimeter, Nano-DSC (Calorimetry Sciences Corp.), with a 1.0 mL gold capillary cell. Measurements were carried out using of 0.20 mg/mL collagen preparations in 20 mM acetate buffer (pH 4.0-5.5) or 20 mM phosphate buffer (pH 6.0-7.5) at a heating rate of 1.0°C/min. An apparent transition temperature (Tm), a halfwidth, and enthalpy were determined from the maximum of the melting peak. The Tm of PHCol and AHCol at pH 4.0-5.0 determined by DSC showed 46.2°C and 46.0°C, respectively. It was almost the same thermogram between the PHCol and the AHCol. In contrast, under neutral pH conditions, AHCol self-assembly was more instable than PHCol. Although the thermograms of these collagen self-assemblies showed different profiles, enthalpy determined by analyzing thermograph was similar values.
Conclusions: Thermodynamic analysis of AHCol revealed that telopeptide domains have a great influence on the thermal stability of collagen self-assembly.

B 154 Structural studies on replication initiation factors involved in plasmid maintenance and propagation

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Background: DNA replication is an absolute requirement for the preservation of genetic information and propagation of the life form it encodes. The trigger for DNA replication initiation is usually a macromolecular switch. In plasmids, this switch often is a single protein that requires the presence of a multicomponent system called the relaxosome. Structural insights of initiator proteins will help block proliferation of plasmids that produce life-threatening virulence and/or antibiotic resistance factors.
Observations: We have solved the structures of three different initiator proteins from plasmids that have been found in Gram-negative and –positive plasmids: two proteins of pMV158 and the protein TrwC of plasmid R388. These proteins are either involved in copy number control or in bacterial conjugation, i.e. the horizontal transfer of genetic material through direct cell-to-cell contact. The multidomain sequences of these three proteins have in common an endonuclease domain that specifically nicks a DNA sequence of the plasmid that encodes each respective protein. However, the surrounding domains have different origins and are unrelated both in sequence and function among the three initiators. The structures of the nuclease domains, either with or without some of the other domains, will be presented, which show that the core of the nuclease domain is conserved in all proteins. However, the periphery clearly differs, consistent with the diversification of the surrounding domains and the interacting partners of the respective relaxosomes. In addition, the structures provide insight into the diversification of the oligomerization behaviour of these proteins, and how this affects the mechanisms.
Conclusions: The structures of three diversified DNA replication initiators are presented, showing the structural conservation among their nuclease domains. Interestingly, they also provide information on where diversification has occurred and how this diversification affects mechanism and function.

B 155 New frontiers in host-pathogen interactions: The viral decoy receptor BARF1 sequesters and inactivates the human cytokine CSF-1 via an unprecedented mechanism

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Background: Epstein-Barr Virus (EBV) is a major human pathogen with a broad disease profile including infectious mononucleosis, lymphomas, and malignant epithelial carcinomas. A pronounced phenomenon associated with EBV pathology concerns the massive secretion of viral BARF1, which in recent years was shown to associate with human Colony-Stimulating Factor 1 (CSF-1), a key cytokine for the development of the immune system. However, the molecular and structural basis of this interaction has remained elusive.
Observations: We employed an interdisciplinary approach from of the structural biology toolbox in combination with a diversity of interaction studies in vitro and in a cellular context, to establish the structural and mechanistic basis of the BARF1-CSF1 interaction. Crystal structures of the BARF1-CSF1 complex complemented by negative-stain EM studies, reveal that BARF1 sequesters three copies of hCSF-1 at the periphery of its toroidal structure. Remarkably, BARF1 binds to a compact epitope at the hCSF-1 dimer interface far from the canonical receptor binding epitope on CSF-1. SAXS studies of the complex and individual protein components in solution show that BARF1 undergoes a concerted domain rearrangement upon binding CSF-1, that locks the complex into a rigid assembly. Mechanistic interaction studies uncovered that BARF1 captures hCSF-1 with ultra-high affinity characterized by very slow dissociation kinetics, in what appears to be one of the tightest protein-protein interactions ever measured. In its BARF1-bound form, CSF-1 does not bind to and is unable to signal through its cognate receptor on human macrophage precursors, highlighting the immunomodulatory aspects of BARF1 activity.
Conclusions: We have established a new paradigm for host-pathogen interactions, whereby a massively secreted viral protein scavenges and inactivates the target human protein with exquisite efficiency and specificity without directly competing with the canonical human receptor for the same binding epitope.

B 156 Crystal structure of Cyt1Aa from Bacillus thuringiensis subsp. israelensis and implications for its membrane-perforating function

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Background: Bacillus thuringiensis subsp. israelensis produces, during sporulation, a mosquito larvicidal protein complex containing several Crystalline (Cry) and Cytolytic (Cyt) subunits. Specific proteolysis of Cyt1Aa, the most toxic Cyt family member, activates it against various cell types including mammalian cells. Although cyt1Aa was the first of the cyt family to be isolated and studied, the attempts to obtain Cyt1Aa diffractive in vitro crystal failed upon its tendency to aggregate and precipitate.
Observations: Here, the activated monomeric form of Cyt1Aa was isolated and crystallized, and its structure was determined, the first time for a Cyt1 family member, at a 2.2 Å resolution. Cyt1Aa adopts a typical cytolysin fold with a single domain of alpha/beta architecture, containing a beta-sheet surrounded by two alpha-helical layers, homologous to the corresponding regions of Cyt2Aa, Cyt2Ba and Volvatoxin-2. The deduced structure of Cyt1Aa and the location of its segments responsible for binding to Cry11Aa, support the previously suggested mechanism for association between the two subunits. Extensive sequence-based structural comparisons confirm a previous suggestion that the toxicity of Cyt proteins is inherent to their primary sequences, and provide possible explanation for the lack of toxicity of Cyt1Ca. The hemolytic activity pattern of Cyt1Aa, resembles that of the pore-forming agents, but differs from that imposed by the ionic and non-ionic detergents.
Conclusions: A comprehensive understanding of the machinery of pore-forming toxins, may help to design better membrane-active drugs. Our structural and experimental results support the mechanisms by which Cyt1Aa undergoes conformational changes prior to membrane insertion, oligomerization and perfotraion.

B 157 Structural characterization of an outer membrane-associated pilot protein from the type III secretion system (T3SS) of Pseudomonas aeruginosa

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Background: Pseudomonas aeruginosa is an opportunistic pathogen mostly involved in nosocomial infections causing severe diseases like septicemia and meningitis. Its main weapon is a Type III Secretion System that acts as a molecular syringe to inject toxins into the host cell causing damages ranging from membrane disruption to oncosis. We are interested in the biogenesis of the needle complex, and more precisely the structure and function of ExsB, a Pseudomonas T3SS pilotin.
Observations: ExsB is a 15 kDa lipoprotein predicted to play a key role in the localization and the biogenesis of the T3SS Outer Membrane Ring (the secretin). We solved the crystal structure of ExsB at 1.8A by using MAD methodologies on a selenomethionylated crystal. The structure revealed a unique fold for a T3SS pilotin which shares no similarity either with MxiM from Shigella flexneri or other secretin-stabilizing lipoproteins. Interestingly, the structure displays key features that could be essential for its activity: a highly basic patch that could potentially play a role in membrane association and a deep cradle that could accommodate an extended region from the secretin substrate.
Conclusions: Due to the conservation of ExsB heterologs in all Ysc-related T3SS (Y. pestis, A. salmonicida, P. luminescens...) our data expands the structural basis towards the understanding the mechanism of action of secretin-associated lipoproteins and by extension formation of the Outer Membrane Ring.

B 158 Structure and Function of the Farnesylated Peroxisomal Import Receptor Pex19p

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Background: Pex19p acts as chaperone and import receptor in the posttranslational transport of peroxisomal membrane proteins (PMPs). It is farnesylated at a C-terminal cysteine residue in the so-called CaaX box, which is conserved from yeast to humans. Recently, studies by Erdmann and co-workers showed that farnesylation-deficient S. cerevisiae cells have defects in peroxisomal protein import. Farnesylation has been found to increase the affinity for cargo proteins in vitro and in vivo.
Observations: We are studying the effects of farnesylation on the structure, molecular interactions and dynamics of the human Pex19p domain using NMR spectroscopy. 1H, 15N correlation NMR spectra indicate an unfolded N-terminal part and a structured C-terminus. Amide chemical shift perturbations and 15N relaxation data as well as solvent paramagnetic relaxation enhancements indicate that the C-terminal domain of Pex19 undergoes a partial conformational rearrangement upon farnesylation that involves the presumed PMP interacting region. We have solved the solution structure of the farnesylated Pex19p C-terminal domain, including numerous distance restraints involving the protein and the farnesyl group. Unexpectedly, we find that the farnesyl moiety is bound in a hydrophobic cavity inside the protein with additional contributions from residues in the PMP binding alpha-helix. Microscale thermophoresis assays confirm an enhanced binding of the farnesylated protein to PMP peptides. Flotation assays demonstrate that PMP binding targets the Pex19p-PMP complex to the membrane.
Conclusions: Our investigations have elucidated a surprising structural impact of Pex19p farnesylation which explains its importance in peroxisomal biogenesis. Furthermore, our biochemical and functional studies suggest a functional role for the farnesyl in PMP binding and membrane attachment.

B 159 The microbial lag phase is a rapidly evolvable trait with complex environmental and physiological determinants

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Background: The microbial lag phase is characterized by a period of slow to non-detectable growth triggered by environmental change. It was first described in detail by Monod in 1941, but still continues to resonate with current research problems today. In this study we examine how gene expression and transiently inherited cellular states (i.e. epigenetic 'memory' such as transcriptional memory of protein carryover) can influence the severity of the microbial lag phase.
Observations: Using shifts between preferred and non-preferred sugars, we directly observe individual cells in the lag phase using time-lapse microscopy. We find that the lag phase of an individual cell is indeed a period of non-detectable growth. Interestingly, the lag phase of individual (and genetically identical) cells within a population is highly variable. Moreover, we find that lag severity is dependent on many factors including the cells' prior growth conditions, strain background and nutrient sources of previous and present environments. In many conditions we find little evidence for a significant role to be played by epigenetic mechanisms such as 'transcriptional memory' or 'protein carryover' of previous environments, and rather find that lag severity in general is a function of how much an organism 'anticipates' a changing environment. Based on our observations, we find that empirically measured parameters of lag severity can be incorporated into a simple model of population growth. Then, informed by our model, we present a paradigm to quickly evolve cells to have beneficial mutations that yield strains with shorter lags. The mechanism of this evolution has yet to be determined.
Conclusions: A cell's lag phase a rapidly evolvable trait; a complex interaction between genes and environment, with large variation even between a population's genetically identical cells. Even proteins directly involved in new nutrient source assimilation are rarely the sole factors required to resume growth.

B 160 Time-resolved Phosphoproteome Analysis of HGF-stimulated DU-145 Cells

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Background: The human receptor tyrosine kinase Met coordinates growth and morphogenesis of different organs. Binding of its natural ligand, the hepatocyte growth factor / scatter factor (HGF/SF), induces signalling cascades that control cell motility and proliferation, but also contributes to invasion and metastases formation in cancer. Although the central Met pathway was studied in detail, our knowledge about nodes for signal integration and the maintaining of physiological signalling is still limited.
Observations: This study used state-of-the-art phosphoproteomics to refine our knowledge of the HGF/Met signalling network. To learn more about the dynamic phosphorylations downstream of the Met receptor, prostate cancer DU145 cells were stimulated with HGF for different time points and the induced phosphorylation patterns were studied in the first 20 minutes. Phosphopeptides were purified from total cell lysates, differentially labelled for time-resolved quantifications (iTRAQ) and analysed by mass spectrometry (Orbitrap Velos). In total, we were able to quantify about 8000 phosphorylated peptides derived from 2600 proteins. Functional clustering revealed 129 Ser/Thr- and 25 Tyr-protein kinases, and 10 Tyr-phosphatases. Additionally, 20 transcription factors, 31 cell junction-associated, 54 actin-binding, 31 ubiquitination and 33 endocytosis-associated phosphoproteins were characterised. Phosphorylations at known signal components downstream of Met, such as Erk1/2 and Akt, exhibited reason-

able regulation profiles serving as proof of concept. Besides known phosphosites numerous unknown phosphorylations were regulated indicating novel nodes and structures of the Met signal network.
Conclusions: Time-resolved phosphoproteomics has identified new phosphoproteins that are regulated in HGF/Met receptor-dependent signalling cascades. We are now focussing on the spatial and temporal properties of these components and define their role in physiological and patho-physiological Met-phenotypes.

B 161 Escherichia coli vs. Salmonella: FRET-based comparative physiology of chemotactic network dynamics

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Background: The pathway mediating chemotaxis in Escherichia coli is one of the best studied signaling networks in biology. All the molecular constituents are known and the signaling transfer functions have been recently characterized using theoretical modeling and in vivo fluorescence resonance energy transfer (FRET) experiments. Because these transfer functions determine bacterial chemotactic performance, it is of interest to ask what parameters are subject to tuning during evolution.
Observations: To get a glimpse of such selection at work, we examine the chemotaxis system of a closely related species, Salmonella typhimurium. We carry out in vivo FRET measurements, while stimulating the bacteria with time-varying temporal inputs. Measurements of the rapid receptor response using step stimuli show that signal amplification at the level of the receptor-kinase complex is much weaker in Salmonella than in E. coli. Sensitivity-tuning is observed in both species; however, receptor cooperativity in Salmonella is low and nearly constant, with a Hill coefficient (nH) of ~1, as compared to E. coli, which reaches nH ~4. Responses to exponential ramp stimuli reveal that the negative feedback near steady state is ~3-fold stronger than that in E. coli, implying faster kinetics of receptor methylation. The frequency response, measured using exponentiated sine waves, shows that the cutoff frequency for low-pass gradient-sensing behavior is higher in Salmonella than in E. coli. We further found that this characteristic frequency is invariant in Salmonella over a >500-fold range of ambient concentrations, implying a background-independent adaptation timescale.
Conclusions: FRET measurements using time-varying stimuli revealed quantitative differences in chemotactic signal processing between E. coli and Salmonella. The considerable differences in receptor cooperativity and methylation kinetics, might relate to the different environments that these two species inhabit.

B 162 neXtProt, a novel human protein knowledge platform

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Background: neXtProt (www.nextprot.org), the new human-centric protein knowledge platform, is developed jointly by the CALIPHO group at the SIB and by GeneBio, and aims to help researchers answer pertinent questions relevant to human proteins. neXtProt is built on a corpus of both curated knowledge – originating mainly from the UniProtKB/Swiss-Prot knowledgebase – and carefully selected and filtered high- throughput data pertinent to human proteins.
Observations: Compared to UniProtKB/Swiss-Prot, neXtProt integrates full exon mapping of isoforms and supplemental SNPs from ENSEMBL, expression data at protein level from the Human Protein Atlas, expression data at mRNA level from the BGee database, and additional proteomics data. Each information is annotated with high precision, using dedicated ontologies and controlled vocabularies, and is accompanied by manually assigned quality tags and references.
Conclusions: Such a data gathering and grading effort is complemented by the development of tools that will allow such data to be analyzed and interrogated at systems-level, in order to gradually be able to answer the challenges of modern research in human protein science.

B 163 Mapping of the human kinome interactome through a reproducible systematic approach

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Background: Kinases have a fundamental role in a great number of biological processes, still a complete picture of the molecular networks associated to this class of proteins is missing. Large scale proteomic approaches based on mass-spectrometry are a powerful tool but generally suffer from low data reproducibility and are too much dependent on the variability of the experimental and analytical strategies used, like purification methods, mass-spectrometer set-ups, and bioinformatic processing of data.
Observations: We decided to apply an unbiased systems level approach to obtain a view of the interplay among the human kinome within its cellular environment, and started by selecting those human protein kinases that show an abundant basal level of expression in the 293HEK cell line: few of the members in this core are well characterized and some are drug targets, while the majority of them are poorly annotated, and may represent new unexplored targets. Each kinase was fused to a Streptavidin-HA tag, a stable HEK-293 cell line was generated for its inducible expression, and a double step purification was performed to isolate its interactors. We used a mass-spectrometric/bioinformatic analytical strategy that allowed us at the same time to generate an exhaustive functional annotation map and to efficiently separate the set of highly reliable data from the most variable one. The proteins assembled around these kinases hint at their functional features: among these interactors, known binding partners were confirmed to be part of the core complexes, and many new ligands were identified.
Conclusions: The proteomic approach applied in this study provides an initial map of the protein complexes associated with

human kinases. The high reproducibility and quality of the data obtained makes us confident in proposing new standards for future characterizations of protein complexes.

B 164 Genome-wide quantification of mRNA and protein and integration with protein turnover in a bacterium

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Background: Biological function and cellular responses to environmental perturbations are regulated by a complex interplay of DNA, RNA, proteins and metabolites inside cells. To understand these central processes in living systems at the molecular level, we integrated experimentally determined organism-wide abundance data for mRNA, proteins, as well as individual protein half-lives from the genome-reduced bacterium Mycoplasma pneumoniae.
Observations: We provide a fine-grained, quantitative analysis of basic intra-cellular processes under various external conditions. Proteome composition changes in response to cellular perturbations reveal specific stress response strategies. Protein abundances are regulated in functional units, such as complexes or pathways, and reflect cellular lifestyles as well as mechanistic properties of molecular machines. The analysis of mRNA-protein correlation revealed that the regulation of gene expression is largely decoupled from protein abundance in M. pneumoniae. Integrating our quantitative data with protein half-life measurements showed that translation efficiency has a higher regulatory impact on protein abundance than protein turnover. Stochastic simulations using in vivo data showed how low translation efficiency and long protein half-lives effectively reduce biological noise in gene expression.
Conclusions: Our study provides a detailed integrative analysis of absolute cellular protein abundances and the dynamic interplay and regulation of mRNA and proteins, the central biomolecules of a cell.

B 165 HLX controls the expression of guidance cues and negatively regulates sprouting of endothelial cells

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Background: The HLX gene encoding a diverged homeobox transcription factor has been found to be upregulated by VEGF-A in endothelial cells. We have now investigated the gene repertoire induced by HLX and its potential biological function.
Observations: HLX strongly increased the transcripts for several repulsive cell guidance proteins including UNC5B, plexin A1 and semaphorin 3G. In addition, genes for transcriptional repressors such as HES1 were upregulated. In line with these findings, adenoviral overexpression of HLX inhibited endothelial cell migration, sprouting and vessel formation in vitro and in vivo, whereas proliferation was unaffected. This inhibition of sprouting was caused to a significant part by HLX-mediated upregulation of UNC5B as shown by shRNA-mediated downmodulation of the respective mRNA. VEGF-A stimulation of endothelial cells induced elevated levels of HLX over longer time periods resulting in especially high upregulation of UNC5B mRNA as well as an increase in cells displaying UNC5B at their surface. However, induction of HLX was strongly reduced and UNC5B upregulation completely abrogated when cells were exposed to hypoxic conditions.
Conclusions: These data suggest that HLX may function to balance attractive with repulsive vessel guidance by upregulating UNC5B and to downmodulate sprouting under normoxic conditions.

B 166 Cochlear Gap Junction plaque is disrupted by Connexin26 mutations, a new molecular pathology of deafness

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Background: Hereditary deafness affects about 1 in 2000 children and GJB2 gene mutation is most frequent cause for this disease in the world. GJB2 encodes connexin26 (Cx26), a channel component in cochlear gap junction. Gap junction in the cochlea provides an intercellular ion transport to maintain high level of the endocochlear potential essential for sensory hair cell excitation.
Observations: We have generated the phenotype of a mouse model carrying human Cx26 with R75W mutation (R75W Tg) and inner ear specific conditional Cx26 deficient mice (Cx26CKO) with partially mosaic deficiency in cochlear tissue. In this study, we analyzed the formation of gap junction in cochlear supporting cells of R75W Tg mice and Cx26CKO. Gap junction composed of Cx26 in wild type mice showed horizontal linear gap junction plaques (GJP) along the cell-cell junction site with the adjacent cells and these formed pentagonal or hexagonal outlines of normal inner sulcus cells and border cells. The GJP in R75W Tg mice did not show normal linear structure, although the round small spots were observed around the cell-cell junction site. Cx26CKO had same phenotype although some of the cells with Cx26 expression due to their mosaicism showed normal linear GJP with Connexin(Cx)30 only at the cell junction site between two Cx26 positive cells.
Conclusions: Our results demonstrated that Cx26 was essential for the formation of linear or planer GJP in cochlea which was not compensated by other cochlear Connexins such as Cx30. In this study, we showed a new molecular pathology of sensorineural deafness.

B 167 Conditional mutagenesis in the mouse intestine – a tool for studying the role of Hic1 (hypermethylated in cancer 1) tumor suppressor in the colorectal cancer

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Background: HIC1 tumor suppressor gene encodes an evolutionarily conserved transcriptional repressor and inhibitor of Wnt/beta-catenin signaling. The Wnt/beta-catenin signaling pathway regulates many aspects of embryonic development and tissue maintenance in the adulthood, while an aberrant activation of the pathway is a hallmark of gastrointestinal cancer. Hic1 is essential for mouse embryonic development and exerts an anti-tumor role in the adulthood.
Observations: Since Hic1-deficient mice die perinatally, we generated a conditional Hic1 null allele using Cre/loxP system. These Hic1 conditional (Hic1flox/flox) mice provide new insights into the Hic1 function in developing and mature tissues. To study the role of Hic1 in the intestine, we ablated Hic1 from the intestinal epithelium (using villin1-CreERT2 mice) and intestinal epithelial stem cells (Lgr5-CreERT2 mouse strain) of Hic1flox/flox mice. Intestines depleted of Hic1 showed altered representation of secretory cells. Increase in Paneth cell numbers occurred at the expense of enteroendocrine cells. Paneth cells are believed to constitute the stem-cell niche; therefore their elevated numbers eventually enhances the stem-cell-containing crypt compartments and could lead to hyperplastic features. Hyperplasia, and subsequent dysplasia, of intestinal crypts has been reported to underline processes leading to progressively growing neoplasias.
Conclusions: Our data gained from the conditional knock-out mice point to Hic1 role in maintenance of the intestinal homeostasis, a crucial phenomenon disrupted during tumorigenesis.

B 168 A Survey for Genes Affecting Synapsis in Budding Yeast

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Background: We have established a systematic genome-wide survey for recording the effect of non-essential genes on chromosome morphology in Saccharomyces cerevisiae. The 4800 non-essential mutants from the Euroscarf deletion collection were crossed to SK1 to generate diploid hybrid strains homozygous for the deletions. 3700 have been analyzed to date by chromosome spreading followed by immuno-staining of Zip1 and Rec8 6 hours after meiotic induction and categorized based on the extent of Zip1 axes.
Observations: Almost all genes known to be required for synapsis were re-identified when analyzed. In total we found no synapsis in 94/3700 (2.5%) of the mutants 20 of which were already known to be essential for synapsis. 19/94 are previously uncharacterized ORFs. 312/3700 (8.4%) of the mutants displayed incomplete synapsis, of these 30 are well known (including e.g. Msh4, Spo16, Spo22 and Ndj1) and 71 candidates of them are uncharacterized ORFs. For validation the candidates are being re-generated in SK1 background. 67 candidate genes have so far been knocked out in SK1 background to be analyzed by Cytology and Southern. 48 of these 67 primary candidates produce a phenotype in the secondary screen. However, in SK1 many candidates show a reduction and delay in pre-meiotic S-phase and synapsis, rather than complete absence of synapsis. 10(14%) are indeed unable to complete SC formation, whereas 56% exhibit significant delays or reductions. At least 50% of mutants producing a strong phenotype showed a prominent S-phase delay or arrest. Some of the very strong phenotypes were caused by mutants, which may alter expression patterns, as they are involved in chromatin remodeling or transcription.
Conclusions: All of the 11 candidates assessed, show at least a delay in crossover and noncrossover formation.(e.g. the irc25Δ displays a specific reduction of crossover (50% of the wild type level) whereas noncrossover is unaffected. We conclude that our survey identifies SC defects with high sensitivity.

B 169 CDK1 phosphorylation governs nuclear proteome redistribution in daughter cells after division: legacy of mother cells

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Background: Eukaryotic cells control many biological processes by regulating the movement of macromolecules into and out of the nucleus. Several nuclear proteins share CDK1 phosphorylation site near their NLS signal which affects their nuclear transport. However in most cases neither the role, nor the structural background of this regulation is poorly understood. We would like to provide deeper insights into these mechanisms through dUTPase and other proteins which are subjected to CDK1 phosphorylation.
Observations: Phosphorylation may abolish nuclear import of the protein if it is properly situated in proximity of its NLS. We have gathered known CDK1 substrates, where this phosphorylation was known to disrupt binding to importin-alpha. We tested new candidates, which were known CDK1 substrates, but the effect of this phosphorylation was not jet investigated regarding their localization (eg. UBA1, p53, UNG). Based on our findings, we here propose a scheme that clearly identifies the exact sequence position where a negative charge will necessarily disrupt NLS function. Using dUTPase as a model protein, we have studied nuclear accumulation dynamics after cell division

of mutants mimicking hyper- or hypophosphorylation and the wild type enzyme. Results showed that after cell division the phosphorylated wild type form may re-enter the nucleus only after a considerable delay as compared to mutants that cannot be phosphorylated. The exact molecular mechanism underlying the disruption of the interaction with importin-alpha and its phosphorylated cargo, has also been investigated with numerous biophysical methods, and X-ray crystallography.
Conclusions: Our data show that the nuclear proteom of daughter cells is determined by phosphorylation events occurring in the mother cell. This could be applied to several known CDK1 substrates. Based on our proposed scheme, further candidates might also be indentified; which is currently still in progress.

B 170 Evolutionary genetics evidence for strong differences in the biological relevance of host microbial sensors

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Background: Pattern-recognition receptors (PRRs) constitute key actors in the detection of pathogens by the host. Detecting how natural selection has targeted their genes allows identifying those that play an essential role in host defence. Here, we studied the levels of naturally-occurring variation of two major families of intracellular PRRs, the NOD-like Receptors (NLRs), mainly sensing bacteria and cellular danger signals and the RIG-I-like receptors (RLRs) more involved in the sensing of viruses.
Observations: We resequenced 24 genes in a worldwide panel (HGDP-CEPH) and performed inter- and intra-specific neutrality tests. One of our most striking results concerned the NALPs, a subfamily of NLRs and probably the least known among PRRs: 9 out of 14 NALPs have been targeted by strong purifying selection, exhibiting highly constrained amino-acid altering variation. This suggests that most NALPs fulfil essential functions, thus constituting excellent candidates to be further studied in the context of infectious and autoimmune diseases. By contrast, the remaining NLRs and the 3 RLRs seem to evolve more adaptively; at least 3 non-synonymous mutations were identified as putative targets of positive selection. More generally, when comparing the results for PRRs – the NALPs, the NODs, the RLRs and the TLRs – a clear pattern emerged. Among the families of PRRs specialised in the sensing of nucleic acids particularly from virus, the endosomal TLRs fulfil a more essential biological role than RLRs. Likewise, among the receptors specialised in the sensing of other pathogens and stress signals, the NALPs appear to fulfil more crucial functions with respect to the NODs and the cell-surface TLRs.
Conclusions: Our data allow us to propose a general hierarchical model, which indicates strong differences in the relative biological importance of PRRs and pave the way for future biochemical, immunological and clinical genetics studies.

B 171 Endocytosis of post-Golgi VAMPs by the clathrin adaptor CALM

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Background: SNAREs facilitate membrane fusion and to function they need to be trafficked to their correct localisation. SNAREs, like all other cargo, must be hence packaged efficiently into transport vesicles. In most cases it is not known how SNAREs are recognized as vesicle cargo. Many SNAREs are retrieved from the plasma membrane in a clathrin-dependent manner. The aim of this project is to investigate whether the clathrin adaptor CALM is a cargo-selective adaptor for the synaptobrevin family members.
Observations: Using a combination of immunofluorescence and a flow cytometry-based endocytosis efficiency assay, we show that three post-Golgi R-SNAREs belonging to the brevin family, VAMPs 2, 3, and 8, are sorted into endocytic clathrin-coated vesicles (CCVs) by the clathrin-associated protein CALM. We found that CALM binds directly to the SNARE domain of VAMPs. Mutations that abolish binding to CALM cause the VAMPs to be mislocalized to the plasma membrane. In contrast, mutations that abolish SNARE complex formation have no effect on the endocytosis of these VAMPs. Two other R-SNAREs, VAMPs 4 and 7, have sorting signals for other adaptors and are relatively unaffected by CALM knockdown; however, when these other sorting signals are removed, they too become CALM-dependent. We also tested the effect of other components of the clathrin machinery on the endocytosis of VAMPs and how CALM depletion affects the endocytosis of other cargo proteins. CALM and AP-2 are cargo-selective adaptors, with CALM facilitating the endocytosis of VAMPs, while AP-2 facilitates the endocytosis of proteins with AP-2-dependent sorting signals, such as the transferrin receptor, which has a YXXphi motif.
Conclusions: The clathrin adaptor CALM facilitates the sorting of several post-Golgi VAMPs. The interaction is unusual; it involves the SNARE domain itself rather than an N-terminal regulatory domain.Therefore CALM-associated VAMPs can only be packaged into CCVs as monomers and not as part of a cis-SNARE complex.

B 172 Immature neurons form tunnelling nanotube-like connections with astrocytes that facilitate Cx43-dependent electrical cell-to-cell coupling

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Background: Neurons and astrocytes cooperate with each other in many different ways. Little is known as to how these interactions are initialized and which signals are involved. We here addressed if tunnelling nanotubes (TNTs, cellular nanotubes), a previously unrecognised form of cell-to-cell communication play a role in establishing crosstalk between neurons and astrocytes. TNTs are intercellular membranous channels, which facilitate the exchange of molecular and electrical signals between cells.

Observations: When freshly prepared hippocampal neurons were seeded on cultured astrocytes, the immature neurons generated short protrusions towards astrocytes resulting in the formation of TNT-like connections. These TNTs were transient structures with a lifetime of approximately 15 min. Characterization by fluorescence microscopy revealed that all analyzed structures were positive for microtubules (n = 20) but only 65% of them contained also F-actin. Furthermore, a significant portion of TNTs (n = 7/20) displayed a punctate staining for the gap junction protein Cx43, which was in most cases localised at the contact site between the TNT and the astrocyte. Using optical membrane-potential measurements combined with mechanical stimulation, we found that many immature neurons were electrically coupled with astrocytes via TNTs after early times of co-culture up to 5 hours but not at later time points around 24 hours. Immunofluorescence analyses with an antibody against Cx43 showed that this marker was strongly expressed by most neurons at 5 hours of co-culture but was absent in nearly all neurons after 24 hours.

Conclusions: Immature neurons form transient TNTs with astrocytes, which result in long-distance electrical coupling. This coupling depends on the presence of Cx43 interposed at the membrane interface between the TNT and the connected cell, and is controlled by the neurons through regulation of the Cx43 level.

B 173 Role of Sec24B in the quality control of mutant cystic transmembrane conductance regulator

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Background: A single phenylalanine (F) deletion at position 508 (DF508) in the cystic transmembrane conductance regulator (CFTR) causes a folding defect in the protein and it is the most common cause of cystic fibrosis. Te defective CFTR is retained in at ER and degraded by the ER associated degradation (ERAD) system. In yeast, CFTR degradation is shown to be assisted by COPII. This observation led us to study the role of COPII proteins in deciding the fate of DF508-CFTR in mammals.

Observations: We have depleted common COPII components by the siRNA approach in Hela cells expressing DF508-CFTR. Sec24B depletion resulted in significant rescue of DF508-CFTR, which shifted from the ER localized form (band B) to Golgi processed form (band C); however, Sec24B depletion had no effect on the transport of the temperature sensitive variant of Vesicular stomatitis virus G protein (VSVG). Also the level of the ER localized form (band B) of CFTR increased, indicating that its degradation was reduced. Interestingly, Sec24B depletion blocked the apparent DF508-CFTR and BCAP31 movement to the juxtanuclear quality control compartment where degradation prone transmembrane proteins are retrotranslocated and degraded by proteasome. When Sec24B was depleted and GFP-DF508-CFTR transiently expressed, resulted a changed localization from ER to plasma membrane (rescue) in 15% of the cells. Also Sar1b depletion had similar effects. The cells that showed rescue of GFP-DF508-CFTR had less numerous exit sites than the cell that did not rescue the protein. The depletion of Sec24B increased the band B and the Golgi processed band C also in CFBE epithelial cells.

Conclusions: We suggest that the misfolded DF508-CFTR must reach the QC compartment before degradation, and sorting proteins to this compartment requires the conventional COPII component Sec24B. Preventing DF508-CFTR from moving to the degradation compartment might help to fold and reach the plasma membrane.

B 174 Munc18-1 tuning of vesicle fusion

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Background: The release of hormones and neurotransmitters, mediated by regulated exocytosis, can be modified by regulation of the fusion pore, which is considered to be stable and narrow initially. Eventually, the fusion pore expands fully (full-fusion exocytosis), or reversibly closes (transient exocytosis). In the latter case, the release of vesicle cargo depends on the fusion pore dwell-time and diameter. However, the molecular events mediating transitions between stages of exocytosis are unclear.

Observations: By using the high-resolution patch-clamp capacitance technique, we studied single vesicles to see if Munc18 protein, interacting with the membrane fusion-mediating SNARE proteins, affects fusion pore properties. We transfected lactotrophs with Munc18-1 mutants to affect the interaction of Munc18-1 with Synt1 (R39C), Rab3A (E466K) and Mints (P242S). Compared with wild-type, Munc18-1 E466K increased the frequency of the fusion event. The latter two mutants increased the fusion pore dwell-time. All the mutants stabilized narrow fusion pores and increased the amplitude of fusion events, likely due to preferential fusion of larger vesicles, since over-expression of Munc18-1 R39C did not affect the average size of vesicles, as determined by STED microscopy. Single molecule atomic force microscopy experiments revealed that wild-type Munc18-1, but not Munc18-1 R39C, abrogates the interaction between Syb2 and Synt1 binary trans complexes. However, neither forms of Munc18-1 affected the interaction of Syb2 with the preformed binary cis Synt1-SNAP25B complexes.

Conclusions: This indicates that Munc18-1 favors tethering of Syb2-containing vesicles to the preformed binary cis complex of Synt1-SNAP25B at the plasma membrane. Munc18-1 and ternary SNARE complex tune fusion pores via multiple converging mechanisms involving Munc18-1 interactions with Synt1, Rab3A and Mints.

B 175 Cytoplasmic distribution and mobility of AQP4-organelles in rat astrocytes

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Background: Brain edema is associated with many neurological disorders and is a major clinical issue. Aquaporin-4 (AQP4) water channel is supposed to have a major role in the development of brain edema and is strongly expressed in astrocytic end-feet membranes. Regulation of the plasma membrane density of AQP4 is unclear. We tested the hypothesis that the plasma membrane localization of AQP4 correlates with the properties of AQP4 vesicle traffic which may be altered in pathologic conditions.
Observations: We identified the plasma membrane localization of AQP4 and the localization of two newly described AQP4 isoforms, AQP4d and AQP4e, in cultured rat astrocytes. These experiments were coupled with quantitative vesicle mobility studies. The results show that AQP4e is mainly present in the plasma membrane, but AQP4d isoform also has a minor plasma membrane distribution. The vesicle mobility studies of the predominant plasma membrane isoform AQP4e were performed in several conditions mimicking pathological states and the results indicate that the plasma membrane distribution of AQP4e correlates with alterations in vesicle mobility. Stimulation with dbcAMP, to induce reactive gliosis, the hallmark of which is overexpression of intermediate filaments, and hypoosmotic stimulation, to mimic edema, significantly affected the mobility of AQP4e carrying vesicles. In reactive astrocytes, AQP4e-vesicle mobility was diminished, but hypoosmotic stimulation triggered a time-dependent biphasic response in AQP4e vesicle mobility: an initial transient reduction followed by an increase in vesicle mobility. Hypoosmotic stimulation also triggered major rearrangements of the vimentin cytoskeleton mesh.
Conclusions: Different responses of AQP4e vesicle mobility in conditions mimicking pathological states, indicate complex vesicle mobility regulation, which may in part be due to alterations of cytoskeleton, as seen in hypoosmotic conditions. Other mechanisms remain to be identified in future studies.

B 176 The FAK-ABCA1 system, a ‘head’ module that couples population context to membrane composition and trafficking

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Background: When cells grow at low local cell density or at the edges of cell islets, Focal Adhesion Kinase (FAK) becomes activated. We want to demonstrate, in this study, that FAK signaling controls the production of the cholesterol transporter ABCA1 in order to couple membrane organization (and downstream activities) to the population context.
Observations: This occurs through PI3K/Akt pathway and subsequent degradation of the transcription factor Tal1. This ‘‘two state’’ system set a low ABCA1 transcription in cells that have active FAK(Low cell density), and a high ABCA1 transcription in cells that have inactive FAK (High cell density). Indeed high levels of ABCA1 lead to cholesterol efflux, low membrane lipid ordering and thus inactive Rac1 and inactive Akt, two key kinases components for signaling to cell spreading and growth. Low levels of ABCA1 lead to the strict opposite behaviour, with in which high levels of cholesterol and sphingolipids lead to activated Rac1 and Akt, and therefore the ability to spread and to grow in size. Remarkably, the loss-of-FAK phenotype in cell spreading and growth can be reversed by inhibiting ABCA1 or by exogenously loading the cell surface with cholesterol, demonstrating that FAK controls these cellular properties to a large extent via the FAK-ABCA1 system.
Conclusions: At an applied level, the system is highly relevant for the process of epithelial-to-mesenchymal transition (EMT), representing a crucial step in the development of metastasis. Knowledge of these system’s design principles will aid our understanding of its deregulation during EMT.

B 177 Imaging of mobile stable nanoplatforms in the live cell plasma membrane

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Background: The plasma membrane has been hypothesized to contain nanoscopic lipid platforms, which are discussed in the context of ‘lipid rafts’ or ‘membrane rafts’. Based on biochemical and cell biological studies, rafts are believed to play a crucial role in many signaling processes. However, there is currently not much information on their size, shape, stability, surface density, composition and heterogeneity.
Observations: We present here a method which allows for the first time the direct imaging of nanoscopic stable platforms with raft-like properties diffusing in the live cell plasma membrane. Our method senses these platforms by their property to assemble a characteristic set of fluorescent marker-proteins or lipids on a time-scale of seconds. A special photobleaching protocol was used to reduce the surface density of labeled mobile platforms down to the level of well-isolated diffraction-limited spots, without altering the single spot brightness. The statistical distribution of probe molecules per platform was determined by single molecule brightness analysis. For demonstration, we used the consensus raft marker glycosylphosphatidylinositol-anchored monomeric GFP and the fluorescent lipid analogue Bodipy-GM1 which preferentially partitions into liquid ordered phases. For both markers we found cholesterol-dependent homo-association in the plasma membrane of living CHO and Jurkat T cells in

the resting state, thereby demonstrating the existence of small, mobile, stable platforms containing these probes.
Conclusions: We further applied the technology to address structural changes in the plasma membrane during fever-type heat shock: at elevated temperatures the mGFP-GPI homo-association disappeared, accompanied by an increase in the expression of the small heat shock protein Hsp27.

B 178 VAMP-associated protein B (VAPB) drives proliferation and invasion of breast cancer cells through regulation of critical vesicle trafficking events

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Background: Tumor cells heavily rely on vesicle-mediated transport for receptor localization and growth factor secretion. Aberrant expression of vesicle trafficking proteins is predicted to enhance tumor growth and metastasis. VAPB (vesicle associated membrane protein; associated protein B) is a putative vesicle-associated protein that is overexpressed in clinical breast cancers and correlates with reduced patient survival.
Observations: We report that VAPB enhances growth and invasion of breast cancer cells through interactions with Rab and Arf small GTPases. Using cell culture and mouse models of HER2-overexpressing breast cancers, we found that shRNA-mediated knockdown of VAPB reduced 3-dimensional colony size and cell proliferation, while VAPB overexpression increased colony size and cell growth. In vivo, inhibition of VAPB expression decreased tumor volume and proliferation. Using a modified Boyden chamber assay, we found that VAPB overexpression increased tumor cell invasion. To mechanistically dissect the role of VAPB in breast cancer, we identified novel VAPB interacting proteins by mass spectrometry-based proteomics (Multidimensional protein identification technology). Specifically, Rab1, Rab8A, Arf1, Arf3, and Arf5 were identified in complex with VAPB. These VAPB-interacting proteins regulate critical vesicle trafficking events between the endoplasmic reticulum-Golgi apparatus to the plasma membrane, a mechanism used by cells to secrete growth factors. Consistent with this finding, we demonstrated that conditioned medium collected from VAPB overexpressing cells increased AKT activation in recipient cells.
Conclusions: These data suggest that VAPB functions as a critical regulator of vesicle-mediated protein trafficking from the ER-Golgi network to plasma membrane, promoting breast tumor cell proliferation and motility and underscores the vesicle-mediated transport pathway as a potential therapeutic target.

B 179 First insight into non-mammalian SLCs: functional characterization of zebrafish (Danio rerio) uptake transporters (Solute Carrier families SLC21 and SLC22)

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Background: Membrane transporters of families SLC21 (Organic anion polypeptides; Oatp) and SLC22 (Organic cation/anion/zwitterions transporters) are involved in uptake of various endogenous and foreign compounds. However, their role has not yet been comprehensively investigated in non-mammalian organisms. The goal of our study was molecular characterization of toxicologically relevant uptake transporters in zebrafish (Danio rerio).
Observations: Using phylogenetic analysis, we annotated 29 zebrafish genes within SLC21 and SLC22 families and conducted tissue expression profiling using qPCR. Using the transiently transfected HEK293 cells and radioactively labeled model substrates, estrone-3-sulfate and p-aminohippurate, we conducted functional characterization of Oatp1d1 (SLC21) and seven organic anion transporters (SLC22). Within vertebrate and invertebrate lineages, we found that Oatp1d subfamily is present only in teleosts fish and consist of two members: Oatp1d1 and Oatp1d2. According to our results, physiological role of zebrafish Oatp1d1 could be transport of specific hormones and bile salts, while it also interacts with a wide range of pharmaceuticals and environmental contaminants. Oatp1d1 significantly differs from four mammalian Oatp1 subfamily genes in terms of its substrate specificity. Within the SLC22 family, two co-orthologs of mammalian OAT1 and OAT3, zebrafish Oat1/3 and Oatlike1, show overlapping substrate range but different affinities. Human OAT2 has 5 co-orthologs in zebrafish, Oat2a-Oat2e, that show differential substrate specificities among each other and in comparison with mammalian orthologs.
Conclusions: In conclusion, our study presents the first functional characterization of non-mammalian SLC genes. We showed that gene orthology relationship within human and zebrafish SLC21 and SLC22 genes are complex with differential substrate range and affinities among species.

B 180 Rab GTPases and mast cell exocytosis

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Background: Mast cells (MC) are specialized secretory cells involved in innate and adaptive immune responses as well as in mediating allergic and inflammatory reactions. These cells are packed with secretory granules (SG), which contain allergic, inflammatory and immunoregulatory mediators. When triggered, multiple signaling events are activated resulting in fusion of the SGs with the plasma membrane (degranulation).
Observations: Adopting screening approaches aiming to unveil stimulus-secretion coupling networks in MC has been limited due to their low transfection efficiency. Hence, genetic manipu-

lations are unlikely to leave an impact on the actual readouts of average secretion measured by conventional methodologies. We established a technology that allows functional and phenotypic-driven screens. Using this technology, we have screened the Rab family of small GTPases. We identified Rabs that localize to the secretory granules, Rabs that coordinate the spatial location of the secretory granules and a network of 28 Rabs that modulate exocytosis. Strikingly, this network includes Rab GTPases that are implicated in endocytic recycling through the pericentrioral endocytic recycling compartment (ERC). We are further analyzing the pattern of individual cell responses to manipulations of the relevant Rabs under defined cellular settings by using a chip-based system.
Conclusions: Our results implicate the ERC as playing a role in controlling MC exocytosis. We combine multidisciplinary approaches including cell and molecular biology, and computational techniques aiming to unveil the cellular machineries involved in the execution and regulation of MC regulated exocytosis.

B 181 Molecular basis for M-Sec mediated tunneling nanotube formation

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Background: Tunneling nanotubes (TNTs) are a novel type of cell-cell communication recently reported. TNTs connecting plasma membranes of remote cells mediate rapid propagation of activation signals including calcium flux. On the other hand, TNTs are exploited by certain infectious agents such as HIV-1 and pathogenic prion proteins for rapid intercellular transmission. Despite accumulating evidence for the biological significance of TNTs, molecular basis for TNT formation remained largely unknown.
Observations: We have recently reported that a myeloid cell-specific protein M-Sec, encoded by Tnfaip2 gene, can promote formation of functional TNTs. Time lapse imaging demonstrated that exogenous expression of GFP-M-Sec in HeLa cells induces de novo formation of numerous membrane protrusions extending from the plasma membrane, some of which physically connect remote cells to form TNT-like structures capable of calcium flux transmission. Furthermore, RNA interference against M-Sec greatly reduced endogenous TNT formation as well as intercellular propagation of calcium flux in a macrophage cell line.Subsequent studies revealed that the molecular interaction of M-Sec with RalA small GTPase and the exocyst complex is required for the M-Sec-mediated TNT formation. N-terminal lysine-rich region of M-Sec is required for the plasma membrane localization of M-Sec, which seems to trigger the formation of TNT-like membrane protrutions. In vitro binding assay suggested that M-Sec interacts with membrane phospholipid. To better understand the molecular basis for M-Sec function, we are now trying X-ray crystallography of M-Sec.
Conclusions: Collectively, our data indicate that M-Sec functions as a key regulator of functional TNT formation by binding to the plasma membrane through interaction with phospholipid, and by recruiting the RalA-exocyst pathway.

B 182 A Human Genome-wide siRNA Screen for Novel Endocytic Machinery

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Background: The mechanisms regulating initiation and fusion of plasma membrane-derived clathrin-coated vesicles remain elusive. To identify novel proteins involved in this regulation, we performed a human genome-wide screen using 21,121 siRNA pools. A fluorescence 96-well plate reader-based assay was used to quantify the cell surface accumulation of CD8 chimeras with two different clathrin dependent trafficking motifs, YXX and FXNPXY, as well as cell surface MHC class I (clathrin-independent cargo).
Observations: About 300 genes were found with a phenotype either similar to the clathrin knockdown or with cargo specific phenotypes. The hits include known components of the machinery (e.g. dynamin), proteins with domains previously implicated in trafficking (e.g. the TBC domain present in RabGAPs), proteins with functions previously not implicated in regulation of clathrin-coated vesicles (e.g. several subunits of the V-ATPase 6), as well as a large proportion of proteins with as yet unknown functions. Pathway analysis of the hits identified novel links between clathrin-mediated endocytosis and basic cellular processes such as cell cycle and mRNA splicing. We are now validating the hits using individual siRNAs, and further characterizing the knockdown phenotypes. For example, we developed a high-throughput immunofluorescence and automated microscopy-based assay to study recruitment of known machinery to clathrin-coated pits at the plasma membrane.
Conclusions: The validation assays should allow us to categorize the hits into functional groups and improve our understanding of how formation of clathrin-coated vesicles is initiated, quality controlled, and how they are targeted to their destination membranes.

B 183 Identification and physiological analyses of mammalian epithelial cell defective in GPI-lipid remodeling

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Background: GPI-anchored proteins (GPI-APs) are a class of lipid-anchored proteins expressed in all eukaryotic cells. In polarized epithelial cells, GPI-APs are localized in the apical plasma membrane. The lipid moiety of GPI-APs is remodeled and this confers on GPI-APs the property to partition into the detergent-resistant membrane (DRM) fraction. This DRM association has been proposed to be involved in the apical targeting of GPI-APs in polarized epithelial cells.
Observations: We recently observed that GPI-biosynthesis is essential for development of cell polarity in drosophila retina. In order to examine the role of GPI-APs in formation of mammalian epithelial cell polarity, we isolated mutant Madin-Darby canine kidney (MDCK) cells whose surface expression of GPI-APs was

specifically decreased to 1 to 10% of wild-type cell level. In mutant cells, the GPI-AP precursor proteins were normally GPI-anchored in the ER, but were not stably associated at the plasma membrane. Instead they were secreted into the medium. In mutant cells, intracellular GPI-APs were not partitioned into the DRM, suggesting that mutant cells were defective in GPI-lipid remodeling. The mutant MDCK cells formed a polarized monolayer on plastic permeable filters and did not show any obvious defect in tight junction integrity. We are currently analyzing several basolateral and apical proteins and lipid markers in order to reveal the potential roles of GPI-APs in the formation of polarized epithelial cells. Interestingly, in mutant cells, GPI-APs were exclusively secreted into the apical medium even though they were not partitioned into DRM fraction.

Conclusions: We report the first mutant MDCK cell that is specifically defective in DRM association of GPI-APs due to lack of lipid remodeling. The apical secretion of GPI-APs in this mutant argues against an essential role for raft incorporation in the apical targetting of GPI-APs in polarized epithelial cells.

B 184 Expression and characterization of rainbow trout (Oncorhynchus mykiss) Bcrp (abcg2) in Sf9 insect cells

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Background: The cellular efflux transporter BCRP (abcg2) belongs to the ATP-binding cassette (ABC) superfamily and plays an important role in elimination of xenobiotics in mammals. To date, its role in fish remains elusive. To investigate and characterize its role we expressed rainbow trout (Oncorhynchus mykiss) BCRP in both Sf9 insect cells and human HEK293 cell line. For direct comparison of fish and mammalian transporters the human BCRP was also expressed in the same protein expression systems.

Observations: We found that fish BCRP is not glycosylated in the same way as its human counterpart. Furthermore, trout BCRP exhibited weaker modulation of transport activity in the presence of cholesterol, a major component of cell membranes that strongly affects the transport rate of human BCRP. Km values for ATP were similar in the absence of cholesterol for both human (0.38 mM) and trout BCRP (0.11 mM). Cholesterol increased both Km 2.5 fold. Although cholesterol did not change the baseline transporter activity, it exhibit differential effect on the transport rate of fish and human BCRP in the presence of the model substrate sulfasalazine. After initial biochemical characterization of the two transporters, we tested 40 model compounds using the ATPase assay in order to determine and compare human and trout BCRP substrates/inhibitors specificity. We found significant correlation ($r2 = 0.64$) in substrates/inhibitors affinity between trout and human BCRP. Among the most interesting findings was the high affinity of trout transporter for the polycyclic aromatic hydrocarbons (PAH), benzo[a] pyren and 2-acetylamninofluorene, showing for the first time that BCRP is important for PAHs excretion in fish.

Conclusions: Substrate affinity of trout BCRP implies its relevance in disposition of physiological compounds and environmental contaminants in fish. Trout BCRP is underglycosylated in HEK293 cells. Contrary to human BCRP, activity of trout BCRP was not significantly affected by cholesterol.

B 185 Exhaustive Protein Interaction Mapping: An Exocyst Case Study

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Background: Yeast two-hybrid (Y2H) protein interaction screening has proven instrumental for the analysis of the interactome of the different model organisms, tissues or cells, mostly thanks to pairwise testing or screening of oligo dT-primed cDNA libraries. However, interaction map completeness has been limited by the use of full-length proteins and C-terminal polypeptide fragments which result in significant false negative rates.

Observations: To circumvent these limitations, we have used a domain-based strategy to construct highly complex, random-primed cDNA libraries. The complexity of these libraries is greater than 10 million independent fragments in yeast, with an average fragment size of 800 bp. To ensure reproducible and exhaustive Y2H results, these libraries are screened to saturation using an optimized mating procedure, allowing to test on average 97 million interactions per screen (10-fold coverage of the library). As a consequence, multiple, independent fragments are isolated for each interactant, enabling the immediate delineation of a minimal interacting domain and the computation of a confidence score. To illustrate the power of this Y2H approach, we have screened all eight subunits of the Homo sapiens and Drosophila melanogaster exocyst complexes against our human placenta and drosophila embryo libraries, respectively.

Conclusions: The results presented here give exciting insights into the regulation of the exocyst by diverse signaling pathways.

B 186 The endosomal protein rabip4' binds beta3 adaptin and mediates the AP-3 pathway of tyrosinase in melanoma cells

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Background: Melanosomes are lysosome-related organelles of pigment cells that synthesize and store melanin. The early endosomal compartment serves as an intermediate for cargo transport to melanosomes. Although many components of the endosomal sorting machinery have been unraveled, the sorting mechanisms to melanosomes are still incompletely understood.

Observations: We here report that the endosomal FYVE domain protein rabip4' interacts with the heterotetrameric adaptor protein complex AP-3 through the beta3 subunit. The interaction is direct and involves the FYVE domain of rabip4' and the hinge region of beta3 adaptin. Rabip4' colocalized with AP-3 on a subpopulation of early endosomes. Knock down of rabip4'

or AP-3 in MNT-1 cells selectively increased the intracellular protein level and DOPA oxidase activity of tyrosinase.

Conclusions: Our results show that rabip4' is associated with the AP-3 transport pathway of tyrosinase in pigmented melanoma cells, possibly by mediating tyrosinase sorting in endosomes.

B 187 A nuclear exclusion signal in the SN4TDR protein in Schizosaccharomyces pombe causes it to be localized in the cytoplasm and interact with moe1 and RPL22

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Background: The SN4TDR protein, which in most eukaryotes has four Staphylococcal nuclease (SNc) domains, a Tudor domain and a variable 5th SNc domain is encoded by SN4TDR in S. pombe. We isolated RNA and used it to synthesize a cDNA containing the full coding sequence complete with a poly(A) tail, thus providing the first evidence that this gene is expressed in fission yeast.

Observations: Several constructs of different regions of this gene were linked to XFP, and they were localized in cytoplasm and were apparently excluded from the nuclei, as observed by fluorescence microscopy. However, constructs lacking the region between the second and third SNc domains were localized to the nucleus, indicating that the region is a nuclear exclusion signal (nuclear export signal) or NES. Using the yeast two-hybrid system in Saccharomyces cerevisiae followed by immunoprecipitation of associated proteins with myc-tagged SN4TDR protein in S. pombe, we identified a strong association of SN4TDR with moe1, a spindle destabilizing factor and subunit of the translation initiation factor, eIF3, as well as a weaker association with the ribosomal protein, RPL22. Moe1 is the zeta subunit of eIF3 and binds directly to RNA and indirectly to microtubules, while RPL22 is a heparin binding protein and the target of small RNAs from Epstein Barr virus. Analysis of a knockout of the sn4tdr gene revealed a minor decrease in the initial growth rate but the gene was not essential.

Conclusions: From these results, we anticipate that SN4TDR may play an important role in initiation of translation in the cytoplasm, but (an additional) role in spindle fiber function during cell division cannot be ruled out.

B 188 COG complex interacts with multiple Golgi SNAREs

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Background: The Conserved Oligomeric Golgi (COG) complex is a ubiquitously expressed membrane-associated protein complex that consists of eight subunits (Cog1-8). The COG complex functions in the retrograde intra-Golgi trafficking through association with coiled-coil tethers, SNAREs, Rabs and COPI. We have previously reported functional interaction between Golgi t-SNARE Stx5 and COG subunits COG4 and COG6.Now, yeast two hybrid (Y2H) assay was used to uncover a complete set of COG-SNARE interactions.

Observations: Interactions between COG6 and Membrin, Stx6 and SNAP29 and between COG8 and Membrin, Stx16 and SNAP29 were found. Co- IP confirmed results of Y2H and revealed that Stx5, Stx16 and SNAP29 interact with the whole COG complex, while Stx6 and Membrin preferably interact with the Cog5-8 subcomplex. COG6 SNARE binding B1 domain was identified. COG6deltaB1p failed to co-IP with SNAREs and lost the capacity to localize to the Golgi, confirming the importance of COG-SNARE interaction. The relocalization of COGs to the mitochondria was used to divert COG-specific vesicular traffic from Golgi. Relocalization of COG4 to the mitochondria specifically diverted GFP-Stx5-containing membranes to that organelle, while relocalization of COG8 specifically diverted GFP-Stx16-positive membranes to the mitochondria region. Diversion of either Sytx5 or Stx16 to mitochondria region caused extensive fragmentation of Golgi membranes. In contrast, relocalization of COG6 to mitochondria did not significantly affect Stx5 or Stx16-positive membranes and Golgi morphology in general. Instead, it was capable of diverting traffic of both GFP-Membrin and GFP-Stx6 positive membranes to the mitochondria region.

Conclusions: In summary, this data indicates that the COG complex and its subcomplexes are decisive elements for organization of three distinct SNARE-mediated Golgi trafficking pathways.

B 189 Oxidative protein folding by an endoplasmic reticulum localized peroxiredoxin

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Background: Endoplasmic reticulum (ER) oxidation 1 (ERO1) transfers disulfides to protein disulfide isomerase (PDI) and is essential for oxidative protein folding in simple eukaryotes such as yeast and worms. Surprisingly, ERO1-deficient mammalian cells exhibit only a modest delay in disulfide bond formation suggesting the presence of an alternative patwhay to ERO1.

Observations: To indentify ERO1-independent pathways to disulfide bond formation, we purified PDI oxidants with a trapping mutant of PDI. PRDX4 stood out in this list, as the related cytosolic peroxiredoxins are known to form disulfides in the presence of hydroperoxides. Mouse embryo fibroblasts lacking ERO1 were intolerant of PRDX4 knockdown. Introduction of wildtype mammalian PRDX4 into the ER rescued the temperature-sensitive phenotype of an ero1 yeast mutation. In the presence of an H_2O_2 generating system, purified PRDX4 oxidized PDI and reconstituted oxidative folding of RNase A.

Conclusions: These observations implicate ER localized PRDX4 in a previously unanticipated, parallel, ERO1-independent pathway that couples hydroperoxide production to oxidative protein folding in mammalian cells.

B 190 Investigating the Formation of Metabolic Bacterial Organelles in Mammalian Cells

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Background: Some heterotrophic bacteria can synthesize polyhedral structures containing metabolic enzymes that are bounded by a unilamellar protein shell. These bacterial microcompartments contain enzymes associated with a specific metabolic process e.g. 1,2-propanediol utilization. There are 21 genes associated with propanediol utilization microcompartments, five of which are thought to be absolutely required to form the proteinaceous shell.

Observations: Work by Warren and colleagues at Kent has shown that if one of these essential genes (pduA) is expressed in E.coli then long, thin organelles are formed in the cell. Further work within the Warren laboratory has also shown that proteins can be selectively targeted to these proteinaceous compartments. This project set out to investigate whether the five essential pdu shell proteins required to form the microcompartment can be expressed in CHOK1 cells or if this is toxic to mammalian cells. The expression of these genes in mammalian cells, and whether they can form proteinaceous structures within such cells, has not previously been reported. If such 'organelles' could be produced in mammalian cells this opens up the possibility of targeting specific processes or proteins into these in mammalian systems. V5 tagged and untagged individual shell protein constructs were generated using a pcDNA3.1 V5 plasmid backbone and in addition stable cells expressing GFP tagged pdu subunits were also generated. The effects of expressing these in CHOK1 cells were determined by Western blotting and fluorescent immunostaining.

Conclusions: To date we have shown that individual pdu shell proteins can be transiently expressed in CHOK1 cells and this does not appear to be toxic to the cell. Work on the stable expression of individual pdu shell proteins and their effects on mammalian cells is also reported.

B 191 Computational analysis of protein oxidative damage: effect of carbonylation on structure, dynamics and aggregability of villin headpiece on the atomistic level

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Background: One of the most important irreversible oxidative modifications of proteins is carbonylation, a process of introducing the carbonyl group in reaction with reactive oxygen species. Importantly, carbonylation increases with cell age and is associated with the formation of intracellular protein aggregates and the pathogenesis of a number of age-related disorders. However, it is still largely unclear how carbonylation affects protein structure, dynamics and aggregability on the atomic level.

Observations: Here, we use classical molecular dynamics simulations to study structure and dynamics of the carbonylated headpiece domain of villin, an important actin-organizing protein. We perform an exhaustive set of molecular dynamics simulations of native villin headpiece together with every single combination containing carbonylated versions of its seven lysine, arginine and proline residues, the quantitatively most important carbonylable amino acids. Surprisingly, our results suggest that high levels of carbonylation, far above those associated with cell death in vivo, may be required to destabilize and unfold protein structure through the disruption of specific stabilizing elements, such as salt bridges or proline kinks, or tampering with the hydrophobic effect. On the other hand, by using thermodynamic integration and molecular hydrophobicity potential approaches, we quantitatively show that carbonylation of hydrophilic lysine and arginine residues is equivalent to introducing hydrophobic, charge-neutral mutations in their place.

Conclusions: By comparison with experimental results, we demonstrate that the nature of carbonylation-induced 'mutations' just by itself significantly increases intrinsic aggregation propensity of both structured, native proteins and their unfolded states.

B 192 Characterization of DNAse activity of starved and refed Tetrahymena thermophila cells

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Background: Deoxyribonucleases (DNAses) are a group of enzyme that breaks down the single or double stranded DNA molecules in a non-specific sequence manner. Single-celled ciliate Tetrahymena thermophila is a well-known model organism which is widely used in experimental biology. Since it has a major macronuclear DNA degradation step, we studied DNase activities of starved and refed T.thermophila cells to determine the most effective DNase by feeding the starved cell with plasmid DNA.

Observations: T.hermophila SB210 cells were grown with PPY medium for overnight. Next day, cells were starved with 50 mM cold Tris-HCI pH 7.5 (SB-starvation buffer) for 24h. Only starved cells (5x105) refed with 1microgram plasmid DNA as inducer. After 1st, 2nd and 3th h cell samples were taken and protein isolation was performed with lysis buffer (20 mM Tris-HCl, 2mM EDTA). The 20microliter nuclease reaction contained 25 microgram of the soluble cytoplasmic proteins and 200 ng substrate were incubated with DNase I (commercial) and DNase II buffer (NaAC pH 4.6) at 37 °C for 60 min and run on agarose gel. A series of DNAse reactions with common DNAse inhibitors and different cations as well as SDS gel activity assay were also performed. We observed that soluble cytoplasmic proteins from starved and refed T.thermophila cells show strong DNase activities and strong inhibition with common inhibitors (i.e. EDTA, Zn + 2 for DNase I and buffer with neutral pH for DNase II). Spectrophotometrical quantitative analysis of DNase activity is currently underway with the help of a commercial DNA binding dye.

Conclusions: Due to the ability of T.thermophila to degrade macronuclear DNA in an apoptosis-like way, we thought that it

might have strong DNase activity. We aimed to induce the starved cells by feeding them with DNA to select the effective DNAse. This study can be the first step for its full characterization.

B 193 Mechanism of action of Plasmodium falciparum CTP:phosphocholine cytidylyltransferase

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Background: Phospholipid synthesis in Plasmodium, the causative agent of malaria, during its intraerythrocytic cycle is essential and constitutes a validated and original pharmacological target. The P. falciparum CTP:phosphocholine cytidylyltransferase (PfCCT) has key regulatory function as it catalyzes a rate-limiting step of de novo phosphatidylcholine biosynthesis. We aim to decipher the mechanism of action of PfCCT, in particular through kinetics and characterization of enzyme-ligand interactions.

Observations: PfCCT constructs with one of the catalytic domains were optimized for E coli expression. Dimeric form and enzymatic activity of the expressed protein was confirmed. Dissociation constant of the PfCCT:CDP-choline (CPDCho) product complex was 50 microM (data from isothermal titration calorimetry), much lower than the Michaelis constants for choline phosphate and CTP substrates. Addition of product, as compared to substrates, provided significantly stronger protection against thermal unfolding (data from Thermofluor). We suggest that the high affinity of PfCCT towards the product CDPCho may be due to its simultaneous interactions with both the CTP and choline phosphate binding conserved motifs. To gain insights into mechanistic details, we adopted a continuous photometric enzyme activity assay. Fluorescence of tryptophane fluorophores within the PfCCT monomer was significantly quenched in CCT:CTP and CCT:CDPCho complexes, indicating that ligand binding is accompanied by conformational changes within the fluorophore microenvironment. Crystallization trials revealed positive effect of the presence of CDPCho on protein crystal growth. Crystals are to be investigated by X-ray diffraction.

Conclusions: Results from independent biophysical techniques showed that PfCCT binds the product CDPCho much stronger than either substrate. CDPCho binding may have a key role not only in enzyme conformation but also in the catalytic equilibrium. Enzyme dynamics is planned to be studied by fast kinetic methods.

B 194 A quantitative cellular sensor for protein aggregation

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Background: Aggregation of the amyloidogenic Abeta peptide produces toxic multimeric species that play a key role in the development of Alzheimer's disease (AD). Compounds that inhibit this aggregation process may prove useful as therapeutics for the prevention and/or treatment of AD. The identification of small molecules that block the aggregation process in vivo poses a significant challenge. Although some aggregation inhibitors have been reported no clinically useful therapeutics have emerged.

Observations: Two major obstacles have held the discovery of compounds, which block A-beta aggregation in vivo, back. First, structure-based rational drug design is impossible due to the lack of a high-resolution structure of Ab. Second, large-scale screening of combinatorial libraries for in vivo modulators of Ab aggregation has been hindered by the unavailability of cost-effective quantitative models of protein aggregation, which replicate the complex cellular environment of mammalian cells, but are also amenable to HTS. We have recently developed a highly sensitive assay, which uses genetically encoded bioluminescence reporters, for monitoring protein aggregation in mammalian cells. This assay is based on chimeric reporters, which consist of a fusion-protein between Ab42 and an intracellular or a secreted reporter enzyme. The misfolding and aggregation of Ab42 reduces the activity of the chimeric reporter by two orders of magnitude relative to a fusion protein between a non-aggregating domain and the reporter. We have tested our system and found that it is an extremely sensitive, quantitative indicator of Ab aggregation in human cell lines, primary neuron cultures and in whole mouse brains.

Conclusions: These assays should facilitate efficient, high-throughput screening of compounds for promoters of protein folding or inhibitors of protein aggregation. To our knowledge, this is also the first and only quantitative sensor for extracellular protein aggregation.

B 195 Understanding the role of TRIM8, a new p53 target gene that modulates the p53 activity, in the progression of glioma

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Background: Impairment of p53 function has a crucial role in tumor evolution. p53 inactivation occurs through inactivation of p53 function by mutations and the abrogation of signaling pathways or effectors molecules that regulate p53 activity. The stabilization and activation of p53 are crucial in preventing cells from becoming cancerous. Thus, one of the most important challenges for the development of improved cancer therapies is the discovery of additional p53-inactivating pathways or p53 modulators.

Observations: We identified TRIM8 as a new p53 target gene that modulates p53 stability and activity. TRIM8 is a member of the Tripartite Motif protein family involved in several pathological conditions. We showed that under stress conditions p53 induces the expression of TRIM8, which in turns stabilizes p53 through their physical interaction. The stabilization of p53 was paralleled by an increase of Ser15 and Ser20 phosphorylated p53

level, two posttranslational modification known to induce cell cycle arrest. Futhermore, overexpression of TRIM8 regulates the p53 transcriptional activity by increasing p21 and GADD45 that results in cell cycle arrest. Concurrently, TRIM8 overexpression decreases MDM2 protein levels suggesting that TRIM8-mediated p53 stabilization occur through the MDM2 degradation. Interestingly, TRIM8 gene maps to 10q24.3, a region associated with rearrangements in gliomas. By using qPCR we showed that TRIM8 expression is significantly downregulated in those tumors and it correlates with the tumor grade. Interestingly, microarray assay and qPCR showed that in glioma samples TRIM8 mRNA level anti-correlates with the miR-17 expression, a microRNA involved in glioma progression.

Conclusions: Collectively, our experiments demonstrate that TRIM8 is as a new key functional modulator of p53 stability and activity that directs the cells towards a program of cell cycle arrest. Further we propose TRIM8 as a novel predictive marker for the clinical progression of gliomas.

B 196 BAG3, together with its multiple partners HSPB8/HSP70/p65, acts as a master regulator of protein homeostasis

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Background: BAG3, together with the small heat shock protein HSPB8, facilitates the degradation of aggregate-prone proteins through autophagy. HSPB8 and BAG3 are both upregulated during aging in the human brain of patients suffering of protein conformation diseases, which was suggested as a compensatory response to impaired proteasomal functioning in order to maintain protein homeostasis.

Observations: Here, we now show that BAG3 in fact may act as a master regulator of protein homeostasis. Besides with HSPB8, BAG3 associates with multiple partners each involved in different aspects of cellular proteostasis. BAG3 upregulation induces proteasomal inhibition, a step that requires its BAG-domain and hence binding to HSP70. This triggers the activation of the eIF2 alpha pathway, a step in which HSPB8 plays an active role and that ensures translational inhibition. Phopshorylation of eIF2 alpha also leads to autophagy activation, which allows the degradation of protein aggregates. Through its PXXP-domain, BAG3 binds to dynein and targets misfolded proteins to aggresomes (Gamerdinger, 2011). Finally, through its C-terminus, BAG3 binds to p65 and suppresses the NF B stress signaling pathway, likely to avoid a premature activation of cell death.

Conclusions: The findings that mutations in BAG3 and HSPB8 are related to neuromuscular/muscular disorders further underscore their importance in protein homeostasis.

B 197 Chaperoning of the Tau protein by the Hsp90 machine

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Background: Protein folding in the cell is assisted by molecular chaperones. The ATP-controlled Hsp90 chaperone machine is selective in choosing its substrates, one of which is the protein Tau. Key features determining this selectivity are unknown: the substrate binding site and the control of substrate interaction by Hsp90's ATPase cycle. Our aims are: 1. Determine the molecular basis for Tau interaction with Hsp90. 2. Identify Hsp90's binding site for Tau. 3. Determine the role of ATP for chaperoning Tau.

Observations: We recently established an NMR approach that allows the analysis of the 170 kDa full length Hsp90 protein and its interaction with ligands. We specifically labelled the isoleucine side chains of Hsp90, acquired methyl-TROSY NMR spectra and assigned resulting signals. We formed a 220 KDa complex of labelled Hsp90 with Tau and acquired NMR spectra in various nucleotide states. Here we show that ATP induces an intimate complex of Hsp90 with Tau substrate, involving its N-terminal and middle domain. The synergistic action of both the substrate and ATP were required for this conformational change. We identified the substrate-binding site of Hsp90 in the presence and absence of ATP. Those data allow interesting conclusions regarding the regulation of Hsp90 by co-factors. We showed by fluorescence spectroscopy that Tau binds with high affinity to Hsp90. Currently we build a model of the Hsp90-Tau complex based on SAXS measurements.

Conclusions: Hsp90 binding to disordered Tau extends the concept of molecular chaperoning to intrinsically disordered proteins. The localisation of the Tau binding site and the ATP-induced structure changes is relevant for the interactions of Hsp90 with other client proteins.

B 198 Bag1 targets conformationally immature BCR-ABL for degradation

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Background: Specific inhibitors of ABL tyrosine kinase have provided a remarkable success in the treatment of chronic myeloid leukemia. However, resistance to the inhibitors by mutation or overexpression of BCR-ABL is still an obstacle. Heat shock protein 90 (Hsp90) inhibitors would be expected to overcome the resistance since BCR-ABL proteins, mutated or not, are ubiquitinated and degraded in their presence by as yet uncovered mechanisms.

Observations: Although imatinib and Hsp90 inhibitors can individually abrogate cell growth of BCR-ABL-expressing leukemic cells, imatinib paradoxically attenuated the inhibitory effect of Hsp90 inhibitors. We hypothesized that imatinib-targeted enzymatically and therefore conformationally mature forms with tyrosine kinase activity are locked in an inactive but stabilized state that may no longer require Hsp90. We screened for molecular chaperones with enhanced binding to BCR-ABL when Hsp90 was inhibited and found a direct in vitro binding of Bag1

to BCR-ABL, which was enhanced by Hsp90 inhibitors and CHIP, inhibited by imatinib and competed with Hsc70. Under Hsp90 inhibition BCR-ABL underwent decreased autophosphorylation and switched its binding partner from Hsp90 and cdc37 to Hsc70 and Bag1. While phosphorylated cdc37 was reported to bind the ATP-binding motifs of a variety of kinases, we found that Bag1 recognized BCR-ABL via BCR 1-413 and the N-lobe with the motif in the ABL kinase domain. Application of an siRNA against the Hsc70-interacting E3 ligase CHIP impaired Hsp90 inhibitor-induced degradation of BCR-ABL. Conversely, induced overexpression of CHIP resulted in BCR-ABL degradation.

Conclusions: We suppose that BCR-ABL proteins consist of an equilibrium of immature versus mature forms and inhibition of Hsp90 causes a conformational shift in BCR-ABL from its mature to immature form that can be recognized by Bag1 for CHIP-mediated degradation.

B 199 Secondary Structure Properties of Activation of Betaine Transporter BetP from Corynobacterium Glutamicum with ATR-FTIR Spectroscopy

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Background: BetP, a secondary glycine betaine transporter protein, protects the bacteria from sudden osmotic stress changes. The structure of BetP (N-terminal truncated mutant) was solved at 3.35 Å resolution, revealing 12 TM helices in an occluded state (Ressl et al., 2009). The N- and C-termini are important in the regulation of activity and solute transport properties of BetP. The structure of the N-terminus and secondary structure alterations in switching from inactive to active state are not yet clear.

Observations: FTIR spectroscopy showed a higher alpha-helical content in the protein than the fraction revealed by the X-ray data that is suggested to originate from the N-terminus. While the X-ray study showed 65% helical structure, IR spectroscopy showed 71.5% and 69% helical structure in inactive and active state, respectively. The results also provide evidence of opening of the protein hydrophobic core upon activation. While 55% of the protein main chain is accessible by buffer solution in the inactive state, an additional 11%, roughly corresponding to 65 a.a., is exposed to buffer upon activation. The less tilted and/or stretched helices (type I-helix) become tilted and/or solvated (type II-helix). BetP also shows changes in intermolecular beta-sheet properties with respect to inactive conformation. This finding supports the proposed mechanism of activation suggesting the reorientation of the four-helix bundle. In this study, the C-terminus of BetP from one monomer is also shown to interact with the loop(s) of another monomer via salt bridges among Arg and Asp/Glu, and thereby constructing the monomer-monomer contact.

Conclusions: ATR-FTIR spectroscopy provides evidence of an opening for glycine betaine transport within the protein in switching from inactive to active state, which in turn affects monomer-monomer contacts. The excess helical content compared to the crystal structure is attributed to the N-terminus.

B 200 Crystal structure and cellular function of the B-repeat of the Listeria monocytogenes invasion protein InlB

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Background: The pathogenic bacterium Listeria monocytogenes employs the surface protein InlB to invade host cells by activation of the receptor tyrosine kinase Met. InlB is a multidomain protein. The N-terminal internalin domain binds to Met. The C-terminal GW domains attach InlB to the bacterial surface and bind host cell proteoglycans. Little is known about the B-repeat, the central domain of InlB. Homologous domains exist in most internalins from L. monocytogenes and many other bacterial proteins.

Observations: We determined the high resolution crystal structure of the InlB B-repeat. The structure is the first representative of this large domain family. The B-repeat has an ubiquitin-like beta-grasp fold but it also shares structural similarity with repeat domains of bacterial mucin-binding and adhesion proteins. This suggests that the B-repeat may be involved in protein-protein interactions and bind a host-cell receptor. Therefore, we tested three different InlB constructs for their ability to stimulate cell motility as a readout for Met activation and for their ability to bind Met. We compared the Met-binding internalin domain to InlB392 (internalin domain + B-repeat) and to the B-repeat alone. In wound healing and colony scatter assays with different primate and canine cell lines, InlB392 activated cell motility whereas the internalin domain or the B-repeat alone did not. Hence, both the internalin domain and the B-repeat are required to elicit a cellular response. In contrast, surface plasmon resonance experiments showed no difference between InlB392 and the internalin domain in their affinities for Met. The B-repeat alone did not bind to Met at all.

Conclusions: The B-repeat probably binds a yet unidentified host cell receptor that functions as co-receptor in Met activation. Taken together our results suggest that other members of the B-repeat domain family may function as spacer or receptor binding domain in bacterial extracellular multi-domain proteins.

B 201 Structural study of Werner syndrome DNA helicase

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Background: The RecQ helicases, a family of DNA-unwinding enzymes conserved from prokaryotes to mammals, play a key role in protecting the genome against deleterious changes. In humans, deficiencies in the members WRN (Werner syndrome protein) and BLM (Bloom syndrome protein) lead to the rare genetic disorders Werner and Bloom syndromes, respectively.

Observations: The diseases are commonly associated with cancer predisposition and/or accelerated aging, and cells derived from patients show high levels of genomic instability such as sister chromatid exchange and telomere shortening. Most RecQs including WRN and BLM share unique regions referred to as an

RecQ C-terminal (RQC) and a helicase-and-ribonuclease D/C-terminal (HRDC) domain. Recently we determined the 3D structures of a WRN RQC domain-dsDNA complex and a WRN HRDC domain. The structures showed how the RQC domain recognizes, binds, and unwinds DNA, establishing the first structural paradigm concerning the DNA structure-specific activities of the RecQ helicases toward recombination and replication intermediates. The beta-wing, an extended hairpin motif within the RQC domain, was used as a 'separating knife' to wedge between the first and second base pairs.

Conclusions: The results advance our understanding of the RecQ helicases in terms of the prevention of cancer and accelerated aging.

B 202 Quantitative modeling of the terminal differentiation of B cells and mechanisms of lymphomagenesis

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Background: Mature B-cell exit from germinal centers is controlled by a transcriptional regulatory module that integrates antigen and T cell signals and ultimately leads to terminal differentiation into memory B cells or plasma cells. Despite a compact structure, the module dynamics are highly complex due to the presence of several feedback loops and self-regulatory interactions, and understanding its dysregulation, frequently associated with lymphomagenesis, requires robust dynamical modeling techniques.

Observations: We present a quantitative kinetic model of three key gene regulators, BCL6, IRF4 and BLIMP, and use gene expression profile data from mature human B cells to determine appropriate model parameters. The model predicts the existence of two different hysteresis cycles associated to two different signaling pathways that can direct B cells through an irreversible transition towards a differentiated cellular state. By synthetically perturbing the interactions in this network, we can elucidate known mechanism of lymphomagenesis and suggest new candidate tumorigenic alterations, indicating that the model is a very valuable quantitative tool to simulate B cell exit from the germinal center under a variety of physiological and pathological conditions.

Conclusions: We have shown how cooperativity between these two different signaling pathways ensures the correct differentiation of germinal center B cells into terminally differentiated plasma cells, and we have suggested a number of additional tumorigenic alterations.

B 203 Specific alterations of the microRNA transcriptome, its target proteome, and global network structure in CRC after treatment with MAPKs inhibitors

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Background: The MAPK pathway has a master control role in different cancer-related biological processes as cell survival, migration, differentiation, apoptosis. This pathway also regulates the activity of many transcription factors, which control the expression and activity of miRNAs and the corresponding biosynthetic machinery. Based on these premises, we decided to investigate the miRNA transcriptome alterations after inhibiting the MAPK pathway in colorectal cancer (CRC).

Observations: By exploiting the TaqMan Low Density Array technology, we performed in biological triplicate the expression profile of 745 miRNAs in three different human CRC cell lines (Caco-2, HCT-116, SW620), before and 12 hours after a drug-induced block of the MAPK pathway: the biomolecular effects of three different inhibitors (FR180204, U0126, WAY265506) were separately analysed. By comparing profiles from each treated and control cell line for each treatment (two-class paired test), differentially expressed (DE) miRNAs were identified through Significance Analysis of Microarrays (SAM). Through this approach, we were able to pinpoint: (i) common DE miRNAs in all CRC cell lines after treatment with a specific MAPKs inhibitor; (ii) DE miRNAs in a single CRC cell line after treatment with all three MAPKs inhibitors. All of these DE miRNAs are regulated by MAPK signalling, directly or indirectly.

Conclusions: Our data demonstrate that miRNA regulation by MAPK signalling is cell line – and inhibitor – specific. Given that DE miRNAs and their molecular targets are involved in processes regulated by ERK signalling, they could be considered as new potential diagnostic biomarkers and therapeutic targets.

B 204 A synthetic genome approach to tag based protein function analysis in metazoans

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Background: The rapid technology developments of the last 10 years have had a dramatic impact on our ability to understand genome function at both DNA and RNA level. Unfortunately the discovery rate at protein level has not changed significantly since the late 1980s. The challenges in unraveling protein function are imposed by the inherent chemical and structural diversity in the protein world, which makes it hard to isolate or localize specific protein targets.
Observations: We have developed a genome wide tag based platform for rapid in vivo function analysis of any C. elegans protein of interest. Using high-throughput recombineering we systematically engineered a ‘synthetic genome’ collection of large genomic fragments caring fluorescent and affinity tagged alleles for each protein coding gene. Upon genome integration these large transgenes behave like additional alleles expressing a tagged version of the protein of interest, which can now be localized, biochemically purified or quantified using generic, reproducible and comparable tagged based assays. Because all cis acting elements are typically included in a construct of this size the endogenous regulation mediated by regulatory proteins and RNAs at the level of transcription, splicing, message turnover and translation are maintained and can reveal fine-grained expression pattern dynamics that are not possible with the traditional cDNA based transgenes or promoter reporter constructs. The transgenes have many additional applications, for example as reporters in loss of function studies. We are currently extending this approach to Drosophila and mammalian tissue culture cells.
Conclusions: The availability of the TransgeneOme resource opens the possibility to uncover the function of thousands of previously unstudied proteins, and would eventually allow us to build a systems wide atlas of protein localizations and interactions within the context of a multicellular model organism.

B 205 Protein ligand interaction studies of alpha-synuclein protein responsible for Parkinson’s disease and chemical compounds of Bacopa monneri (Brahmi)

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Background: Parkinson’s disease (PD) is a neurodegenerative disorder characterized by progressive loss of dopaminergic neurons of substantia nigra .It has been discovered that tyrosine hydroxylase (TH) converts L-dihydroxyphenylalanine to dopamine by an enzyme AADC (aromatic amino dicarboxylase). alpha-Syn(alpha synuclein),an over expressed protein in PD reduces AADC activity that may be responsible for lowering the level of dopamine. Therefore probably inhibiting the alpha-Syn may increase the AADC activity.
Observations: Bacopa monneri has been in use since early as a brain tonic to enhance memory development, however how the components of Bacopa monneri functions is yet unknown. In this paper an attempt has been made to establish the component’s activities insilico with alpha-Syn that is over expressed in PD. We found that among the screened four components, Wogonin interacts potentially with alpha-Syn and has passed all the properties of drug with drug likeliness 1.71, drug score 0.83, highly soluble, highly permeable, non mutagenic, non tumerogenic and non irritant.
Conclusions: Using structure based drug approach, the drug designed in this paper was non mutagenic, nonirritant, non tumerogenic and low molecular weight. The drug created thus has immense potential based on in silico parameters.

B 206 Structure and mechanism of antifungal action of the antimicrobial peptide papiliocin 2

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Background: Papiliocin 2 is a 36-residue antimicrobial peptide which was isolated from the swallowtail butterfly papilio xuthus larvae. This peptide was shown to contain potent antimicrobial activities against several human pathogenic bacterial and fungal strains, but its structure and exactly mechanism of action are unknown. We investigated the structure and mechanism of action towards human pathogenic fungi.
Observations: Circular dichroism analyses revealed that papiliocin 2 was disordered structure in water but folded to form a relatively high alpha-helical structure in membrane-mimetic HFIP, SDS, DPC and LPS Solutions. This finding is further verified by NMR spectroscopy. The tertiary structure of papiliocin 2 revealed that show a typical alpha-helical amphipathic structure in a micellar environment, a structural prerequisite in permeabilization of the bacterial membrane. To understand the antifungal mechanisms of papilocin 2, flow cytometric analysis using propidium iodide (PI) staining and 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence analysis were conducted against

Candida albicans cells. The results demonstrated that papiliocin 2 perturbed and disrupted the fungal plasma membrane. Furthermore, we confirmed that C. albicans cells treated with papiliocin 2 showed several diagnostic markers of yeast apoptosis, such as accumulation of intracellular ROS, phosphatidylserine exposure, active metacaspase, dissipation of the mitochondrial membrane potential. In addition, nuclear fragmentation, which is important marker of late stage apoptosis, was seen by DAPI and TUNEL assay.

Conclusions: Papiliocin 2 exerts its antifungal activity by a membrane-active mechanism, and leads to apoptosis in C. albicans through ROS accumulation and activating the metacaspase. These evidence strongly support antifungal property of papiliocin 2 by promoting apoptosisi in C. albicans.

B 207 Poorly soluble proteins are over-represented in neurodegenerative disease pathways

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Background: A fundamental requirement for life is that proteins remain soluble in the cell. When protein homeostasis fails to maintain solubility, toxic protein aggregates can overwhelm our natural defense systems, causing Alzheimer's, Parkinson's, and other diseases. In environments of compromised protein homeostasis, hundreds of proteins can aggregate. This problem is particularly serious given the recent observation that proteins are often expressed at levels at which they are only marginally soluble.

Observations: We used bioinformatics methods to determine how factors contributing to protein insolubility affect the proteome in health and disease. We found that functionally important proteins have stringent solubility requirements, whereas pathways involved in disease are populated by poorly soluble proteins. Two critical determinants of whether a species will be soluble are its chemical solubility, which we estimate with a predictor of aggregation propensity (the Zyggregator algorithm), and its abundance, which we estimate with mRNA expression. Essential E. coli proteins and E. coli and human proteins with more binary protein-protein interactions have, on average, a lower propensity to aggregate. This is true even at comparable levels of expression or disorder. We also identified cellular pathways at risk for disruption by misfolding. In a search of all KEGG pathways for over-representation of poorly soluble proteins, we identified the Alzheimer's, Parkinson's, and Huntington's disease pathways. Proteins in these pathways have, on average, higher expression levels than expected given their aggregation propensities. This is also true for the AlzGene, PDGene, and HDGene databases.

Conclusions: Functionally important proteins seem to have evolved to avoid misfolding and maintain core cellular activities. Other processes are disrupted in neurodegenerative diseases. Our results suggest that this is a dysfunction of susceptible cellular pathways composed of poorly soluble proteins.

B 208 A chemical proteomics approach for the detection and analysis of protein 3-nitrotyrosine

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Background: Protein tyrosine nitration is a posttranslational protein modification that is found in disease states such as Lung Cancer, cardiovascular disease and diseases associated with oxidative stress. Modified tyrosine residues have been reported to affect protein structure and function however current methods have experienced difficulties in isolating nitrated proteins for further mass spectrometric analysis due to lack of sensitivity and specificity.

Observations: A chemical proteomics approach has been developed that enables the direct probing of protein nitrotyrosines in clean and complex samples. The approach enables a highly specific conversion of 3-nitrotyrosine into 3-azidotyrosine through a series of polypeptide compatible reduction and diazotransfer chemical derivatisation steps. Using model amino acids, peptides, proteins and cell lysate samples, it was shown that 3-nitrotyrosine was selectively and covalently biotinylated with commercial alkyne biotin reagents via the Cu(I)-catalysed [3 + 2] cycloaddition reaction or phosphine-biotin via the Staudinger ligation. Tandem Mass spectrometry with electron transfer dissociation showed highly specific ligation on 3-nitrotyrosine and absence of non-specific ligation on lysine or arginine. Extracted Ion Chromatograms exhibited excellent yields in all conversion steps. Furthermore visualisation of 3-nitrotyrosine derivatised proteins in complex samples via strepavidin-HRP blotting was also demonstrated.

Conclusions: This methodology enables the previously incapable, specific enrichment of protein 3-nitro-tyrosines from complex samples. In future, bronchoalveolar lavage fluid and blood samples in smokers will be analysed to further elucidate tobacco smoke oxidative damage in lung tissue.

B 209 Heatless PCR: new technology and paradigm

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Background: PCR is the most widely used tool in biological and molecular investigations. Since it was first conceived, PCR require temperature controls to treat DNA. Although some progresses have been made in optimizing the heating steps, developing a PCR system that reduces heating temperature, use of reagents and time is required. Thus, improving the fidelity of the amplified DNA, avoiding damage of DNA, and to make it a more cost-effective technique, is necessary.

Observations: With the aim to renew the concept of DNA amplification we developed a new technology, able to detect and amplify DNA without using thermocycler, in a higher concentration and purity using less time (15-30 minutes) and reagents (50-65%), also preventing DNA damage, as compared to conventional PCR. The denoted technology is able to amplify DNA up to 4,5 fold the conventional PCR method. The positive results in developing this novel PCR was due to the use of paramagnetic

principles of the bonding dissociation of DNA. thus, different levels of micro currents based on voltage and/or amperages were used to run the PCR. A micro current of 900 mV and 450 mA was the best set of in order to have higher DNA amplification and purity within a short time (35 minutes). Effective DNA amplification was achieved in different types of DNA samples, including linear and coiled plasmids with a starting concentration of DNA of 5 ng/ul. Also, genomic DNA was successfully amplified. Amplification was confirmed by using cDNA technique. Efficacy of the new heatless PCR was also demonstrated by the successful use of the product obtained from the heatless PCR by digestion, and cell transformation methods.

Conclusions: The heatless PCR demonstrated to be highly efficient and cost-effective for amplification of DNA. It also brings further understanding not only on DNA, but perhaps it also sets up the beginning of a new biological paradigm based on paramagnetic effects on biological systems, including genetics.

Poster Abstracts Session C

Tuesday 13 September 12:30–14:00

C 001 Role of Sequoia, a Zn finger transcription factor, in photoreceptor axon targeting in Drosophila

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Background: Neuronal differentiation causes dramatic changes in cellular morphology, e.g. growing an axon to the correct synaptic regions in the brain. Several differentiation processes are intrinsically regulated by transcription factors coordinating interplay between cell fate and axon growth. Sequoia, a Zn finger transcription factor, affects axon and dendrite development in many Drosophila neuron types. We are interested in understanding its role in regulating axon targeting of photoreceptor neurons.

Observations: Sequoia was identified in eye specific mosaic screen designed to identify genes affecting photoreceptor axon targeting. R8 and R7 photoreceptors show consecutive peaks of elevated Sequoia expression, which correspond to their sequential target-layer innervation. Loss of Sequoia leads to down regulation of Ncad and Dlar and causes R7s to project to the R8 layer, whereas a prolonged expression in R8 induces over projection of their axons into the R7 layer. Interestingly, cross-talk mechanisms between neighboring R cell axons, in addition to the endogenous Sequoia level, seems to determine the final targeting position. To learn more about the downstream targets of Sequoia we are using ChIP-seq approach. The transcriptional targets identified can be then tested using genetic modifier screen to confirm their functional relevance in Sequoia regulated axonal targeting. We are also interested in functional characterization of Sequoia to understand which domains of Sequoia are responsible for its function.

Conclusions: Sequoia mediates photoreceptor axon targeting in a temporal fashion. Identification of Sequoia downstream targets would give a better understanding of Sequoia mediated photoreceptor axon targeting. This study would help us in understanding genetic regulation of neuronal circuit assembly.

C 002 Atypical intercellular bridges and short-term germ cell cysts in early oogenesis in the bat, Carollia perspicillata

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Background: In majority of animals the oogenesis begins with the synchronous mitotic divisions of the specific oogonial cell, termed the cystoblast. The divisions of the cystoblast lead to the formation of the cyst of sister cells (cystocytes) interconnected by intercellular bridges. The bridges are formed as a result of incomplete cytokineses and in many mammalian species become subsequently reinforced by characteristic electron-dense rims.

Observations: The ovaries of early embryos (40 days after fertilization) of Carollia perspicillata bat contain numerous germ-line cysts which are composed of 10 to 12 sister germ cells (cystocytes). The variability in the number of cystocytes within the cyst and between the cysts (that defies the Giardina rule) indicates that in this bat species the mitotic divisions of the cystoblast are asynchronous. The serial section analysis showed that cystocytes are interconnected by atypical, strongly elongated and short lived intercellular bridges lacking of electron-dense rim but rich in microtubule bundles and microfilaments. It seems very likely that these cytoskeletal components are the remnants of the midbody. During later stages of embryonic development (44-46 days after fertilization) the somatic cells penetrate the cyst, and their cytoplasmic projections separate individual oocytes. Separated oocytes surrounded by the single layer of somatic cells constitute the primary ovarian follicles.

Conclusions: The presence of germ-line cysts and intercellular bridges (although non canonical) in fetal ovaries of C. perspicillata indicate that the formation of germ-line cysts is the evolutionarily conserved phase in the development of the female gamete throughout the animal kingdom.

C 003 Coordination of patterning and growth in the spinal cord

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Background: In the developing spinal cord, several types of neuronal progenitors are specified and spatially arranged along the dorso-ventral axis in response to the morphogen gradient of Sonic hedgehog (Shh). This process of establishment of the neural tube pattern involves the transcriptional specification of progenitor identity, as well as proliferation and terminal differentiation. How these processes are coordinated is poorly understood.

Observations: To address this problem, we measured the spatiotemporal dynamics of gene expression, proliferation and differentiation rates during three days of development. We found that the sizes of distinct progenitor domains change over time at different rates. These differences are not due to differences in proliferation, since our measurements showed that the proliferation rate is spatially uniform. In contrast, the differentiation rate progressively increases in time and differs between progenitor types. Before the onset of differentiation, the changes in ventral domain sizes correlate with the increasing amplitude of the Shh gradient and occur faster than the tissue grows, implying that progenitor identity is actively specified in response to signaling. At later times, the change in domain sizes can be explained by the proliferation and differentiation of different progenitor types alone.

Conclusions: Our data suggests a 2-phase model of patterning: early domain size depends on switches of cell identity, but late only on proliferation and differentiation. We are now using this

approach to study conditions where growth or signaling is altered, and to compare mouse and chick neural tube patterning.

C 004 Cdc42 is required for the maintenance of oocyte fate in Drosophila

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Background: Cell polarity is essential in all living organisms for numerous developmental and cellular processes and depends on key players highly conserved between species. Small GTPases are pivotal proteins in cell communication. The small GTPase Cdc42 regulates actin dynamics through its effector proteins WASp and Arp2/3, but it has also been directly linked to polarity proteins and to cell polarity establishment and maintenance in several cell types and organisms.
Observations: The Drosophila oocyte provides an excellent model for studies of cell polarity. It develops in an egg-chamber consisting of the oocyte and 15 interconnected nurse cells. Although polarity proteins are needed for oocyte polarity maintenance, the only contribution of Cdc42 in oogenesis so far described is restricted to minor changes in actin protrusions in the nurse cells. We show that Cdc42 is indeed needed for oocyte polarity maintenance. Loss of Cdc42 in the germline leads to egg-chambers with 16 nurse cells, which results in arrest of oogenesis. Oocyte markers are first correctly localized, but do not display the typical polarized translocation within the oocyte and are lost at later stages. Although correctly localized, the levels of several other polarity proteins are reduced. In the germline Cdc42 itself localizes to the cytoplasm and at the anterior cortex of the young oocyte, which underlines its role in maintaining oocyte polarity.
Conclusions: We establish a role for Cdc42 in oocyte polarity. While most polarity mechanisms rely on interdependent regulation of polarity proteins, this mechanism is altered in the Drosophila oocyte, indicating that polarity regulation in stem cell derived tissues might differ from that in other tissues.

C 005 Quantification of molecular oscillations during mesoderm patterning using real-time imaging in mouse embryos

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Background: In vertebrates, the segmentation of paraxial mesoderm into somites, the precursors of vertebrae, involves the activity of a molecular oscillator, commonly referred to as segmentation clock. This oscillator operates in the yet unsegmented, presomitic mesoderm (PSM) and involves periodic activity of the Wnt, Notch and Fgf-signaling pathways. In order to determine fundamental oscillator properties, a quantification employing real-time imaging is required.
Observations: To this end, we developed and further modified a two-photon, fluorescent imaging setup that enables the direct quantification of molecular oscillations (period = 2.5hrs.) in developing mouse embryos. In addition, we are generating a series of novel mouse reporter lines for the quantification of oscillatory activity both at transcriptional level, but importantly also at the level of protein dynamics. We use homologous recombination in embryonic stem cells to introduce reporter molecules directly into the endogenous genomic locus of candidate genes, such as Axin2 (a bona fide Wnt-target gene).Combining these approaches, we address a basic feature of PSM-oscillations -- the phase-delay between neighboring cells. We characterize this phase-delay, and the resulting phase-waves along the PSM, and present our recent findings addressing the nature and mechanism underlying this particular synchronization.
Conclusions: Using two-photon, real-time imaging we quantify fundamental PSM-oscillator properties and discuss their role during mesoderm patterning in mouse embryos.

C 006 Formation of Germ Cell Clusters During Zebrafish Embryogenesis

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Background: Zebrafish germ cells (GCs) migrate towards the site where the gonad develops, forming two bilateral clusters by 24 hours post fertilization (hpf). In casanova (cas) mutants, which lack endoderm, GCs initially form two separate clusters. However, at around 24hpf, two clusters come close together and by 28hpf they fuse at the midline. In Drosophila 2 lipid phosphate phosphatases (wunens) repel GCs and this repulsion is necessary for GC dispersal and equal sorting into two gonads during migration.
Observations: To determine the basis of the observed GC phenotype in cas mutants, we first studied the GC specification and differentiation in these embryos, showing that those processes proceed normally. Knocking down of two of the zebrafish wunen homologs results in fusion of the germ cell clusters. These wunen homologs are expressed in the endoderm and endoderm development in the knocked-down embryos is not affected. Using a digital-scanned light sheet microscope the dynamics of GC cluster relative to the endoderm was investigated and we found that GCs upon touching the endoderm, sharply change their direction and proceed away from the endoderm.
Conclusions: We propose that these two wunen homologs function as a repulsive signal in cooperation with the endoderm and thereby maintaining the separation of the two germ cell clusters in zebrafish.

C 007 Adhesion and Cell Fate Specification: unraveling the interplay during embryonic development

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Background: During embryonic development, cell fate specification is often accompanied by profound changes in cell adhesion. These changes in adhesion are thought to facilitate progenitor cell segregation and assembly into distinct tissues. However, it is still unclear how cell fate specification and cell adhesion depend on each other: are changes in cell adhesion a mere secondary

consequence of cell fate specification or is cell adhesion also interfering with cell fate specification?

Observations: To address these questions, we are using gastrulating zebrafish embryos as an assay system as they allow concurrent analysis of cell fate specification and cell adhesion in live embryos. Making use of transgenic reporter fish lines, we are able to follow cell fate specification during the course of gastrulation. Furthermore, we are using a dual micropipette aspiration assay to characterize the adhesive properties of different progenitor cell types by measuring the de-adhesion force between individual cells. The combination of these tools allows us to correlate cell fate specification with changes in cell adhesion and, by interfering with cell fate specification and/or cell adhesion, determine their mutual dependencies.

Conclusions: Our preliminary findings suggest that embryonic cells considerably modulate their adhesive properties before being committed towards a specific cell fate. Currently, we are studying how these changes in cell adhesion impact on cell fate specification and vice versa.

C 008 Mechanical forces driving zebrafish epiboly

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Background: The molecular and cellular mechanisms by which tissues take shape are fundamental to many biological processes. While the genetic pathways controlling tissue morphogenesis have been intensively analyzed, its mechanical principles are poorly understood. An excellent assay system to study the biophysical basis of tissue morphogenesis is zebrafish gastrulation, where within a few hours major morphogenetic changes result in the formation of germ layers and the establishment of the body axis.

Observations: To obtain insight into the biophysical basis of tissue morphogenesis during gastrulation, we study enveloping cell layer (EVL) epiboly, the spreading of a squamous epithelium over the yolk cell. A circumferential actomyosin band within the yolk syncytial layer (YSL) has been proposed to act as a purse string pulling on the EVLmargin. However, direct evidence supporting this hypothesis has been missing. Using laser ablation to measure tension within the actomyosin band, we find an anisotropic tension distribution with highest tension parallel to the EVL margin. Notably, there is also tension perpendicular to the EVL margin, indicating that the actomyosin band is not free to constrict in this direction. To understand how anisotropic tension within the actomyosin band controls EVL epiboly, we have started with a hydrodynamic description of this process, modeling the actomyosin cortex as an active, viscoelastic gel. Initial quantifications of cortical flows within the EVL and YSL are consistent with predictions from our theory, supporting the general plausibility of our theoretical approach.

Conclusions: Based upon initial results, we propose a new mechanism for the actomyosin band in EVL epiboly: in addition to function as a geometry-dependent purse string, it exerts a friction-based pulling force. Future experiments will have to analyze the generation of this force and its contribution to epiboly.

C 009 Quantitative study of signal transduction by TGF-beta pathway during development

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Background: We aim at understanding how the TGF-beta (TGFb) signaling cascade allow the cell to integrate multiple, and contradictory signals. We have recently shown that smad2 and smad4 translocation into the nucleus in response to TGFb stimulation does not have the same dynamics and that target gene expression temporal profile coincides with the one of smad4 (Warmflash et al.), putting emphasis on the importance of following dynamics of both smad2 and smad4 to understand how cells read TGFb signals.

Observations: we use a microfluidic cell culture chip developed by the Quake Lab in Stanford University, allowing precise temporal control of extracellular environment combined with time-lapse fluorescence microscopy to follow the real time response of Smad2 and Smad4 to stimulation by TGFb ligands, by recording both the nuclear-translocation of the Smad transcription factors and subsequent gene expression. As a model system we use C2C12 cells transfected with fluorescent fusion Smad proteins. Consistently with dish experiments, continuous TGFb stimulation for 12h shows that smad2 remains in the nucleus as long as signaling is going on while smad4 has a more complex dynamics. With 1hour-pulses TGFb stimulations we measure rise and fall time of both smad2 and smad4, and ramps of TGFb concentration revealed that smad2 response is graded while smad4 has essentially an adaptive behavior. As the volume of the microfluidics culture chambers is very small, (40nL) secreted factors are much less diluted than in regular cell culture condition and the cell density can be precisely controlled in each chamber independently. This allows us to study the effect of cell density on smad dynamics.

Conclusions: Further developments, including correlation of history of smad response to expression of TGFb targets at the single cell level using immunofluorescence and combinatorial stimulation of the two branches of the pathway, should reveal how the smad cascade encodes extracellular information.

C 010 The transcription factor myocyte enhancer factor 2C acts as an inhibitor of endothelial cell sprouting

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Background: Angiogenesis, the new formation of blood vessels, is stimulated by the growth factor VEGF. We have previously defined by microarray analysis genes specifically up-regulated by VEGF, but not by the pro-inflammatory IL-1 or the more general growth factor EGF. Among the specifically VEGF-regulated transcription factors was MEF2C.

Observations: The selective induction of MEF2C by VEGF was confirmed by realtime RT-PCR and by Western blotting.

Induction of MEF2C by VEGF-A was mediated via VEGFR-2 not 1, since we found MEF2C to be inducible by VEGF-E but not PlGF. MEF2C was further induced by pro-angiogenic bFGF. To investigate function of MEF2C during angiogenesis a spheroid sprouting assay was performed following overexpression or dominant-negative inhibition of MEF2C in HUVECs. We found MEF2C effectively inhibited angiogenic sprouting and inhibition of MEF2C augmented sprout formation. In an in vitro wounding assay MEF2C overexpressing cells exhibited reduced migratory capacity. In contrast, proliferation appeared unaffected supporting a specific inhibitory effect on migration and sprouting. To define target genes of MEF2C potentially responsible for the inhibition, gene profiling of cells overexpressing MEF2C was performed. One of the most prominently induced genes was alpha-2-macroglobulin. A2M is an effective protease inhibitor and scavenger molecule for growth factors like VEGF or bFGF. Our data further show VEGF indeed up-regulates A2M at normoxic conditions, whereas its induction is largely reduced by hypoxia.
Conclusions: We therefore propose that MEF2C via up-regulation of A2M functions in a negative feed-back loop to prevent sprouting under normoxic conditions.

C 011 Simplet controls cell proliferation and gene transcription during zebrafish caudal fin regeneration and is a required for beta-catenin-dependent Wnt signal transduction during zebrafish embryogenesis

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Background: Morphogenesis requires the coordinated contribution of progenitor cells that proliferate and pattern. We show the gene simplet (smp) is required for cell proliferation and patterning during early embryogenesis and adult tissue regeneration: knockdown after amputation of the fin inhibits its regeneration, but increases shh expression and causes ectopic bone formation. Knockdown in the embryo truncates posterior structures. The mechanisms through which smp regulates these phenomena are unknown.
Observations: Morpholino knockdown of smp in zebrafish embryos reduced activation of transgenic Wnt-dependent reporters and of endogenous Wnt-target genes in vivo, and this effect was associated with the loss of nuclear localization of beta-catenin. Other signaling pathways were not affected. smp knockdown also prevented the induction of the Wnt-dependent reporters despite overexpression of wnt8 or a stabilized version of beta-catenin. However, smp did not inhibit reporter activation by Lef1 overexpression. Co-expression experiments showed that smp enhanced the activity of different members of the Wnt cascade, including beta-catenin. Expression of Smp-GFP in cultured cells and embryos showed its localization both in the nucleus and in the cytoplasm, and the nuclear localization of Smp-GFP coincided with the nuclear localization of endogenous beta-catenin. Smp contains a single nuclear localization signal (NLS) and mutation of this signal prevented the nuclear localization of Smp and of endogenous beta-catenin. Furthermore, loss of the NLS in Smp reduced Wnt signaling and phenocopied the loss-of-function wnt and smp phenotypes as well as prevented Wnt overexpression gain-of-function phenotypes.
Conclusions: These results demonstrate that smp is required beta-catenin-dependent Wnt signal transduction by regulating beta-catenin nuclear localization during early embryonic development and possibly during tissue regeneration.

C 012 Primitive endoderm differentiates via a three-step mechanism involving Nanog and RTK signalling during mouse preimplantation

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Background: Pre-implantation development in the mouse is characterised by the differentiation of two extraembryonic lineages – the trophectoderm and primitive endoderm (PrE) – and of a pluripotent cell population – the epiblast – that gives rise to the embryo proper. The epiblast cells are marked by Nanog whereas PrE cells are expressing Gata6, Gata4 and Sox17.
Observations: To analyse the function of Nanog in preimplantation embryos we have used KO mouse and a new method of in vivo RNAi by plasmid electroporation. We show that inactivation of Nanog in all the cells or in a subset of cell cell-autonomously activates Gata6 expression, priming the cells toward a PrE identity. However Nanog mutant embryos cannot activate the expression of Sox17 or Gata4 which are later markers of the PrE. We show here that Fgf4 expression is restricted to Nanog expressing cells and absent in Nanog mutant embryos. By administrating exogenous Fgf4 to cultured Nanog mutant embryos, were were able to rescue the expression of Sox17 and Gata4. This result demonstrates that Nanog cell autonomously activates Fgf4 which then activates Sox17 and Gata4 in a non cell-autonomous manner. Moreover, using Fgfr and Mek inhibitors in Nanog mutant embryos cultures at different time-windows, we were able to show that the expression of Gata6 first depends on a direct activation by the RTK pathway but subsequently is maintained only by the absence of Nanog.
Conclusions: Our results reveal a three-step mechanism for PrE differentiation: 1- Gata6 is directly activated by the RTK pathway, 2- Gata6 expression is maintained by the absence of Nanog and 3- Sox 17 and Gata4 are activated by both Gata6 and Fgf4 which is secreted by epiblast cells.

C 013 Characterization of the zebrafish ortholog of a human orphan protein

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Background: Several thousand human proteins lack functional characterization. Some of these proteins are conserved in the zebrafish, suggesting they have roles in vertebrate development and/or physiology. The CALIPHO group (Computer Analysis and Laboratory Investigation of Proteins of Human Origin) proposes to characterize such 'orphan' proteins using different biological tools, including the zebrafish model.
Observations: Using RT-PCR test we identified a small group of zebrafish genes that show increased post-maternal expression. To assess the potential developmental function of Cal110 -one of these uncharacterized proteins- morpholinos were injected into single cell zebrafish embryos. Early cell divisions were slowed in injected embryos, compared to control embryos. Moreover, in morphants, spindles are less stained and multi-nucleated cells were observed. At pharyngeal stages, Cal110 morpholino-injected embryos showed neural tube defects and a curved tail. Using in situ hybridization we detected Cal110 in the olfactory bulb and neural tube at 48 hpf. These results were recapitulated using transgenic reporter fish expressing EGFP under the control of the Cal110 promoter region. Transgenic embryos showed EGFP expression very specifically in ciliated cells. Cal110 has a C-terminal sequence that resembles the dimerization domain of PKA regulatory subunit. Injection of mRNA encoding this domain mimics the effects of Cal110 knock-down, whereas the mRNA mutated at two critical residues has no effect. This suggests that Cal110 is functional as a homodimer, and points towards potential binding partners.
Conclusions: All together these results show that Cal110 may be involved in cell division and cilia formation where microtubules are key molecules. Our study is an example of how the zebrafish can assist in the characterization of 'orphan' human proteins.

C 014 Unraveling the mechanism of Dead end protein function in maintenance of the zebrafish germline

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Background: The primordial germ cells (PGCs) of the zebrafish are specified early in development and express a specific set of molecules responsible for the establishment and maintenance of the germline and for the behavior of the germ cells. Many PGC-specific mRNAs are targeted by miRNA-controlled degradation in somatic tissues, while being protected in the germline. Identification of those protected RNAs should provide important clues concerning the control over germ cell fate and behavior.
Observations: We identified the RNA binding protein Dead end (Dnd) as major player in controlling mRNA stability and translatability, by direct binding to some of the known germ cell specific mRNAs like tdrd7, nanos1 and hub. Specifically, loss of Dead end results in degradation of those germ cell specific genes, culminating in severe defects in germ cell behavior and subsequent death of the PGCs. To identify additional mRNAs to which Dnd binds and are thus important for germline development, we have established an in vivo, co-immunoprecipitation-based scheme for isolating Dead end-bound endogenous mRNAs from sorted zebrafish PGCs. Combining this method with 'next generation' sequencing, we aim at identifying a large set of yet unknown Dnd targets, providing insights into the processes controlled by Dnd. In preliminary experiments we were able to demonstrate the efficiency of the method in isolating known Dnd targets.
Conclusions: The combination of in vivo experiments and in silico analysis will be utilized to identify the additional Dnd targets. Combined with functional analysis of the isolated mRNAs, we hope to gain new insights into processes like germline specification, maintenance and migration.

C 015 Behaviour of induced mammalian adipogenic stem cells (ASCs) after transplantation into mouse brain

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Background: Adipogenic stem cells (ASCs), incubated in medium with BDNF and 5-azacytidine as inducers, gained neural features. Neural induction of ASCs displayed expression of four neural genes: eno2, map2, beta-3-tub, and nestin. The induction effect of BDNF was mediated by receptor TrkB, mRNA of which was detected within initial ASCs population.
Observations: GFP expressing ASCs were induced with BDNF and 5-azacitidine for 3 days. The cells were injected into striatum of Black6 mice. By the 11th day after injection the control cells disappeared from the brain sections, whereas induced cells remained alive. These data suggest that the effect of BDNF accompanied with 5-azacytidine not only promotes neural differentiation of ASCs and increases the expression of neural genes, but also increases the survival of cells after transplantation into brain tissue. Immunohistochemical staining of sections containing the transplanted cells showed that induced by the developed method ASCs expressed dablkortin. The preliminary experiments on transplantation of induced cells into the mouse striatum after Endotelin I injection had shown that in the ischemic brain they behaved more actively, then control uninduced cells. In the former case the active migration of the induced cells into brain parenchyma occurred. The cells migrated mainly along the blood vessels, and there are also some free migrating cells. The induced cells survived in the ischemic brain up to the 14 days while uninduced cells were completely dead at that time.
Conclusions: ASCs induce nerve migration via BDNF and effect can be enhanced by cell exposure to differentiation mediumTransplantation studies demonstrated unusually prolonged

survival of induced cells transplanted into mouse brain, as well as high migration ability of the cells toward ischemic zone of brain.

C 016 Shh and Neo1 relationship during cerebellar development and tumorigenesis

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Background: The canonical Shh/Gli pathway plays multiples roles during embryonic development and adulthood. By using different genomic approaches, we have recently uncovered several new direct and indirect Shh targets. One of these targets, Neo1, classically known as a Netrin1 receptor and recently involved in many processes during Central Nervous System (CNS) development, has emerged as an interesting candidate.
Observations: Here, we used Chromatin Immunoprecipitation (ChIP) in mouse embryonic CNS and luciferase reporter assays to demonstrate in vivo and in vitro direct binding of Gli transcription factors to the neo1 promoter. Neo1 is expressed in CNS, namely rostrocaudal migratory stream, cortex, and cerebellum. In particular in the developing cerebellum, its expression is located in the proliferative outer External Germinal Layer (EGL). The Shh pathway activation seems to be relevant to drive and regulate Neo1 expression and, more importantly, to contribute thereby to GCP proliferation since EGL precursors that undergo active migration and differentiation do not longer express the Neo1 marker.
Conclusions: Taken together, our results show that Neogenin1 is regulated by the canonical Hh signaling in the CNS, and may play a prominent role in the development of medulloblastoma, an observation essential for improving anticancer pharmacology.

C 017 Bmp and Hedgehog signaling pathways converge to generate and expand/maintain hematopoietic stem cells in mouse embryo

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Background: In mouse midgestatation, the aorta-gonad-mesonephros (AGM) region generates the first hematopoietic stem cells (HSCs). Interactions between endoderm and mesoderm are required for hematopoietic induction. We hypothesize that endodermally-produced hedgehog (Hh) and BMP4 in mesenchymal tissues ventral to the aorta trigger the cascade of events leading to the induction/expansion of HSCs. This study aims to examine the molecular sequence/interface between these two pathways during hematopoiesis.
Observations: To identify the cells activated by BMP signaling, we used transgenic BMP-response element (BRE)-gfp mice, in which gfp expression is correlated with the activation of the BMP pathway. The affects of the addition of BMP4, Hh or both growth factors on HSC induction/growth was investigated in our ex vivo AGM explant model in serum free media. After explant culture, cells were flow cytometrically sorted based on BRE-gfp expression and transplanted into recipient irradiated mice. Preliminary data show that AGMs from E11 embryos exhibit significant hematopoietic reconstitution when treated with BMP4 compared to serum-free control, suggesting that BMP4 is required for maintenance and/or expansion of HSCs. In the presence of BMP4, all AGM derived HSCs are BRE-gfp expressing. The sorted BRE-gfp negative fraction does not reconstitute hematopoiesis. Moreover, culture of AGM with a combination of both BMP4 and Shh highly supports HSC maintenance and expansion in the E11 AGM. Interestingly, the premature induction of HSCs was observed at early E10. Gene expression analysis on highly purified hematopoietic, endothelial and mesenchymal cell populations is ongoing.
Conclusions: Serum-free culture allowed us to study affects of morphogens BMP4 and Hh on HSC development. Both pathways affect HSCs. Their actions appear to be additive and to converge at the HSC induction/expansion stages. Future studies will focus on molecular interfaces of the pathways in pre-HSCs and HSCs.

C 018 Investigating the role of Abnormal Spindle Protein Asp in neuroepithelial development

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Background: Mutations in ASPM (abnormal spindle-like microcephaly-associated) cause primary microcephaly in humans, a disorder characterized by reduced brain size without any other abnormalities outside the nervous system. The Drosophila homolog Asp, is a microtubule-associated protein, first identified by phenotypic characterization of a larval lethal mutation that display defects in spindle morphology, mitosis and meiosis, with neural progenitors arresting in a prometaphase-like state.
Observations: Here we use the larval Drosophila brain as a model system to ascertain the role of Asp during neural development. We have found that asp mutant larvae and pharate adults display reduced brain and head size, with extensive lost of cells in the optic lobe. Interestingly, this structure in the fly brain develops from neuroepithelia (NE) like the vertebrate nervous system, where symmetrically dividing epithelial cells expand the pool of neural progenitors, that later divide asymmetrically when neurogenesis begins. By characterization of the NE in asp mutant brains, we have identified a variety of defects including cell adhesion and polarity abnormalities that induce the extrusion of some NE cells to ectopic regions of the brain where they ultimately undergo apoptosis. Analysis of the dynamics of the protein in vivo showed that Asp displays a cell cycle-dependent localization. However, during mitosis, it specifically localizes to spindle microtubules and spindle poles. Remarkably, in the NE Asp also localizes to the apicolateral region in interphase cells where it possibly interacts with adhesion, polarity and/or other proteins that determine its specific function in NE development.
Conclusions: Overall, mutations in Asp impair expansion of progenitor cells, induce loss of tissue architecture and ultimately, impair neurogenesis. Our work suggests that apart from main-

tenance of spindle integrity, Asp has an additional role in the organization and maintenance of the NE during development.

C 019 PICK1 is a novel interactor of NEPH/nephrin proteins regulating cell recognition and morphogenesis

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Background: Diseases of the glomerular filter of the kidney are a leading cause of end-stage renal failure. A major constituent of this filter is the slit diaphragm, a highly specialized cell-cell contact of podocytes, the visceral epithelial cells of the glomerulus. The slit diaphragm plays a critical role for the size-selective filtration barrier of the kidney. The immunoglobulin superfamily proteins NEPH1-3 and nephrin contribute to the structure and the signalling capacity of the slit diaphragm.
Observations: Using yeast-two hybrid screens we identified the PDZ/BAR domain protein PICK1 as a novel interactor of NEPH/nephrin proteins. Here we provide further biochemical evidence for this interaction, as well as evidence for a role of PICK1 as regulator of the endocytosis of the NEPH/nephrin protein complex. NEPH/nephin proteins are evolutionarily highly conserved. In the fruit fly D. melanogaster as well as in the nematode C. elegans it has been shown, that this set of proteins is critically involved in developmental processes that require highly regulated cell-cell recognition and formation of cell-cell contacts. Among these processes in Drosophila is the development of the regular pattern of the fly eye. Our data demonstrate that RNAi mediated knock-down of PICK1 interferes with eye development in a way, that is highly reminiscent of the phenotype that occurs when interfering with NEPH/nephrin-like proteins. Thus, our findings suggest that NEPH/nephrin proteins and PICK1 are indeed involved in the same biological pathway.
Conclusions: We identified PICK1 as a novel interactor of NEPH/nephrin proteins. These proteins act together in eye development of the fly, a process that requires highly regulated cell-cell recognition and formation of cell-cell contacts. Further studies will have to clarify the role of PICK1 in the kidney.

C 020 Identification of the maternal transcription factor Glis1 as a novel reprogramming factor of somatic cells

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Background: Induced pluripotent stem cells (iPSC) are generated from somatic cells by the transgene expression of three transcription factors Oct3/4, Sox2, and Klf4 (OSK). iPSC technology in therapy is greatly expected, however, there are problems to be overcome before clinical application. We focused on three points; the low efficiency of iPSC generation, the tumorigenicity of the resulting iPSC, and the unclear molecular mechanisms underlying the reprogramming process.
Observations: In this study, we screened transcription factors to identify novel factors which promote reprogramming and accumulate knowledge of factors involved in reprogramming process. We evaluated a library consisting of 1,437 transcription factors for their ability to replace Klf4 or Oct3/4 during iPSC generation. We found that 18 factors can reproducibly replace Klf4 during iPSC generation; we failed to identify any factors that replaced Oct3/4. Among the 18 above factors, we analyzed Glis1 in detail. Glis1 is enriched in unfertilized oocytes and one cell-stage embryos. We found that Glis1, when expressed together with OSK, markedly enhanced the generation of iPSC. Moreover, Glis1 specifically promotes the generation of iPSC but not partially reprogrammed cells or transformed cells. In addition, the DNA microarray analyses revealed that Glis1 promotes multiple pro-reprogramming pathways, including Myc, Nanog, Lin28, Wnt, and Esrrb. Glis1 might thus be a link between reprogramming during iPSC generation and reprogramming after nuclear transfer.
Conclusions: We screened 1,437 transcription factors to identify novel reprogramming factors. The identification of Glis1 and other novel factors from the screening might be beneficial for understanding mechanisms that underlie iPSC generation and future application of iPSC technology.

C 021 Evolving pluripotency

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Background: Embryonic pluripotency in the mouse is established and maintained by a gene regulatory network under the control of a core set of transcription factors that include Oct4, Sox2 and Nanog. While this network is largely conserved in eutherian mammals, very little information is available regarding its evolutionary conservation in other vertebrates.
Observations: We have compared the embryonic pluripotency networks in mouse and chick by means of expression analysis in the pre-gastrulation chick embryo, genomic comparisons, and functional assays of pluripotency-related regulatory elements in ES cells and blastocysts. We find that multiple components of the network are either novel to mammals or have acquired novel expression domains in early developmental stages of the mouse. We also find that the downstream action of the mouse core pluripotency factors is largely mediated by genomic sequence elements non-conserved with chick. In the case of Fgf4, we find that elements driving expression in embryonic pluripotent cells have evolved by a small number of nucleotide changes that create novel binding sites for core factors.
Conclusions: Our results show that the network in charge of embryonic pluripotency is an evolutionary novelty of mammals that is related to the comparatively extended period during which mammalian embryonic cells need to be maintained in

an undetermined state prior to engaging in early differentiation events.

C 022 Analysis of the transcription factor network underlying neural tube patterning using a novel graphical visualization technique

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Background: During vertebrate neural tube development, the morphogen Sonic Hedgehog (Shh) induces five progenitor domains that generate distinct neuronal subtypes. These domains are distinguished by the expression of different combinations of transcription factors (TFs) embedded in a gene regulatory network (GRN). We aim to systematically define the transcriptional states corresponding to the progenitor domains and decipher the underlying GRN.
Observations: To accomplish this we have perturbed the GRN in vivo by exposing chick neural tube cells to different levels and durations of Shh signalling and assayed their transcriptomes. To define sets of co-regulated genes and identify patterns of gene expression, we have applied a non-linear dimensionality reduction technique, t-statistic Stochastic Neighbour Embedding (t-SNE), combined with a novel technique, we term 'nearest neighbour plots'. These approaches offer a visualization of gene expression relationships that provides a straightforward and intuitive means to explore and interrogate transcriptome data.
Conclusions: Together, we introduce a novel and simple way to analyse the large and complex data sets generated by high throughput gene expression studies. We have used this technique to start deciphering the transcription programme of progenitors in the neural tube.

C 023 Oct4 kinetics predict cell lineage patterning in the early mammalian embryo

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Background: Transcription factors are central to sustaining pluripotency, yet little is known about transcription factor dynamics in defining pluripotency in the early mammalian embryo. Here, we establish a fluorescence decay after photoactivation (FDAP) assay to quantitatively study the kinetic behavior of Oct4, a key transcription factor controlling pre-implantation development in the mouse embryo.
Observations: FDAP measurements reveal that each cell in a developing embryo shows one of two distinct Oct4 kinetics, before there are any morphologically distinguishable differences or outward signs of lineage patterning. The differences revealed by FDAP are due to differences in the accessibility of Oct4 to its DNA binding sites in the nucleus. Lineage tracing of the cells in the two distinct sub-populations demonstrates that the Oct4 kinetics predict lineages of the early embryo. Cells with slower Oct4 kinetics are more likely to give rise to the pluripotent cell lineage that contributes to the inner cell mass. Those with faster Oct4 kinetics contribute mostly to the extra-embryonic lineage.
Conclusions: Oct4 kinetics, rather than differences in expression levels, are identified as a predictive measure of developmental cell lineage patterning in early mouse embryos. The FDAP assay may offer an important tool for assessing the developmental potential of somatic cells reprogrammed into pluripotency.

C 024 Role of slow as molasses (slam) in membrane invagination in the Drosophila embryo

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Background: Drosophila slow as molasses (slam) is required for plasma membrane invagination and organisation of Rho signalling during cellularization. Slam localizes to the prospective site of invagination and to the tip of the invaginating membrane, the furrow canal (FC) as well as cytoplasmic particles during cellularisation. The dynamics of localisation is shown by both the protein and RNA, suggesting a function as a RNA-protein particle.
Observations: We have generated a slam deletion mutant lacking both the maternal and zygotic contribution. In contrast to the previously reported zygotic and partial loss-of-function mutants, we observed a complete lack of membrane invagination in the slam null mutants. Whereas Cadherin-GFP, RhoGEF2 and Patj did not accumulate at the prospective site of the furrow canal, Nullo, Dia and F-actin localisation was not obviously affected, suggesting redundent functions of Slam and Nullo in organisation of F-actin. To investigate how the site of invagination between adjacent nuclei is defined and how slam accumulates specifically at this site, we analysed the dynamics of GFP-Slam protein and slam-MS2 RNA in comparision to other markers of the furrow canal and by FRAP. We find that Slam is an early marker accumulating before F-actin and RhoGEF2. By FRAP we find that both protein and RNA show slow and incomplete exchange during cellularisation and before mitosis 14 in case of GFP-slam. During a short period of less than 5 min at the onset of interphase 14, however, both protein and RNA are completely exchanged within about 2 min.
Conclusions: Our data suggest that two redundant pathways triggered by Slam and Nullo control formation of the furrow. Analysis of slam dynamics indicate that membrane domains forming the furrow canal are defined within a narrow window at the onset of cellularisation when slam RNA and protein accumulate.

C 025 Mouse knockout of the cholesterogenic Cytochrome P450 Lanosterol 14-alpha demethylase (Cyp51) – animal model for Antley-Bixler syndrome

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Background: Antley-Bixler Syndrome (ABS) is a rare genetic disorder with distinctive malformations of the craniofacial area and additional skeletal abnormalities. Defects in demethylation of lanosterol catalysed by cholesterogenic enzyme Cytochrome P450 lanosterol 14-alpha demethylase (CYP51), appears to be the cause for some forms of ABS but the causative CYP51-ABS relationship has not yet been established.
Observations: To test whether Cyp51 is involved in development of ABS-like phenotype we developed a full knockout mouse model. Ablation of Cyp51 locus in knockout embryos was confirmed at the RNA and protein level resulting in accumulation of lanosterol and 24,25-dihydrolanosterol, immediate CYP51 enzyme substrates. Sterol intermediates downstream of CYP51 enzymatic step were not detected indicating a complete blockade of de novo cholesterol synthesis. Cyp51-deficient embryos exhibited several skeletal characteristics of ABS and died at E15 due to heart failure. Hearts of knockout embryos displayed several abnormalities including hypoplasia, ventricle septum, epicardial and vasculogenesis defects, suggesting important role of Cyp51 in embryogenesis with emphasis on heart development and coronary vessel formation. Alterations in sonic hedgehog and retinoic acid signaling pathways, involved in development of heart and skeleton, were identified as the most possible downstream molecular mechanism leading to ABS phenotype.
Conclusions: The Cyp51 knockout mouse model presented in this study resembles many features of ABS and can serve as an animal model for this syndrome. As our study also developed a conditional Cyp51lox allele this will enable future examination of tissue or time specific Cyp51 effects in development and disease.

C 026 Molecular Mechanisms of Primitive Endoderm Formation

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Background: The mouse embryo has three distinct cell populations at the late blastocyst stage; the extraembryonic trophectoderm (TE), and an inner cell mass comprising the pluripotent epiblast (EPI) and extraembryonic primitive endoderm (PE). The individual cells in the inner cell mass are thus separated into two populations with distinct developmental fates.
Observations: Since the PE lines one surface of the EPI, it is thought to have some role in the patterning of the EPI as development progresses. A number of transcription factors have been described to be involved in the specification and function of the PE. In this study, we look at one of these factors from the Sry-related family of genes, Sox17. Here, we use high throughput genomic methods on a differentiating cell line, F9, to provide us with a broad overview of the switch from pluripotent cell to differentiated PE as well as the role of Sox17 in this transition. At the same time, the employment of detailed studies such as RNAi knockdowns in the mouse embryo provides us with spatial and temporal resolution of its expression pattern as well as role in PE formation. We demonstrate that the absence of Sox17 results in the reduced capability of cells to differentiate towards PE in in vitro cell lines as well as influencing the sorting and numbers of PE cells in the mouse embryo in vivo.
Conclusions: These findings highlight the importance of Sox17 in PE formation at a stage earlier than the Sox17-null mouse. In addition, the data generated from single cell and genome-wide expression experiments have allowed for the selection of other interesting candidate genes involved in PE formation.

C 027 Interaction between Nodal-, FGF- and Integrin- signaling in progenitor cell fate specification during zebrafish gastrulation

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Background: Nodal-, FGF- and Integrin- signaling play key roles in progenitor cell fate specification during zebrafish gastrulation. However, it remains unclear how the spatiotemporal interaction between these pathways modulates their cell fate specifying activities.
Observations: To determine cross-regulation between the Nodal-, FGF- and Integrin signaling pathways, we first examined the expression level of integrin beta1 (itgb1) in Nodal- and FGF-signaling deficient embryos during gasrulation. We found that both Nodal- or FGF- signaling is required for expression of itgb1. To examine functional interactions between these pathways, we analyzed how Integrin is required for Nodal- and FGF- function. We found that Nodal-induced endoderm cell fate specification depends on itgb1 expression and that FGF-induced mesoderm specification is inhibited by itgb1. These results suggest that concurrent signaling by Integrins and Nodals/FGFs is required for induction of endoderm and inhibition of mesoderm cell fate specification during gastrulation.
Conclusions: Activation of Integrin- signaling in Nodal/FGF-induced mesendoderm progenitor cells triggers these cells to become endoderm instead of mesoderm. We are analyzing how spatiotemporally coordinated activation of Integrin- and Nodal/FGF- signaling regulates endoderm versus mesoderm fate specification.

C 028 Spalt mediates an evolutionarily conserved switch to fibrillar muscle fate in insects

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Background: Somatic muscles are composed of different muscle types with distinct contractile properties. How these different muscles are specified and molecularly constructed remains elusive. Similarly, insect muscles are not all the same. Many insect species possess asynchronous indirect flight muscles that enable fast and powerful wing oscillations. To achieve these specific parameters, flight muscles contain stretch-activated myofibrils that display a unique fibrillar morphology.
Observations: In a genome-wide muscle-specific RNAi screen we have identified the transcription factor spalt-major (salm) as a master regulator of fibrillar flight muscle development in Drosophila. Knockdown of salm results in the transformation of fibrillar flight muscles into tubular muscles, which are the synchronous Drosophila body wall muscles. We could show that Salm expression is induced specifically in the myoblasts that will form the indirect flight muscles by the transcription factor vestigial (vg). However, in contrast to vg, salm is not only required but also sufficient to induce the fibrillar muscle fate when expressed ectopically. Moreover, we performed microarray analyis of dissected muscles revealing that salm is responsible for all features characteristic to fibrillar flight muscles; in particular, it regulates the expression and splicing of various sarcomeric proteins that execute the fibrillar muscle fate. Finally, we disrupted spalt expression by RNAi in Tribolium, which similarly results in the transformation of fibrillar into tubular muscles demonstrating that Spalt's function in switching to fibrillar muscle fate is evolutionarily conserved.
Conclusions: We identified the transcription factor salm as a key regulator of fibrillar flight muscle fate, as salm is not only required but also sufficient to induce the fibrillar muscle program. Strikingly, this function of Spalt is conserved in insects over 120 million years of evolution.

C 029 Spatial Proteomics at the wing imaginal disc: Maintaining the Positional Information at the proteomics level

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Background: Position of cells and maintenance of their position by the presence of boundaries is a striking feature of many developmental systems. These phenomena in the Drosophila wing imaginal disc, for example, are poorly understood. A detailed and quantitative description of the proteome of these cells is important for a comprehensive understanding of the biological processes that drive patterning, growth and morphogenesis of the wing imaginal disc.
Observations: We developed and applied spatial proteomic technologies in order to gain a quantitative understanding of the wing imaginal disc at the protein level. By using genetically manipulated flies, expressing GFP in a wing disc compartment-specific manner, we were able to sort the cell populations based on their fluorescence via flow-cytometry and subsequently applied proteomics on these subsets of cells. This workflow enabled us to obtain the first compartment-specific proteomes of the wing disc and revealed positional-related protein profiles from this complex tissue. We then performed surface protein analysis of the wing disc tissue by using glycocapture enrichment methods and obtained an enhanced map of the expressed N-glycoproteins. Combining the two approaches has provided us with a number of candidate proteins to test for their involvement in compartment boundary maintenance. A possible mechanism to explore, for example, would be the contribution of the candidate protein in the transmission of the signal derived from mechanical tension that exists at the boundaries.
Conclusions: Our study provides the first detailed proteomics data associated with the position of cells in the tissue. Our positional information-based proteomics approach will shed light into how boundary maintenance is achieved and will help to model the processes that mediate wing disc development.

C 030 Retinoic acid regulates planar cell polarity signaling during neural tube closure in vertebrates

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Background: Retinoic acid (RA) is the active form of vitamin A, which place a major role in development by modulating differentiation, proliferation, apoptosis and cell migration. In the vertebrate embryo, the main source of RA is Aldh1A2 (Raldh2). Raldh2-/- embryos exhibit a short, limbless trunk, an open neural tube and die at midgestation due to severe cardiovascular defects. The molecular mechanism underlying the pathogenesis of neural tube closure in Raldh2-/- embryos is not known.
Observations: Here we show that Raldh2-/- mouse embryos exhibit a craniorachischisis-like (CRS) neural tube defect due to improper closure of the neural tube at the hindbrain-spinal cord junction. Whole mount in situ hybridisation experiments with probes directed against PCP core genes showed a dramatic down regulation of Vangl2 and Fzd3 in the hindbrain and spinal cord. Targeted inhibition of RA signaling in the neural ectoderm of Xenopus laevis embryos using a dominant negative retinoic

acid receptor (dn-RAR) construct also leads to an open neural tube defect, suggesting that the RA involvement in neural tube closure is conserved in vertebrates. Furthermore, blocking RA signaling with dn-RAR drastically abrogates the elongation of animal caps neuralized by over-expression of Brain Factor-2 (BF2). This indicates that RA is required for the Convergent Extension (CE) of neural epithelia in vertebrates.

Conclusions: Our data identify RA as the first long-range, non-Wnt signal required for PCP signaling and CE of neural epithelia in vertebrate embryos, and thus include Raldh2 into the exclusive list of genes (Vangl2, Ptk7, Celsr1, etc.) shown to generate a CRS phenotype when mutated.

C 031 Evaluation of the effects of STZ-induced diabetes on in vitro fertilization and early embryogenesis processes

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Background: Diabetes mellitus is a multisystemic disorder with high mortality/morbidity rates that effects on infertility. We aimed to investigate the effects of diabetes on germ cells and in vitro fertilization in experimental diabetic rat models.

Observations: Diabetic in vivo rat model was generated by applying 50 mg/kg streptozotocin. Precondition will be taken that the test animals are held in optimal conditions in the experimental surgical laboratories during the time (15 day) detrimental effects due to diabetes can evolve. The test animals will be divided into 4 groups: Group 1 (Control group, 20 animals: 10 female and 10 male) group of healthy animals; Group 2 (30 animals: 15 female and 15 male) group of diabetic female and healthy males; Group 3 (30 animals: 15 female and 15 male) group of healthy female and diabetic males; and Group 4 (30 animals: 15 female and 15 male) group of diabetic animals. After germ cells have been obtained from the test animals, the cells will be left in maturation media until they get ready for fertilization. Next to the in vitro fertilization procedure and the day assignment of the created embryo, early and late blastocyst stages was followed by light microscopy and cell adhesion molecule e-cadherin and gap junction protein Connexin 43 was evaluated immunohistochemically. We found that fertilization rates decreased in group 2 and 4, reaching blastocyst stage range also decreased in group 2, 3 and 4.

Conclusions: Connexin-43 expression decreased significantly in group 2, 3 and 4; e-cadherin expression decreased in groups 3 and 4 when compared control group. Decreased expressions of gap junction and cell adhesion proteins affects adversely in vitro fertilization and blastocyst success rate in diabetes.

C 032 Role of barricade in the regulation of Drosophila neural stem cells

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Background: Stem cells have the capacity to self-renew and generate complex lineages made of distinct cell types. To study how fate decisions are regulated in such lineages, we use the Drosophila larval neuroblast where the stem cell self-renews and produces intermediate neural progenitors (INPs) through asymmetric cell division. Newly generated INPs need to undergo lineage progression before they can generate terminally differentiated cells. The precise mechanisms underlying this progression remain unknown.

Observations: In order to identify all genes required for the progression of these lineages we conducted a genome-wide RNAi screen in the Drosophila larval neuroblast. One of the most interesting candidates that caused an increase of neuroblasts upon knockdown is the previously uncharacterized gene CG6049/ Barricade. Barricade is the orthologue of human Tat-SF1, a gene that has been involved in transcription elongation and splicing. It is particularly interesting because increasing evidence show that elongation control is a critical regulatory step of transcription.

Conclusions: We are interested in understanding how Barricade and transcription elongation regulate stem cell lineages.

C 033 Quercetin is a proteasome inhibitor and induces autophagy and inhibition of mTOR signalling

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Background: The bioflavonoid quercetin has long been known to exert anti-tumour effects, although the mechanisms remain unknown. Investigation of the potential interference of this antioxidant with the efficacy of cell stress-inducing anti-cancer drugs revealed extensive intracellular vacuolation induced by quercetin in epithelial cancer cells that eventually led to apoptosis. The underlying mechanisms have been investigated.

Observations: Application of quercetin to epithelial human cancer cells induced a marked intracellular vacuolation that led to cell cycle arrest and ensuing apoptosis. Accumulation of biomarkers of autophagy, including fluorescent autophagy markers and acidotropic dyes characterized these vacuoles as phagolysosomes. Prior to the formation of autophagosomes, an immediate and pronounced inhibition of the autophagy-controlling mTOR activity in quercetin-treated cancer cells occurred, accompanied by a marked reduction in the phosphorylation of the mTOR substrates 4E-BP1 and p70S6 kinase. Assessment of cellular proteasome activity revealed an effective and immediate inhibition of all three enzymatic activities of the proteasome by quercetin in cancer cells.

Conclusions: Proteasome inhibition by quercetin can be regarded as a major cause of quercetin-induced cancer cell death. Since proteasome inhibitors represent a highly effective group of anticancer drugs, these results suggest potential new applications for quercetin in cancer treatment.

C 034 Repression of STAT3, STAT5A and STAT5B expressions in the chronic myelogenous leukemia cell line K–562 with unmodified or chemically modified siRNAs and induction of apoptosis

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Background: STAT3, S5A, S5B that take part in JAK/STAT pathway are assigned in signal transduction and in the activation of genes whose expressions increase in malignancy with playing role in the development of leukemia. In the current study, transfection of anti-cancer target STATs specific modified/unmodified siRNAs to K562 cells for 12 days long, induction of apoptosis by supressing their expressions both at mRNA and protein levels and evaluating JAK/STAT pathway members expressional changes were aimed.
Observations: According to XTT results, the used siRNA concentrations were not cytotoxic. As for gene supression results at mRNA level; with anti-S3-FU/FC siRNA, STAT3 expression was reduced to 20,60% (p = 0.001) and 16,51% (p = 0.041) on 10th-12th days. Using anti-S5A-FU/FC siRNA, STAT5A expression was reduced to 45,26% (p = 0,041) and 23,75% (p = 0.00) on 8th-10th days, 17,33% with S5A-CHL-siRNA at day 12. STAT5B expression was reduced to 59,05% (p = 0,009), 44,47% (p = 0,005), 14,06% (p = 0,001) with S5B-FU/FC-siRNA on 8th-12th days. According to Western Blot results, while STATs expression levels were still strongly downregulated by modified siRNAs, unmodified siRNAs lost their efficiency on 10th-12th days. While apoptotic cell rate was high in modified siRNA transfected group at 72th-96th hours (29%-98%); unmodified siRNAs caused low rates of apoptosis (2%-12%) and no apoptosis was seen after 96 hours. According to array results, when STAT3 was supressed, A2M and CEBPB expressions were decreased; when STAT5A was supressed while STAM, SOCS4 expressions were decreased; SOCS1 was increased. Supression of STAT5B resulted with IL20, STAM, SOCS4, SOCS5 expressional downregulation since PIAS2 was upregulated.
Conclusions: STATs were supressed at mRNA/protein levels, leukemic cell apoptosis was induced, modified siRNAs were found to be more effective in long term gene silencing, gene expression profiles of the JAK/STAT pathway components were evaluated and new candidate target genes were identified for CML therapy.

C 035 Quantification of mesenchymal stem cell differentiation by electrochemical impedance sensing

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Background: Because of their mesodermal differentiation potential mesenchymal stem cells (MSCs) are a model system for the investigation of cell differentiation processes. We established electrochemical impedance sensing in live cell chips which might help to identify of new biomarkers relevant for cell differentiation. It also has relevance for the quality control of MSCs for innovative cell therapies approaches and for risk assessment in cancer research (Angstmann et al. (2011) Cytotherapy, in press).
Observations: Impedance profiles were recorded with MSCs derived from bone marrow and adipose tissue, either non-induced or induced for osteogenesis or adipogenesis. These were correlated to differentiation markers assessed by qRT-PCR and western blot.The impedance profiles of osteogenic induced MSCs revealed an initially rapid and continuous rise corresponding to the formation of mineralised matrix. Conversely, adipogenic induction caused shallower initial slopes and eventually declining profiles, corresponding to more compact, adipocyte-like cells with numerous lipid vacuoles. Importantly, impedance recordings correlated well to the extent of differentiation evaluated by histochemical staining, protein and RNA analysis and were capable of discriminating well differentiating from poor differentiating MSCs. The same assays now are used to characterize stemness in the stem cell niche vs. migration and differentiation on extracellular matrix molecules. By simulating signaling processes it is not only possible to investigate differentiation pathways on the molecular level, but also to optimize cells for therapeutic applications and estimate their tumorigenic potential upon different influences.
Conclusions: Adhesion to the extracellular matrix is an important parameter during MSC differentiation. Impedance profiling offers a basis for standardized real time, non-invasive screening of MSC properties. Ongoing experiments with different substrates might help to identify signals relevant for MSC behavior.

C 036 A double-stranded DNA virus tool to deal with autophagy and cell death

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Background: Autophagy is a relevant cellular defense mechanism that directly eliminates intracellular pathogens and has a crucial role for innate and adaptive immune responses. Some viruses have developed tools to counteract this cellular response. A179L, the viral Bcl2 homolog (vBcl2) of African swine fever virus (ASFV), interacts with pro-apoptotic Bcl2 family proteins to inhibit apoptosis.
Observations: We found that this vBcl2 homolog is also able to manipulate autophagy by interacting with the Bcl2 family protein Beclin 1 through its BH3 homology domain. At subcellular level, A179L colocalized with Beclin 1 at mitochondria and the endoplasmic reticulum. Both A179L transient expression and virus infection inhibited autophagosome formation in cells. Autophagy inhibition might be crucial to avoid degrada-

tion of newly assembled virions by lysosomal fusion. Moreover, ASFV did not induce autophagy in infected cells, as analyzed by LC3 activation and/or autophagosome formation. The virus uses the endocytic pathway to enter and traffic infected cells at early infection stages until it builds its replication site near the nucleus. Surprisingly, starvation conditions and rapamycin-induced autophagy prior to or at the time of infection severely decreased infected cell numbers, and few cells harboring viral factories were found in cultured cells. Therefore, these results suggest that some components of the autophagic pathway act as host factors in the early viral replicative cycle.

Conclusions: Some viruses can benefit autophagy and membranous supplies provided from early autophagy induction while others should inhibit it. For ASFV is essential to keep autophagy-related components in a non-activated status for a successful infection through the interaction of vBcl2 A179L and Beclin 1.

C 037 The effect of p38 pathways on FHIT expression in DNA damage induced apoptosis

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Background: Cancer is the most common cause of death in the world. The previous studies have shown the loss of FHIT (Fragile histidine triad)gene in many human cancers. FHIT is a tumor suppressor gene and its function is to induce apoptosis. FHIT signaling pathway and mechanisms involved in the tumor suppressor activity is unknown.

Observations: In present study, we investigated the signaling cross talk of p-p38 on FHIT expression in cancer and noncancerous cells. MCF-7 and HEK293 FT cells were cultured and DNA damage induced cell death was initiated by etoposide. Protein expression was evaluated by western blotting technique. Pretreatment of cells were done with SB202228 (p38 inhibitor) and blocking FHIT expression using siRNA. Cell proliferation was determined by MTT assay.

Conclusions: P38 inhibitor had no effect on FHIT expression but FHIT siRNA transfection caused decrease of p-p38 activity, this reduction was significant in HEK293FT. This finding may provide the identification of the exact signaling pathway of FHIT and helps finding possible targets for cancer therapy.

C 038 Autophagy and apoptosis are responsible for the remodeling of silkworm larval tissues

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Background: In Lepidoptera the larval midgut consists of a monolayered epithelium formed by columnar, goblet and stem cells. During metamorphosis stem cells give rise to the midgut of the adult, while the larval midgut epithelium degenerates. At present there is not yet a clear understanding of the death mechanisms that lead to the disappearance of these cells. The aim of this work is to analyze the remodeling process in midgut tissues during fifth larval instar and pupation in the silkworm, Bombyx mori.

Observations: Light and electron microscopy observations revealed that the midgut epithelium starts to degenerate since the spinning stage. Columnar and goblet cells are sloughed into the lumen of the new pupal midgut, where they undergo progressive degeneration until disappearance. A detailed cellular and molecular analysis was performed to assess the occurrence of apoptosis and autophagy in larval midgut cells. We analyzed the presence of ultrastructural features typical of these two processes and performed Real-time quantification of autophagy and apoptosis-related genes. In addition DNA fragmentation and caspase activation were evaluated to detect apoptosis, while intervention of autophagy was assessed through Atg8 processing, as well as measure of lysosomal enzyme activity. Both autophagic and apoptotic markers showed appreciable changes at different developmental stages of the midgut renewal process, mainly in relation to ecdysone levels in the hemocoel.

Conclusions: The degeneration of the larval midgut cells is a gradual process due to the concerted action of apoptosis and autophagy. Autophagy intervenes just after the larva ceases to feed as a pro-survival process to gain energy from larval cells that are no more useful to the animal.

C 039 Bcl-2 functionally compensates for down-regulation of ubiquitin ligase CHIP in breast cancer cells and establishes drug resistance

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Background: Carboxy terminus of Hsp70p-Interacting Protein (CHIP) is a ubiquitin E3 ligase which maintains cellular homeostasis by regulating degradation of misfolded and excess proteins. Previously we have discovered that CHIP suppresses breast cancer cell growth and metastasis. Since it is found that intensity of CHIP expression in breast cancer shows positive correlation with survival after chemotherapy, we hypothesize that CHIP represses formation of drug resistance of breast cancer cells.

Observations: In this study, we find that down-regulation of CHIP induces resistance to anti-cancer agents in breast cancer. We generated CHIP knocked-down (KD) cells and found that CHIP KD cells were resistant to various anti-cancer agents both in vitro and in vivo. By analyzing protein expression profile we identified Bcl-2 among proteins which were up-regulated in CHIP KD breast cancer cells. Since Bcl-2 is known as an anti-apoptotic protein, we hypothesized that up-regulation of Bcl-2

protects breast cancer cells from anti-cancer agent-induced apoptosis. As drug sensitivity is recovered by Bcl-2 knock-down in CHIP KD cells, up-regulation of Bcl-2 is implicated to be responsible for the formation of drug resistance. To further understand the mechanism in which downregulation of CHIP resulted in increased Bcl-2 expression, we analyzed cells at the onset of CHIP knock down. Unexpectedly at the onset of CHIP knock down Bcl-2 protein nor mRNA were not increased but most cells showed apoptosis. From these observations, we hypothesize that apoptotic state caused by downregulation of CHIP leads clonal selection in which cells expressing high level of Bcl-2 can survive selectively.

Conclusions: It is shown that downregulation of CHIP in breast cancer leads resistance to various anti-cancer agents via up-regulation of Bcl-2, which is resulted from clonal selection of cells that highly express Bcl-2. The mechanism of the clonal selection will be discussed.

C 040 Polycomb protein EZH2 controls Rhabdomyosarcoma formation

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Background: Rhabdomyosarcoma (RMS) is a pediatric tumor arising from muscle precursor cells. RMS cells fail to complete both the differentiation program and the cell cycle arrest, resulting in uncontrolled proliferation and incomplete myogenesis. RMS expresses several markers of early muscle differentiation, as MyoD, but displays limited differentiated cells which do not form myotubes. The aim of the study was to analyze the role of EZH2, member of Polycomb Repressive Complex 2, in RMS formation.

Observations: EZH2 is involved in various differentiation processes and in cancer progression. Protein levels of differentiation markers and EZH2 were examined by Immunoblot in myoblasts and in RMS cells under differentiation conditions for 96h. Pellets were collected every 24h. Compared to myoblasts, RMS cells showed lower levels of myogenesis markers and higher levels of EZH2. In order to understand if EZH2 was responsible of muscle-specific genes silencing, a stable EZH2 knockdown RMS cell line was generated. Analysis of mRNA and protein expression by Real Time PCR and Immunoblot showed that EZH2 silencing results in a significant increase of myogenesis markers levels. Cdk9 is a co-activator of MyoD fundamental for the completion of myogenesis. In order to detect a potential inhibition EZH2-dependent of Cdk9 activity, Cdk9-mediated RNA polymerase II CTD phosphorylation was investigated in both wild type and EZH2 knockdown cells. The analysis by Immunoblot and ChIP showed that EZH2 inhibits the RNA polymerase II phosphorylation. Moreover, Co-IP analysis of protein-protein binding, by Co-IP, demonstrated interaction between CDK9 and EZH2, suggesting a role of EZH2 in kinase activity inhibition.

Conclusions: These data suggest that EZH2 expression may participate in RMS formation and progression. Clarify the epigenetic alterations of muscle-specific genes and the mechanism of myogenesis inhibition will be extremely useful to find new therapeutic agents able to induce the reversion of RMS phenotype.

C 041 (Glyco)proteomic analysis of induced pluripotent stem cells during differentiation

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Background: Differentiation of stem cells highly depends on cell-cell interactions mediated by surface glycoproteins, whose glycosylation patterns are known to change upon differentiation. This study aims at the understanding of these changes in order to identify novel glycan biomarkers. The availability of reliable sets of selection markers for specific cell types would be very useful for future therapeutic applications of stem cell derived tissue.

Observations: Differentiation was induced by cultivation of human induced pluripotent stem cells (iPSCs) in differentiation medium causing the cells to aggregate into embryoid bodies (EBs). From these EBs cells can differentiate into various cell types of all three embryonic germ layers. Surface proteins were purified by a biotin-avidin-based procedure and analysed by 2-dimensional difference gel electrophoresis (2-D DIGE). Differences in the protein pattern of iPSCs and EBs occurred as early as one day after EB formation. The observed changes in post-translational modifications were most likely caused by changes in glycosylation. Mass spectrometry (MS) showed that surface protein preparations still contained contaminations from highly abundant cytosolic proteins. In order to remove these contaminations and to enrich for specifically glycosylated proteins lectin immunoprecipitation (IP) was applied. For example, sialylated glycoproteins were purified with LFA, a sialic acid binding lectin. 2-D DIGE analysis showed a very different spot pattern than non lectin-purified surface proteins. This indicates that lectin IP can help to enrich for underrepresented proteins and to identify novel biomarkers.

Conclusions: Methods for purification and analysis of iPSC (glyco)proteins have been developed. Upon differentiation significant (glyco)proteomic changes occurred. Further analysis was performed by lectin IP. This helps to break down the vast diversity of glycoproteins and make subsets accessible for MS.

C 042 DNA damage leads to senescence and astrocytic differentiation of neural stem cells

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Background: Tissue homeostasis failure and ageing are thought to be the manifestations of the loss of somatic stem cell activity, which in turn is considered to happen due to accumulation of DNA damage in stem cells. Upon DNA damage, DNA damage response (DDR) pathways, studied predominantly in tumour cell lines and fibroblasts, are known to cause cell cycle arrest, apoptosis or senescence. Here, we addressed the role of DDR in adherently growing embryonic stem cell-derived murine neural stem cells (NSC).

Observations: We have discovered that DNA damage by X-rays leads in these cells to a swift cell cycle arrest and senescence, which is strikingly associated with transcriptional downregulation of DDR, a feature we revealed to be common to terminally differentiated astrocytes. Upon irradiation, NSC also lose the expression of their stem cell markers such as Nestin, Sox2 and Pax6. Moreover, irradiated NSC acquire the expression of typical astrocyte markers such as GFAP and S100b, while still being cultured in NSC proliferation medium, without addition of any known astrocyte differentiation stimuli. We also studied the mechanisms behind this phenomenon. Strikingly, inhibition of key DDR factors such as ATM and p53 in fact profoundly promotes the astrocytic differentiation of X-ray irradiated NSC. Instead, the onset of astrocytic differentiation is strongly dependent on the activation of JAK/STAT and BMP/SMAD signaling pathways. Their inhibition prevents upregulation of GFAP, yet does not allow bypass of senescence or cell cycle re-entry of irradiated NSC.
Conclusions: We propose a two-stage model of DNA damage effect on NSC: 1) rapid cell cycle arrest and senescence, associated with the loss of stem cell features 2) acquisition of astrocyte-similar characteristics. This may account for somatic stem cells failure in pathological conditions of genotoxic insult.

C 043 Molecular and cellular networking underlying the ex-vivo expansion of human mesenchymal stem cells (MSCs) revealed by 2-DE based quantitative proteomics

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Background: Human mesenchymal stem cells (MSCs) have become one of the most promising candidates for tissue engineering and regenerative medicine applications. In order to meet the clinically relevant cell dosage, a fast ex-vivo expansion process is required, which can result in loss of MSCs proliferative potential. Quantitative proteomics based on two-dimensional gel electrophoresis (2-DE) can contribute to elucidate the mechanisms underlying MSCs expansion and differentiation governed by protein networks.
Observations: Bone marrow MSCs were expanded and collected at P3 (day 7) and P7 (day 35) passages. Immunophenotypic characterization and multilineage differentiation assessment were performed at the end of each passage. P3 and P7 proteomes were separated by IEF/SDS-PAGE 2-DE and stained with fluorescent dye. A total of 79 proteins whose content was statistically different in P3 and P7 were identified by mass spectrometry. Of these, 30 are unique protein forms while the remaining correspond to multiple isoforms, namely the cytoskeleton components beta-actin (7) and vimentin (24) presumably resulting from alternative splicing or post-translational modification. The differently expressed proteins belong to the categories: Structural Components and Cellular Cytoskeleton, Folding and Stress Response Proteins, Energy Metabolism and Apoptosis. The proteins with an increased content in P7 compared to P3 belong to the network 'Cell death and skeletal and muscular disorders' while 'Gene expression, cell death, cellular growth and proliferation' include the less expressed proteins. The transcription factors MAZ, SP1 and AP1 are the suggested regulators of genes encoding the proteins present in the dataset.
Conclusions: This study provided insights into human MSCs culture dynamics suggesting that the loss of MSCs proliferative potential relates with increased apoptosis and decreased cell proliferation, increasing our understanding of the networking underlying ex-vivo expanded MSCs for therapeutic applications.

C 044 Comparison of modulation of membrane permeability transition pore opening of heart mitochondria in normal and Type II diabetic rats using Manihot esculenta leaf extract

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Background: The aim of this study is to compare the modulatory effect of M. esculenta leaf extract on the mitochondrial membrane of the heart in normal and diabetic conditions, using Type2 diabetes rat model. The model was developed by the combination of high fat diet and low dose streptozotocin (35 mg/kg). Diabetes is a lifestyle disease with many complications that affect the kidneys and heart of subjects by free radical generation, oxidative stress and lipid peroxidation with toxic products in the blood.
Observations: Using the method of Mela and Seitz (1969) with modifications, low ionic mitochondria were prepared using various buffers made of mannitol, sucrose, HEPES, EGTA, BSA. Teflon homogeniser was used to grind the heart tissue in 9% of the appropriate buffer. The method of Lapidus and Sokolove (1993) was used for the swelling assay with CaCl2 as inducer and Spermine as inhibitor. The mitochondrial was incubated in the buffer containing rotenone for 3-3.5 minutes before being energized by Sodium succinate. Decrease in absorbance was recorded over a period of 12 minutes using a UV-Spectrophotometer at 540nm. The crude ethanolic extract dissolved in DMSO, was tested in four concentrations of 200, 600, 1000 and 1400 microgram/ml. 600 microgram/ml (29.63%) and 1000 microgram/ml (42.60%) significantly induced ($p < 0.05$), while 200 microgram/ml (35.94%) and 1400 microgram/ml (51.56%) inhibited pore opening compared to standard inhibitor, Spermine (20.3%). In normal rats however, 200 microgram/ml (102.78%) and 1000 microgram/ml (69.44%) showed significant induction, while 600 microgram/ml (33.85%)and 1400 microgram/ml (76.92%) showed significant inhibition ($p < 0.05$) in a concentration-dependent manner. Inhibition was found to be concentration-dependent.

Conclusions: The extract showed antagonistic effect when compared with results obtained for DMSO only in both normal and diabetic rats. Standardized dosage of the extract may be useful in the managment of diabetic cardiomyopathy and other mitochondria-related complications associated with Type2 diabetes mellitus.

C 045 AATF acts as a phosphorylation-dependent switch to modulate the quality of the p53 response

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Background: In response to DNA damage cells activate a complex signaling network to prevent further cell cycle progression. Activation of this signaling network, which is collectively referred to as the DNA damage response (DDR), provides time for DNA repair, recruits repair machinery to the sites of genotoxic damage, or, if the lesions are beyond repair capacity, leads to the activation of additional pathways mediating apoptosis.
Observations: We and other groups have identified AATF, a transcriptional regulator and RNA pol II binding protein, as a key protein involved in DDR pathways. Here we show that in resting cells AATF resides in the cytoplasm where it interacts with Myosin Regulatory Light Chain 3 (MRLC3). After genotoxic stress this cytoplasmic MRLC3:AATF protein complex is disrupted through phosphorylation of AATF on Thr-366 by the p38MAPK activated checkpoint kinase MK2 causing subsequent nuclear re-localization of AATF. In the nucleus, AATF binds to the PUMA promoter region to repress p53-driven Puma expression and hence negatively regulates the p53 response towards apoptosis. AATF-depleted xenograft tumors display a significantly increased doxorubicin sensitivity, compared to control tumors expressing an unspecific shRNA. The protective effect of nuclear AATF signaling is also reflected by a panel of human lung cancer cell lines, which were stratified solely based on their p53 status and AATF copy number. In these experiments focal copy number gains in the AATF locus were associated with a significant protection from cisplatin-induced apoptosis in p53-proficient cell lines only.
Conclusions: Here we show that AATF acts as a phosphorylation dependent molecular switch to modulate the p53 response after DNA-damage, regulated by p38MAPK/MK2 kinase signaling. Hence this pathway emerges as an attractive drug target for DNA-damage-sensitizing therapeutic regimens.

C 046 Detection of 5-hydroxymethylcytosine in genomic DNA of breast cancer samples

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Background: Analysis of 5-methylcytosine (5-mC) patterns in DNA for the identification of epigenetic dysregulation in cancer is well established whereas elucidation of the role of 5-hydroxymethylcytosine (5-hmC) has yet to be unravelled. Tet proteins convert 5-mC, usually found in CpG context, to 5-hmC which is assumed to be involved in gene expression regulation and might prove to be intriguing for cancer diagnostics. In this study we aimed at the detection of 5-hmC in breast cancer DNA.
Observations: DNA samples from breast cancer tissue ($n = 6$) and blood samples from healthy patients ($n = 6$) were glucosylated with 5-hydroxymethylcytosine glucosyltransferase and digested with the restriction endonuclease MspI. As control reaction each sample was treated with MspI without prior glucosylation. Thus, the selective glucosylation of 5-hmC to glucosyl-5-hydroxymethylcytosine (glu-5-hmC) followed by digestion with glucosyl sensitive restriction endonucleases (GSREs), like MspI, enabled us to distinguish between 5-hmC and methylated or unmodified cytosine. After digestion the samples were analysed for 5-hmC in 325 gene loci by a targeted microarray, Through this approach the detection of three potential gene loci with a significant difference ($P < 0.05$) between the glucosylated samples and the control group was achieved. These markers, LZTS1, SOCS1 and TP53 were validated with qPCR. Thereof LZTS1 as 5-hmC marker could be confirmed in the DNA of all six breast cancer samples ($P < 0.001$) but in none of the blood samples.
Conclusions: Our detection of 5-hmC DNA in cancerous tissue might contribute to understand possible functions of 5-hmc as an epigenetic regulator thus setting aims for cancer diagnostics. These preliminary results suggest to perform further hmC screenings for elucidation of the role of 5-hmC in cancer.

C 047 Synthetic lethality screen to develop personalized lung cancer therapy

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Background: Lung cancer is the leading cause of cancer-related deaths worldwide. Large scale genome sequencing of lung tumor samples has shed light upon the mutation repertoire most frequently found in lung cancer patients. It has also revealed that some mutations co-exist in the same tumor concurrently with the others, whereas some other mutations are mutually exclusive.
Observations: To mimic the situation present in patients also in the cell culture system, we immortalize human primary lung epithelial cells by knocking down p53 and introducing TERT. These immortalized cells are then transformed with one of the mutually exclusive key mutations, namely with the mutant EGFR, mutant KRAS, or the knock-down of LKB1 or NF1. Each of these four background cell lines is transduced with one of other 25 most frequent alterations found in lung cancer, thus rendering a total of 100 isogenic cell lines carrying the mutation profiles found in patients. As most of the affected genes cannot be directly targeted by a drug, we exploit the fact that cancer cells become dependent on other genes (non-oncogenes) and that perturbation of these genes in the presence of cancer-associated mutation results in cell death (so called synthetic lethality). Such synthetic lethal interactions are sought by screening these 100 isogenic cell lines against a panel of drugs that are being clinically tested in lung cancer patients or against inhibitors of

clinically relevant groups of proteins like kinases and chromatin-modifying enzymes.

Conclusions: Identified synthetic lethal interaction partners are used to develop personalized therapy for the patients carrying the respective mutation (or set of mutations). In addition, mechanisms of drug resistance are identified.

C 048 Detection of aberrant DNA methylation using cell free serum DNA in breast cancer patients for minimal-invasive diagnostics

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Background: Changes in DNA-methylation patterns are an early event in cancer development. Many CpG island associated promoter regions of tumor suppressor genes or oncogenes change their methylation status due to growth advantages of the cancer cell. In this study we investigated epigenetic aberrations in cell free serum DNA from breast cancer patients for elucidation of potential biomarkers for minimal invasive diagnostics.

Observations: DNA methylation analyses were performed by methylation sensitive restriction digestion followed by qPCR detection. Methylation analyses of 360 gene targets by a high throughput micro fluidic qPCR device (Biomark Fluidigm) elucidated aberrantly methylated DNA markers ($n = 30$, $p < 0.05$) to distinguish between normal and cancerous tissue. Those 30 gene loci were tested for their methylation status in normal blood and a subset of 12 markers, which were confirmed methylation negative in DNA from blood, were re-evaluated on a larger series of breast cancer tissue DNA ($n = 82$) and healthy controls ($n = 10$) by qPCR. Seven markers enabled 85% correct classification of cancerous versus healthy breast tissue ($p < 0.05$, AUC = 0.84). Of those, 3 markers could be detected and confirmed aberrantly methylated in serum of breast cancer patient ($n = 62$) and serum of healthy controls ($n = 32$; $p < 0.05$, 73% correct classification, AUC = 0.7). Comparing the number of methylated gene loci in biopsies versus serum, we deduced that a fraction of 30% methylated DNA fragments prevailing in cancerous tissue can be detected in serum too.

Conclusions: Although deducing tumormarkers for minimal-invasive diagnostics from methylation profiles of cancer-tissues is a promising strategy, methylation testing of serum DNA analyses remains difficult because less than 1% of serum DNA shows a tumor specific methylation pattern.

C 049 Cdc37 is expressed on the surface of MDA-MB-231 breast cancer cells and is implicated in extracellular HSP90-driven cancer cell invasion processes

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Background: In the past, a pool of HSP90 was identified on the surface of cancer cells where it was shown to participate in invasion processes. We have previously shown that Cdc37, a key component of the HSP90 machinery is localized on the surface of HER2 positive MDA-MB-453 breast cancer cells. The aim of this study was to examine the localization of Cdc37 in a HER2 negative breast cancer cell line named MDA-MB-231, and to investigate its possible involvement in breast cancer cell invasion processes.

Observations: Immunofluoresence experiments showed that Cdc37 is expressed on the surface of MDA-MB-231 cells. This was confirmed by western blot analysis of membrane fractions of these cells. Since HSP90 was previously detected on the surface of these cells, we next examined a possible cell surface interaction of HSP90 with Cdc37. Indeed, co-immunoprecipitation experiments performed in MDA-MD-231 membrane fractions revealed a physical association of HSP90 with Cdc37 on the cell surface. We next examined the possible involvement of surface Cdc37 in cancer cell invasion processes. To this end, wound healing assay was performed in the presence of anti-Cdc37 antibody that specifically binds on the cell surface. It was shown that indeed presence of anti-Cdc37 antibody in the culture medium strongly inhibited MDA-MB-231 cancer cell invasion. We next examined the possible interaction of Cdc37 with EGFR as well as its association with the activation of downstream signaling kinases. Co-immunoprecipitation experiments revealed an interaction of surface Cdc37 with EGFR, while presence of the anti-Cdc37 antibody in MDA-MB-231 cultures, impaired the activation of MEK kinase in EGF-stimulated cells.

Conclusions: Cdc37 is expressed on the surface of MDA-MD-231 breast cancer cells where it is found in association with HSP90 and EGFR. Moreover, inhibition of surface Cdc37 leads to reduced cancer cell invasion and decreased activation of MEK kinase which participates in downstream signaling.

C 050 Y-box binding protein-1 (YB-1) reprograms the epigenome to promote a breast cancer stem cell phenotype and drive tumour progression

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Background: The genome is highly dynamic and plastic. In cancer, the epigenetic mechanisms that maintain cell fate are deregulated. Notably, expression of Polycomb group (PcG) proteins alters the histone methylation landscape to promote a stem cell phenotype. These cells have unlimited proliferative potential and survive therapeutic insult to regenerate the tumour. In this study we demonstrate that YB-1 instigates remodeling of the epigenome to favour a stem cell program that primes cells for malignancy.

Observations: To delineate the earliest events that drive malignancy, non-tumourigenic human mammary epithelial cells (HMECs) were engineered to conditionally express the YB-1 oncogene. This was associated with enhancement in the expression of the stem cell markers, CD44 and CD49f, mammosphere formation, self-renewal capacity, and invasiveness. Isolating CD44+/CD49f+ cells revealed the population to be resistant to chemotherapeutics. To ascertain the importance of these stem cells in tumour progression, we modeled breast acini three-di-

mensionally in vitro. Induction of YB-1 promoted luminal filling and invasion by CD44-expressing cells. We uncovered that the oncogene governed the emergence of stemness through the PcG gene BMI1. In turn, this yielded histone H2A-K119 ubiquitination, silencing of the INK4a locus, and ultimately permissiveness through the G1/S checkpoint. In MDA-MB-231 breast cancer cells, inhibiting YB-1 was sufficient to repress BMI1 and rescue p16INK4a. Concurrent with BMI1-driven epigenetic changes, we also detected an increase in p300 and, consequently, histone H3 acetylation. The resulting chromatin relaxation may expose promoters of genes that instigate tumour-initiation.

Conclusions: Our work reveals an underappreciated role for YB-1 in reprogramming the epigenome to promote a stem cell phenotype that drives malignancy. Targeting the YB-1/BMI1/p300 axis represents a promising strategy to reverse the stem cell fate thereby making cells amiable to therapeutic intervention.

C 051 Evaluation of the mechanism of action of a novel anti cancer compound

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Background: A novel quinoxalinhydrazide derivative, originally developed as an HIV-1 integrase inhibitor, was identified as a promising drug candidate for the treatment of cancer due to its strong cytotoxic activity. Preliminary studies that showed similarity to substances inhibiting topoisomerases and the compound's structure suggest that it targets DNA binding or processing enzymes, but the main mode of action remains unknown to date. The project aims at elucidating the compound's mechanism of action.

Observations: The compound was included in an anti-cancer activity screen by the National Cancer Institue (NCI-60 Screen) where it was tested against 60 different human tumor cell lines. This screen revealed very strong anti-cancer activity in tumor cell lines of all cancer types with a mean IC50 of 200 nM. Based on these data a computer analysis of drug activity profiles (NCI COMPARE) was performed in order to detect compounds with a similar cytotoxicity profile in the NCI-60 screen. Most of the hits resulting from this analysis are compounds that have a mode of action related to DNA damage induction and are interacting with or inhibiting topoisomerases. Together with the similarity to Camptothecin and Mitoxantrone (topoisomerase I and II inhibitors, respectively) that was suggested by preliminary gene expression studies, this points towards a DNA damage-inducing mode of action of the investigational compound. Indeed, in immunofluorescence stainings gamma-H2AX was found to be elevated upon treatment, an observation further underlined by elevated p-ATM protein expression levels.

Conclusions: In conclusion, first impressions of the mechanisms of action of this potent drug candidate suggest that this drug is a potentially new DNA damage-inducing agent. However, the exact nature of DNA damage and its mode of action still need to be investigated in more detail.

C 052 Genetic analysis of three-generation family with multiple manifestation of celiac disease and type 1 diabetes

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Background: We investigated the three-generation family affected by celiac disease (CD) and type 1 diabetes (T1D) sharing a part of genetic risk, alleles encoding HLA-DQ2 and HLA-DQ8 molecules. We hypothesize that impairment of negative regulation of immune response can be associated with PTPN22 gene and activation of immune response can be influenced by expression of toll-like receptors and IL12B genes.

Observations: We genotyped HLA alleles, C1858T polymorphism in PTPN22 gene and A1188C polymorphism in IL12B gene and measured expression of TLR-2 mRNA and protein in all members of family. Peripheral blood DNA was used for HLA typing by the PCR-SSP and for detection of PTPN22 and IL-12 polymorphisms by the PCR-RFLP techniques. TLR2 protein and mRNA expression was assessed using flow cytometry and real-time PCR. We detected a linkage between HLA-DQ2 molecule and manifestation of CD and T1D ($P = 0.0004$, $OR = 107.7$, $CI = 3.844 - 3016$) with the strongest effect of HLA-DQB1*0201 allele ($P = 0.0029$, $OR = 63$, $CI = 3.320 - 1196$). We have not found any linkage of either C1858T polymorphism of PTPN22 or A1188C polymorphism of IL12B genes with autoimmune diseases. We have found no correlation of both, TLR2 protein and mRNA expression with autoimmune markers.

Conclusions: In the analyzed family we found a genetic predisposition to CD and T1D represented by alleles encoding HLA-DQ2 molecule. We suppose influence of other genes involved in ethiopathogenesis of CD or T1D. Additional testing is required for better understanding genetic predisposition in this family.

C 053 Statins affect ATP-binding cassette B1 transporter turnover in human neuroblastoma cells

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Background: ATP-binding cassette (ABC) transporters are upregulated in almost all tumors correlating with chemoresistance and bad prognosis. We have previously shown that statins co-administrated with doxorubicin led to an enhanced caspase 3 and 9 activity in neuroblastoma cells. Doxorubicin, a substrate for ABCB1, is less extruded from the cells in the presence of statins by a direct inhibition and reduction of glycosylation of ABCB1. Consequently, we investigated the turnover of ABCB1 affected by statins.

Observations: Using rt-PCR analyses, increasing concentrations of simvastatin led to a significant inhibition of ABCB1 transporter in mRNA level. Moreover, this inhibition was augmented upon longer exposure times. This is compatible with our previous ob-

servation that in Western blots the ABCB1 transporter content is reduced in SH-SY5Y neuroblastoma cells. Possibly, the decline in ABCB1 transporter is compensated by other ABC transporters. However, a compensation of ABCB1 on mRNA and protein level was not seen for ABCC1, ABCC4, and ABCG2 transporters.

Conclusions: We conclude that simvastatin is able to inhibit and downregulate the ABCB1 transporter directly and immediately which is not compensated by other transporters. Based on these findings, simvastatin is a promising candidate for adjuvant chemotherapy to reduce transporter mediated multidrug resistance.

C 054 Molecular basis of hereditary renal hypouricemia in 15 Czech patients

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Background: Renal hypouricemia is a heterogeneous inherited disorder characterized by impaired tubular uric acid handling in the renal tubules. Patients are usually asymptomatic; however, some may experience urolithiasis and/or acute renal failure. Diagnosis is based on hypouricemia and increased fractional excretion of uric acid, therapy is not available.Type 1 is caused by a loss-of-function mutation in the SLC22A12 gene (OMIM 220150), type 2 is caused by defects in the SLC2A9 gene (OMIM 612076).

Observations: We have selected eight patients (repetitive serum uric acid max. 60 micromol/l and fractional excretion of uric acid min. 43%) for analysis of the SLC22A12. Mutation analysis revealed 3 sequence variations in the promotor and 5´-UTR region and 5 in exonic regions. 3 transitions (G366R, T467M and previously published R477H) and one deletion (A416_L418del), yet unpublished, were found in four homozygous, four compound heterozygous and seven heterozygous subjects. Functional and immunocytochemical analysis of URAT1 mutants was performed in Xenopus laevis oocytes. All mutants (except for the R477H) showed significantly decreased urate transport activity. Oocytes expressing wild-type or R477H mutant exhibited similar immunostaining of URAT1 in the cytoplasm and at the plasma membrane. Immunostaining of mutants G366R or A416_L418del was present only in the cytoplasm leaving plasma membrane negative. Oocytes expressing T467M mutant did not show any immunodetectable signal of the URAT1.

Conclusions: We identified 3 unpublished sequence variants in SLC22A12 gene resulting in loss of function and one previously reported mutation that did not alter protein function. Detailed studies concerning uric acid transporters could clarify the pathogenesis of acute kidney injury in renal hypouricemia.

C 055 Mutations in KIF7 link Joubert syndrome with Sonic Hedgehog signaling and microtubule dynamics

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Background: Joubert syndrome (JBTS) is a rare mostly autosomal recessively inherited developmental disorder characterized by a specific brain malformation with various additional pathologies. JBTS results from mutations in at least 10 different genes, all of which play a role in the formation or function of primary cilia. Primary cilia are essential for vertebrate development, and mutations affecting this organelle underlie a large group of diseases referred to as ciliopathies.

Observations: By positional cloning, we identified mutations in KIF7 in a consanguineous JBTS family and subsequently in additional patients with JBTS. KIF7 is a homolog of the Drosophila kinesin Costal2 and a known regulator of Sonic Hedgehog signaling. KIF7 co-precipitates with another JBTS protein, nephrocystin-1. Knockdown of KIF7 expression in retinal pigment epithelial (RPE) cells causes defects of cilia formation and induces abnormal centrosomal duplication and fragmentation of the Golgi network. The Golgi phenotype affects all three compartments of the Golgi apparatus, and can be rescued by over-expression of full-length KIF7. In contrast, over-expression of a KIF7 truncation lacking the predicted motor domain led to the same Golgi phenotype as the knockdown of KIF7 did. Interestingly, we observed an increase of tubulin acetylation and changes in cell shape. Consistently, KIF7 was found to interact with the histone deacetylase HDAC6 and the polarity complex protein PAR3.

Conclusions: Abnormal tubulin acetylation and microtubule dynamics could be causative for the additional cellular pheno-

types observed in KIF7 knockdown cells. Changes in microtubule stability and growth direction might function as underlying disease mechanisms impacting cilia, centrosomes and Golgi.

C 056 Reversal of multiple drug resistance by inhibition of 5'-ectonucleotidase activity in high-grade glioma

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Background: Glioblastoma Multiforme (GBM) is a tumour with a high rate of proliferation and degree of vascularization, which is extremely resistant to chemotherapy. This is mainly a result of the overexpression of multiple drug resistance (MDR) transporters such as Mrp1. The activity of ecto-5'-nucleotidase (CD73) is essential for production of extracellular adenosine from AMP hydrolysis and there is a correlation between the overexpression of CD73 and MDR transporters.

Observations: Our goal was to investigate the role of CD73 in maintenance of the MDR phenotype and to determine whether a particular adenosine receptor (AR) is implies in chemoresistance in this tumour. We detect an increased expression and activity of CD73 in GBM tumour tissue samples. AOPCP (50 uM), which inhibits CD73 activity, decreased the rate of proliferation in the T98G cell line and in GBM primary culture (50 +/- 6.2%). Exposure of tumoural cells to AOPCP reduced transcript and protein levels of the ABCC1 gene that codes for Mrp1. In addition, expulsion of an Mrp1-specific substrate was inhibited (56 +/- 4.5%) on exposure of the GBM cells to this inhibitor. In a similar manner, inhibition of A3 AR activity, by a specific antagonist (MRS1220, 10 uM), decreased Mrp1 expression (50 +/- 2.6%) and activity (60 +/- 2.0%). GBM cells were chemoresistance to anticancer drugs vincristine and taxol. However, when anticancer drugs were used in conjunction with AOPCP the cell viability was significantly decreased.

Conclusions: We recognized to adenosine as a physiological modulator of MDR phenotype in GBM cells. The use of CD73 and/or A3 AR inhibitors may be useful for decreasing tumoural growth and reversing GBM resistance to chemotherapy.

C 057 The protein expression pattern of CD133+ colon cancer cells indicates activation of the Wnt pathway and potential alteration of splicing mechanisms

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Background: Cancer stem cells (CSCs) theory represents a breakthrough in cancer research. CSCs are able to self-propagate in vitro, they divide asymmetrically and are tumorigenic. These cells can initiate tumor formation, elude treatment and allow tumor reformation after initial successful treatment.

Observations: We have characterized the protein expression pattern of CSCs to identify specific intracellular pathways in these cells. Specifically, we studied two colon cancer cell lines: CaCo-2 and HCT116. Putative CSCs were separated from differentiated cancer cells by flow cytometry using CD133 as stemness marker. Total protein extracts of CD133+ cells were compared to protein extracts of CD133- cells using 2D-DIGE. Proteins were identified by MS: 17 proteins were identified in HCT116 and 22 in CaCo-2, some of which are involved in the Wnt/beta-catenin pathway that leads to activation of the transcription of genes responsible for cell survival and proliferation. Interestingly, we observed up-regulation of the splicing factor SRp20, a newly identified target gene of the Wnt pathway. SRp20 has also recently been shown to play a critical role in cell proliferation and tumor induction and maintenance in various tumor types. To determine whether SRp20 accumulation is a downstream effect of Wnt pathway activation, we stimulated the pathway and we observed SRp20 expression, thus demonstrating a direct cause-effect relationship between Wnt pathway activation and the increased level of SRp20.

Conclusions: In conclusion, our demonstration of activation of the Wnt pathway by CD133+ cells and of up-regulation of SRp20, which is implicated in tumorigenesis, raises the possibility of a sequential series of molecular events occurring in connection with this process.

C 058 Polymorphism in the CHRNA3 Gene Contributes to Early-Stage Non-Small-Cell Lung Cancer Survival

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Background: Recently many genome-wide association studies (GWASs) were reported that polymorphism rs6495309 in the CHRNA3 was associated with the risk of lung cancer disease. This study was conducted to investigate whether this polymorphism may affect survival outcomes of early stage non-small cell lung cancer (NSCLC) patients.

Observations: Five hundred eighty-seven consecutive patients with surgically resected NSCLC were enrolled in this study. The polymorphism rs6495309 in the nicotinic acetylcholine receptor gene 3 (CHRNA3) was investigated for the associations with overall survival (OS) and disease free survival (DFS). The polymorphism rs6495309 was associated with increased OS and DFS early stage NSCLC. Patients with the CHRNA3 rs6495309 CT+TT genotype exhibited better OS and DFS than the rs6495309 CC genotype (adjusted hazard ratio [aHR] for OS = 0.57, 95% CI = 0.42-0.76, P =.0002; aHR for DFS = 0.61, 95% CI = 0.48-0.79, P = .0001). In particular, the rs6495309 polymorphism was significantly associated with OS and DFS in ever-smokers (aHR for OS = 0.58, 95% CI = 0.42-0.81, P =.001; aHR for DFS = 0.58, 95% CI = 0.43-0.77, P = .0002), squamous cell carcinoma

(aHR for OS = 0.53, 95% CI = 0.35-0.79, P = .002; aHR for DFS = 0.65, 95% CI = 0.46-0.93, P = .02).

Conclusions: The CHRNA3 rs6495309 polymorphism not only associated lung cancer risk, also may affect survival in NSCLC. This polymorphism in CHRNA3 gene can help identify patients at high risk of a poor disease outcome.

C 059 The role of B-Raf in Ras mediated skin carcinogenesis

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Background: The Erk/MAPK pathway consists of a protein kinase cascade (Raf-Mek-Erk) that can regulate growth, differentiation and apoptosis. Ras activates the first kinase in this cascade, Raf. Three Raf family members are known: A-, B- and C-Raf. A-Raf knockout mice are viable, despite gastrointestinal and neurological defects. In contrast, ablation of B- or C-Raf in mice results in embryonic lethality. Both Ras and Raf are often found mutated in human tumors.

Observations: The effect of B-Raf in Ras-driven epidermal tumorigenesis was investigated, using a transgenic and a chemical approach. The transgenic approach used a constitutive active form of SOS expressed under the control of the promoter of the basal layer-specific Keratin 5, resulting in constitutive activation of the Erk/MAPK pathway. As a chemical approach we used the widespread DMBA/TPA carcinogenesis protocol. Wildtype mice expressing SOS-F developed tumors 3-5 weeks after birth with a penetrance of 100%; in the DMBA/TPA model, wildtype mice developed papilloma within 7 weeks of treatment with a penetrance of 100% within 16 weeks of treatment. Ablation of B-Raf in the intact murine epidermis shows no obvious phenotype, but led to an impressive reduction in tumor formation, growth and size in both the chemical and the transgenic carcinogenesis model. Histological analyses of tumor sections revealed an increase in differentiation and a decrease in proliferation in B-Raf-deficient tumors, but no changes in apoptosis. Western blot analyses of primary keratinocytes confirmed SOS-F expression, B-Raf deletion and reduced activation of downstream pathway members.

Conclusions: B-Raf plays an important role in Ras-driven tumor formation but seems to be dispensable for normal skin homeostasis, underscoring the therapeutic potential of antitumor strategies targeting B-Raf and the ERK/MAPK pathway.

C 060 Crm1: A Potential Therapeutic Target in Human Melanoma

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Background: Chromosome Region Maintenance1 (Crm1) along with small GTPase protein Ran, Ran associated GTPase activating protein 1(RanGAP1) and Ran binding protein 1(RanBP1) are major players in the nuclear export pathway. Elevated Crm1 expression with subsequent aberration in nuclear protein localization is linked to tumorigenesis. Since potential of Crm1 as a viable target in melanoma remains unexplored, we set forth to systematically evaluate Crm1's prospect as a therapeutic target in human melanoma.

Observations: cDNA micro-array analysis revealed melanoma metastasis-associated significant overexpression of Crm1, RanGTPase, RanGAP1 and RanBP1. Crm1 inhibition, using a highly specific Crm1-inhibitor, Leptomycin-B (LMB) and siRNA mediated Crm1 silencing, triggered extensive apoptosis in metastatic melanoma cell lines. In contrast, immortalized melanocytes and primary lung fibroblasts exhibited unusual resistance towards LMB induced apoptosis. Activation of both the extrinsic and intrinsic apoptotic pathways, with dispensability for the extrinsic pathway was observed in all the cell lines, but one. Crm1 inhibition dramatically induced p53, exclusively in wild-type p53 melanoma cells, however p53 knockdown established its dispensability in LMB mediated apoptosis in both wild-type and mutant-p53 cell lines. Rb-protein hypophosphorylation and accompanying G1-cell cycle arrest potentially precede LMB induced apoptosis. Interestingly, Crm1 inhibition, expected to subdue Mek-Erk signaling activity paradoxically resulted in Erk activation. Ultimately, combining LMB and Mek-inhibitor, U0126, we rule out Mek-Erk signaling involvement either in mediating or resisting LMB induced apoptosis.

Conclusions: Crm1 overexpression in metastatic melanomas, coupled with extreme sensitivity of melanoma cell lines to Crm1 inhibition posits Crm1 as a potential therapeutic target in human melanoma. Therapeutic potential of RanGTPase, RanGAP1 and RanBP1 though anticipated, need to be investigated.

C 061 Na+K+ATPase activity might be the crucial factor for selective inhibition of tumor stem cells by the ionophore salinomycin

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Background: In 2009 Gupta et al. did show that some ionophores for monovalent kations could act as selective inhibitors for tumor stem cells with salinomycin beeing the most active compound. As experimental system they used an epithelial breast cancer cell line and a subclone that gained tumor stem cell like properties by silencing E-cadherin.

Observations: In a different cellular model system we could show a very similar selective inhibition of tumor stem cell proliferation by salinomycin. An isolated stem cell fraction from the human breast cancer cell line MDA 231 was checked against an epithelial subclone of this cell line generated by the transfection of keratin 18. A first approach to clarify the underlying mechanism revealed that the Na + K + ATPase might play a role in this selective inhibition: the dose dependent inhibition of stem cells by salinomycin was approximated in epithelial cells if additionally the Na + K + ATPase was partially inhibited by increasing concentrations of hellebrin. However, qRT-PCR did not reveal any difference in the expression of the enzyme on the

transcriptional level but a significantly lower content of free ATP was observed for the stem cells.
Conclusions: The selective inhibition of tumor stem cells by salinomycin might be caused by a disturbed Na+/K+ gradient originating more likely from energy deficiency than from a less active Na+K+ATPase in these cells.

C 062 Peroxisomal Alterations in Alzheimer's Disease

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Background: In Alzheimer's disease (AD), lipid alterations are present early during disease progression. As some of these alterations point towards a peroxisomal dysfunction, we investigated peroxisomes in human postmortem brains obtained from the cohort-based, longitudinal Vienna-Transdanube Aging (VITA) study. Based on the neuropathological Braak staging for AD on one hemisphere, the patients were grouped into three cohorts of increasing severity (stages I–II, III–IV, and V–VI, respectively).
Observations: Lipid analyses of cortical regions revealed accumulation of C22:0 and very long-chain fatty acids (VLCFA, C24:0 and C26:0), all substrates for peroxisomal beta-oxidation, in cases with stages V–VI pathology compared with those modestly affected (stages I–II). Conversely, the level of plasmalogens, which need intact peroxisomes for their biosynthesis, was decreased in severely affected tissues, in agreement with a peroxisomal dysfunction. In addition, the peroxisomal volume density was increased in the soma of neurons in gyrus frontalis at advanced AD stages. Confocal laser microscopy demonstrated a loss of peroxisomes in neuronal processes with abnormally phosphorylated tau protein, implicating impaired trafficking as the cause of altered peroxisomal distribution. The study design allowed a direct correlation between the biochemical findings and the amount of neurofibrillary tangles (NFT) and neuritic plaques, quantified in adjacent tissue sections. Interestingly, the decrease in plasmalogens and the increase in VLCFA and peroxisomal volume density in neuronal somata all showed a stronger association with NFT than with neuritic plaques.
Conclusions: These results indicate substantial peroxisome-related alterations in AD, which may contribute to the progression of AD pathology.

C 063 Genome-wide shRNA screen for tumor suppressors in the context of oncogene-induced senescence

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Background: During recent years increasing evidence has been provided that oncogene-induced senescence (OIS) is a potent tumor-suppressive mechanism. We have previously shown that human oncogene-expressing melanocytic nevi show hallmarks of OIS. These benign lesions are rather stable structures and rarely progress to melanoma, suggesting that additional (epi) genetic alterations are required for malignant progression. To date, only few mediators of OIS have been identified.
Observations: We performed a genome-wide shRNA OIS bypass screen to identify critical mediators of this process. For this screen, cells (BTR) that allow for conditional induction of RAS-induced senescence were transduced with a genome-wide shRNA library (TRC) and analyzed for their ability to bypass of OIS. We screened for enriched shRNAs by quantitative high throughput DNA sequencing. Technical replicates were used to determine the quality of PCR amplification and sequencing. The analyses revealed that highly abundant shRNAs could be reproducibly detected. Importantly, shRNAs targeting Cdkn2a and p53, genes known to be involved in OIS were identified among the top outliers of the screen. We are currently validating our top 25 candidates.
Conclusions: With this approach we aim to gain further insight into the molecular mechanism of OIS and to identify signaling pathways that contribute to malignant transformation.

C 064 Analysis of the hypertrophy related genes of spontaneously hypertensive rats

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Background: The hypertension promotes the development of cardiovascular disease. The etiology of hypertension is not well-defined, as the hypertension is caused by the complex factors based on genetically and environmentally predispositions. Continuation of high blood pressure develops the remodeling of the cardiac myocytes and the hypertrophy followed cardiac failure. To clarify the mechanisms of hypertension, we analyzed the gene related to hypertrophy of the spontaneously hypertensive rats.
Observations: Total RNAs were isolated by the TRIzol from a variety of tissues of SHRs (SHR/kpo, SHRSP/kpo, and M-SHRSP/kpo) and from WKY/kpo normotensive rat. The genes related to hypertrophy (calcineurin (Cn), Ca-dependent phosphatase; Camk, Ca-calmodulin dependent protein kinase; GATA, transcription factor etc.) were amplified by RT-PCR methods and determined the expression levels of those genes of each tissue of heart, lung, liver, brain, and kidney. Some genes show resemble expression pattern between the rat strains and the tissues. Although, the blood pressure levels and severity of disease of

SHRs were different (M-SHRSP was worst), those of expression levels of the hypertrophy related genes to pathological conditions were not related. It was known that overexpressions of Camks related to hypertrophy, results from the expression of those genes in heart of SHRs and WKY are not so simple. Cloning and sequencing analysis of genes of SHRs showed there were many genetic variants of blood pressure related and hypertrophy related genes. In the variants of Cn and calmodulin, calcium binding sites may change and will be alter each physiological activity.

Conclusions: We cloned and analyzed levels of hypertension related genes to formulate molecular network of diseases and to loom out cross-point of risk-factors of metabolic syndrome. We need those results to reveal pathophysiology of entity morbid organism suffered multifactorial disease such as hypertension.

C 065 Molecular classification of benign and malignant thyroid nodules

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Background: Thyroid nodules are very common and need to be assessed somehow to rule out malignancy, even if only a small percentage is actually malignant. A key component of the diagnosis is fine-needle aspiration (FNA) biopsy followed by cytology, which leads to an indeterminate diagnosis in 15 – 30% of FNA specimen. Histological evaluation of these nodules shows that a large proportion is actually benign. This highlights the need for more accurate tests for pre-operative evaluation of thyroid nodules.

Observations: We conducted microarray analysis of 49 thyroid tumour nodules including all major histological classes. From this data we calculated inference statistics and employed different feature selection algorithms for classification of (1) malignant vs. benign and (2) FTA vs. FTC. This yielded two set of genes comprising of 20 and 22 genes, respectively. Classification accuracies in our data were 100% and 92%, respectively. Then, these gene sets were tested on seven different publicly available datasets (totalling 197 samples), two of those included FTA and FTC nodules. The classification accuracy of the FTA/FTC gene set was: 96% and 100%; the accuracy of the benign/malignant gene set was: 92%, 87%, 90%, 92%, 100%, 94% and 100%. Data on further evaluation of these gene sets on independent samples using qPCR is presented. DNA methylation: We used an in-house developed assay, based on methylation sensitive restriction digestion of DNA followed by microarray readout to assess the methylation status of 360 selected CpG islands. As little as two CpG islands were sufficient to distinguish between follicular adenoma and carcinoma.

Conclusions: We have shown that molecular analysis may improve the diagnostic accuracy of thyroid nodules. We currently test these signatures on fine needle aspiration biopsy material to prove their clinical validity.

C 066 Effect of C/T -13910 cis-acting regulatory variant on expression and activity of lactase in Indian children and its implication for early genetic screening of adult-type hypolactasia

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Background: Intestinal lactase activity declines during childhood in majority of human populations leading to adult-type hypolactasia,limiting the use of fresh milk. Aim of this study was to correlate lactase expression and activity with C/T -13910 variant in Indian children, determine the age of onset of down-regulation of lactase activity and assess the applicability of the C/T -13910 variant as a diagnostic marker for identifying children genetically inclined to develope adult-type hypolactasia.

Observations: Intestinal biopsies were obtained from 176 children aged 1-16 years undergoing routine endoscopy (ethically approved).The biopsies were assayed for lactase activity and genotyped for C/T -13910 variant using PCR-RFLP analysis and DNA sequencing.The functional effect of the C/T -13910 variant on expression of lactase mRNA and protein was examined using reverse- transcription PCR and western blotting.Among the 176 children investigated in our study, 56.8% carried the C/C -13910 genotype, which has been associated with the onset of adult-type hypolactasia, while 40.9% carried the C/T -13910 genotype and 2.3% the T/T -13910 genotype. There was a significant correlation between lactase activity and C/T -13910 variant ($P < 0.001$). The mean level of lactase activity among children with C/C -13910 genotype was 15.9 U/g protein and with C/T and T/T -13910 genotypes was 30.9 U/g protein.C/C -13910 genotype was associated with low expression of lactase mRNA and protein compared with C/T genotype.Considering lactase activity of 10 U/g protein as gold standared, predictive value of genetic test based on C/T -13910 variant for adult-type hypolactasia was 100% in children > 8 years of age.

Conclusions: C/T -13910 cis-acting regulatory variant located ≈14 kb upstream of lactase gene (LCT) completely correlates with lactase phenotype in Indian children. The genetic testing for the C/T -13910 variant may be helpful in the early diagnosis of adult-type hypolactasia in Indian children.

C 067 HTS approach to identify chemicals inhibiting EMT in cancer

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Background: Epithelial-to-mesenchymal transition EMT and the inverse MET are normal events of tissue remodelling in development. Differentiated cells undergo EMT, migrate and undergo MET giving rise for an epithelium therein. Similarly in metastasis, cancer cells become motile, migrate, extravasate and

start growing in a new site. Indeed, gaining metastatic capacity appears to be linked to EMT. Furthermore, EMT is linked to formation of cancer stem cells (CSC), drug resistance and poor prognosis.

Observations: CSCs have the ability to self-renew and to differentiate giving rise to the cellular heterogeneity of the initial tumour. Thus CSCs have a potential role in cancer relapse after therapy, and make an attractive target for drug development. In this study, an EMT/CSC model system is established to allow for high-throughput drug screening of chemicals selectively affecting CSCs. EMT is induced in breast cancer cells and formation of CSC-like cells is confirmed by assessment of acquisition of mesenchymal markers (vimentin, N-cadherin), and loss of epithelial ones (E-cadherin), by immunofluorescence and Western blotting. Both CSC-like and the mock-treated cells are subjected to screening.

Conclusions: Currently we are optimizing the cell model, and first results of the screening will be presented in the meeting.

C 068 The role of p70S6K in the autophagy induced by azathioprine in HepG2 cells

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Background: Almost 5% patients treated with the immunosuppressor azathioprine (Aza) induces hepatotoxicity, which is associated with intracellular glutathione levels reduction, ROS production increase and stress activated kinases increase (JNK and p38) leading to necrosis or apoptosis. However, there are not available data about Aza effect on autophagy. To characterize the molecular mechanism involved, we used fluorescence microscopy, flow cytometry, [gamma-32P]ATP for kinase assay and Western blot.

Observations: Previously, we showed HepG2 cells treated with Aza and BSO (buthionine sulphoximine; a cytosolic GSH synthesis inhibitor) induced cell death due to GSH depletion and oxidative stress activation. However, in absence of BSO, HepG2 cells remain resistant to Aza effect. In the present report, we show that the treatment of cells with Aza does not induce cell death but activates an autophagic-like process. Autophagy is an evolutionary conserved homeostatic process that allows the bulk degradation of long-lived proteins and organelles. This process is characterized by autophagosomes and lysosomes fusion, thus forming the so-called autophagolysosomes. In this study, we show that Aza increases the fragmentation of pro-microtubule-associated protein light chain 3 (LC3) in LC3-I and LC3-II fragments, a biochemical hallmark of autophagosomes formation. This LC3 fragmentation is associated with activation of p70S6K measured by phosphorylation (Thr421/Ser424) and [gamma-32P]ATP kinase assay. In parallel, we observed that the thiopurine was able to increase the lysosomal activity (detected by acridine orange staining).

Conclusions: The autophagy induced by azathioprine on HepG2 cells could be useful to explain the mechanisms of hepatotoxicity reported in some patients. Our results support that the involvement of p70S6 kinase could explain the adverse effects of Aza.

C 069 Determination of genetic markers for kidney complications of diabetes mellitus

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Background: The VDR gene, located on chromosome 12q, is comprised of 11 exons. Allelic polymorphism of the VDR gene includes B/b and A/a alleles present in intron 8 and T/t allele present in exon 9 and F/f polymorphism in start codon. Parathyroid hormone (PTH) belongs to the most significant regulators of calcium and phosphate metabolism. Its corresponding gene is located on chromosome 11p. Allelic polymorphism of the PTH gene includes B/b and D/d alleles present in exons.

Observations: Pacients with diabetes, diabetic nephropaty, non-diabetic nephropaty and controls were gonotyped using polymerase chain reaction (PCR) and restriction fragment lenght polymorphism (RFLP), for four restriction sites in the VDR gene, BsmI, Fok I, ApaI and TaqI. Expression of VDR gene was tested for diabetic nephropaty pacients. PTH genotyping was performed using Bst BI and Dra II restriction enzymes.

Conclusions: Our study shows that patients with ff genotype for the VDR gene are susceptible to diabetes and diabetic nephropaty. A allele seems to be a predisposition factor for both, diabetic and non-diabetic nephropaty. DD genotype is risk factor for diabetes, diabetic and non-diabetic nephropaty.

C 070 Altered DNA methylation patterns in osteoarthritis

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Background: Aberrant DNA methylation plays a role in the development of many different diseases. Recent results indicate that they are also associated with the pathogenesis of osteoarthritis, a disease characterized by progressive loss of cartilage, but exact mechanisms as well as genes involved are not yet defined.

Observations: To investigate methylation changes on a genome-wide scale, a specific amplification protocol has been established based on methylation-sensitive restriction digestion. This amplification enriches the methylated DNA and therefore enables whole genome promoter methylation screenings. This protocol was then used to analyse the DNA methylation status of arthritic and non-arthritic cartilage samples taken from knees of arthritis patients on Agilent 2x244k 'Human Promoter Whole Genome' microarrays. From those chip-experiments a group of 1214 gene

targets was identified, exhibiting significantly different methylation patterns between arthritic and non-arthritic samples. Functional and pathway analyses of these genes were performed through the use of 'WebGestalt' (WEB-based GEne SeT AnaLysis Toolkit). Enriched numbers of these genes were found to be associated with the cartilage metabolism, including cytoskeletal and ECM (extracellular matrix) formation, degradation, organisation and inflammation.
Conclusions: 'WebGestalt' analysis of potential osteoarthritis marker genes confirmed their roles in functional categories and biochemical pathways associated with cartilage degradation. Thus biological relevance of these genes has been approved indicating reliable microarray data for further investigations.

C 071 Characterisation of serum- and glucocorticoid-regulated kinase 1 in adipose tissue

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Background: The serum- and glucocorticoid- regulated kinase 1 (Sgk1) is an early transcriptional target of glucocorticoids that is activated via PI3K-dependent phosphorylation by insulin and IGF-1. Sgk1 stimulates cellular glucose uptake by up-regulation of GLUT1 and GLUT4 cell surface expression and accelerates intestinal glucose absorption via SGLT1. Here we investigate the expression and regulation of Sgk1 in human obesity, diet-induced murine obesity and adipogenesis in 3T3-L1 cells.
Observations: Diet-induced obesity in mice was associated with a significant up-regulation of Sgk1 mRNA expression in gonadal adipose tissue. RT-qPCR and immunohistochemistry experiments revealed that Sgk1 levels are highest in the macrophage-rich stromal vascular fraction and lower in adipocytes. In humans, Sgk1 mRNA was significantly up-regulated in both visceral and subcutaneous adipose tissue of 20 obese patients compared to 20 age- and gender-matched controls. Sgk1 expression in subcutaneous adipose tissue correlated with serum parameters of inflammation and insulin resistance. Sgk1 transcription in 3T3-L1 preadipocytes was strongly induced by dexamethasone and its phosphorylation was enhanced by insulin. However, induction of adipogenesis in 3T3-L1 cells by dexamethasone and insulin led to a progressive reduction in Sgk1 levels. Sgk1 mRNA expression correlated negatively with PPARgamma expression during adipogenesis, whereas Sgk1 overexpression in 3T3-L1 adipocytes did not influence PPARgamma levels.
Conclusions: Since Sgk1 functions in increasing cellular glucose uptake, its down-regulation in adipocytes could play a crucial role in insulin resistance. Simultaneously, increased levels of Sgk1 in macrophages imply a potential function of Sgk1 in obesity-associated adipose tissue inflammation.

C 072 Nuclear factor kappa B (NFkappaB) and its inhibitor (IkappaB) in relation to diabetic nephropathy in type 2 diabetes

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Background: Diabetic nephropathy /DN/ is severe complication of diabetes mellitus /DM/. Family clustering, different ethnic prevalence and fact that only part of diabetics develops DN suggest individual genetic predisposition of DN. NFkappaB is redox-sensitive transcription factor participating together with its inhibitor /IkappaB/ on the genes activation involved in immune processes or apoptosis. The aim of the study is to find association between NFkappaB, IkappaB and development of DN in type 2 diabetic /T2D/ patients.
Observations: In total 540 patients /pts/, divided in 3 groups of diabetics with (n = 111), without DN (n = 78), with non-diabetic renal disease /NDRD/ (n = 40), and 2 control groups represented by healthy controls (n = 159) and pts with systemic lupus erythematosus /SLE/ (n = 152), were genotyped by fragment analysis and RLFP method. CA repeat polymorphism /CA rp/ of the NFkappaB1 gene and the A/G point variation in the 3'UTR region of the NFKBIA gene were investigated for their role in microvascular complications of DM, such as DN. The chi2 test was used for statistical evaluation. 10 out of 18 previously described alleles and 32 genotypes were identified for the CA rp of the NFkappaB1 gene. No significant difference between tested groups was found. Significant differences were observed in the frequencies of NFKBIA genotypes. There was significantly increased occurrence of AA genotype in all tested groups compared to controls, predominantly in diabetics without DN (or = 3.59, p = 0.0005), where presence of GG genotype (or = 0.40, p = 0.02) was significantly decreased, too. No difference was found between SLE and controls. Frequency of heterozygote AG genotype (or = 0.20, p = 0.0001) was significantly decreased in NDRD pts.
Conclusions: Even though we did not find any association of the polymorphisms in the genes for NFkappaB and IkappaB with DN alone, in the case of NFKBIA gene, we observed increased frequency of AA genotype in all groups of type 2 diabetics. Thus the presence of AA genotype could present risk genotype for this disease.

C 073 The role of HDAC1 and HDAC2 in epidermal development and tumorigenesis

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Background: HDACs catalyze the deacetylation of histone tails which leads to a tighter chromatin conformation and are therefore considered as transcriptional corepressors. The class I deacetylases HDAC1 and HDAC2 are not only essential for development and unrestricted proliferation, they are also frequently overexpressed in various cancer types. A prerequisite for the application of HDAC inhibitors in cancer therapy is the knowledge about the role and activity of individual HDACs in different tissues.
Observations: In order to investigate the function of HDAC1/2 in the skin we conditionally deleted the enzymes in the murine epidermis. Even though the deletion of HDAC1 is associated with a slight increase in epidermal proliferation, mice lacking HDAC1 in the epidermis do not show a severe phenotype. Interestingly the proliferative effect observed in HDAC1 knockout epidermis is not detected upon loss of HDAC2. But strikingly combined loss of HDAC1 and HDAC2 has a strong impact on tissue homeostasis and inflicted severe pathological changes like hyperkeratosis, loss of hair and hyperproliferation. Since HDAC1 is associated with cancerogenesis and its deletion also enhanced tumor malignancy in murine teratoma we decided to analyze tumor development in the presence and absence of HDAC1. In order to investigate the role of HDAC1 in epidermal tumorigenesis we crossed K5-SOS skin tumor mice with K5-Cre HDAC1 knockout mice and analyzed tumor formation. Strikingly epidermal tumor onset was strongly accelerated and tumor growth was significantly increased upon loss of HDAC1.
Conclusions: Our results underscore on the one hand the crucial role of HDAC1 and HDAC2 in epidermal development and homeostasis. In addition to that we identified HDAC1 as a crucial regulator of epidermal tumorigenesis, showing hereby that loss of HDAC1 significantly enhances tumor formation.

C 074 Identification the role of autophagy in tubular cells in the kidney

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Background: Autophagy is a cellular bulk degradation pathway that is responsible for long-lived proteins and damaged organelles. Recently, several groups indicated upregulation of autophagy in proximal tubular cells in the kidney under stress conditions. However, its function seems to be controversial.
Observations: To determine the role of autophagy in tubular cells, we generated Atg5 flox/flox: Ksp-Cre mice, in which Atg5, a gene essential for autophagy, is deleted in distal tubular cells, and Atg5 flox/flox: Pax8 rtTA: Teto-Cre mice, in which the Atg5 is deleted in the whole tubular system under doxycyclin administration.
Conclusions: Tubular Atg5 deletion results in accumulation of p62-positive protein aggregates and age-dependend decrease of kidney function. Currently we are investigating the role of autophagy under stress conditions like ischemia/reperfusion injury and cisplatin injection.

C 075 Bortezomib Induces Apoptosis by Interacting with the STAT Proteins in K–562 Leukemic Cells

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Background: STATs are transcription factors that are involved in regulation of cellular activities such as cell growth, differentiation, and survival. STAT3, 5A and 5B also play an important role in the development of leukemia. Bortezomib is a proteasome inhibitor which is used for treatment of multiple myeloma. The aim of this study is to investigate the apoptotic effect of bortezomib on leukemic cells and also clarify the potential relationship between bortezomib-induced apoptosisis and JAK/STAT pathway.
Observations: To evaluate the role of STATs in bortezomib-induced apoptosis of leukemic cells, relative expression levels of target STATs were analyzed at mRNA level by using real-time qRT-PCR. Cytotoxicity was assessed by using XTT assay and bortezomib was found to be cytotoxic in time and dose dependent manner and also IC50 value was determined as 177 microM for K-562 cells with XTT assay. Apoptosis was evaluated by using AnnexinV method under a fluorescent microscope and the number of apoptotic cells were found to be increasing in time depenent manner as almost 100% of the cells treated with IC50 dose of bortezomib were found to be apoptotic at 72 to 96th hours. RT-PCR results have revealed that all of the STATs' mRNA expressions were highly downregulated in time dependent manner. STAT3, 5A and 5B mRNA expressions were found to be as 2.98%, 0.92% and 7.92% respectively.
Conclusions: In this study, we have evaluated the probable role of bortezomib in leukemic cells upon STAT3, 5A and 5B we have clearly demonstarted the cytotoxic effect of bortezomib in K562 cells in time and dose dependent manner which is associated by prominent decrease of STATs mRNA expression.

C 076 An Attempt to Replicate the Results of Genome-Wide Association Studies on Lung Cancer Susceptibility Loci in a Korean Population

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Background: Genome-wide association studies (GWASs) have identified three chromosomal regions at 5p15, 6p21, and 15q25 as being associated with lung cancer risk in European popula-

tions. This study was performed to confirm these associations in Korean patients with lung cancer.
Observations: The genotypes of rs2736100, rs402710, rs401681, and rs31489 at 5p15, rs9295740 at 6p22, and rs2036534 and rs6495309 at 15q25 were determined in 1094 lung cancer patients and 1100 healthy controls frequency-matched for age and gender. The single nucleotide polymorphisms (SNPs) at 5p15 and 15q25 were significantly associated with lung cancer risk. The magnitude of effect was similar to that reported in previous studies and the association was in the same direction. The effect of SNPs at 5p15 region on the risk of lung cancer was significant only in adenocarcinoma. The two SNPs at 15q25 region were significantly associated with lung cancer risk in ever-smokers and squamous cell carcinoma. However, there was no association between the SNP at 6p22 and lung cancer risk.
Conclusions: We confirmed the association between SNPs at the 5p15 and 15q25 regions and the risk of lung cancer in a Korean population.

C 077 FABLE regulates c-ABL activity in the oxidative stress response

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Background: c-Abl is a tightly regulated tyrosine kinase ubiquitously express in vertebrate cells. In its active state c-Abl is hyperphosphorylated and shuttles between nucleus and cytoplasm. Its localization determines either a negative or positive regulation of cell growth and apoptosis. In particular c-Abl was shown to induce apoptosis in response to DNA damage or oxidative stress.
Observations: Conducting a functional screening in S.Pombe, we identified a novel interactor of c-Abl, a Ring Finger E3 ubiquitin ligase that we call FABLE (Finger-Containing Abl Enhancer). FABLE over-expression in mammalian cells dramatically increases c-Abl phosphorylation, while mutations in the FABLE RING domain that abolish E3 ligase activity do not affect c-Abl activity. FABLE positively regulates nuclear c-Abl activity via K63 ubiquitin chains while it does not change the activation status of cytoplasmic c-Abl or BCR-Abl. We observe that FABLE is essential for the activation of c-Abl after oxidative stress mediated by H2O2, suggesting that FABLE is a key regulator of c-Abl kinase activity in the oxidative stress response.
Conclusions: We identify a new mechanism of activation of the tyrosine kinase c-Abl operating in response to oxidative stress. We are now focusing on FABLE-dependent alterations in the transcriptome and proteome networks in response to c-Abl-mediated oxidative stress.

C 078 Laminin-derived peptide C16 regulates gene expression and enhances invasion of metastatic MDA-MB-231 breast cancer cells

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Background: Breast cancer constitutes a worldwide health problem. Cancer cells are engaged in a complex interplay with extracellular matrix. Evidences have shown that peptides derived from laminin are involved in tumor behavior. Among them, C16 (KAFDITYVRLKF), derived from laminin-111 gamma-1 chain, increases cell migration, enhances metastasis, and promotes angiogenesis. These findings prompted us to investigate C16 regulation of gene expression in metastatic breast cancer cells (MDA-MB-231).
Observations: By microarray, we analyzed role of C16 regulating gene expression in MDA-MB-231 breast cancer cells. We also investigated effect of this peptide on cancer-related phenomena, such as cell proliferation, apoptosis, and invasion. MDA-MB-231 cells were treated by C16 (100microgram/ml) or C16SX scrambled peptide control. Total RNA was extracted, biotin-labeled cDNA was generated and hybridized to Human Gene 1.0 ST Array (Affymetrix). GeneChips were stained and scanned. Expression Console software converted gene fluorescence intensities into numerical values. MDA-MB-231 cells expressed 80 genes significantly regulated ($p < 0.05$) by at least 1.2-fold in response to C16 peptide. Among them, 33 were cancer-related genes (validated by real-time qPCR). These cancer-related genes were mostly associated to invasion, proliferation and apoptosis. To correlate gene expression with function, we analyzed whether C16 would regulate invasion, proliferation and apoptosis in MDA-MB-231 cells. Functional assays indicated that C16 significantly increased cell invasion in Boyden chambers, but had no effect on cell proliferation and apoptosis.
Conclusions: We conclude that the laminin-derived peptide C16 regulates gene expression and enhances invasion of metastatic breast cancer cells.

C 079 Blockade of adenosine A2B receptor prevents glomerulosclerosis in streptozotocin-induced diabetic rats

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Background: Diabetic nephropathy (DN) is characterized by renal alterations leading to progressive proteinuria, glomerulosclerosis and loss of renal function. It is the first cause of renal transplants worldwide. Notably, patients suffering from DN exhibit increasing plasma levels of adenosine. In streptozotocin (STZ)-induced diabetic rats, the extracellular glomerular adenosine concentration is increased. Our aim was evaluate the possible pathogenic role of adenosine receptors (AR) in the DN.
Observations: We performed in vitro studies in rat mesangial cells in order to evaluate the role of AR in myofibroblast transdifferentiation (MTD). The increment in the expression of alpha-smooth muscle actin (alphaSMA) and fibronectin were used as markers of MTD. The use of an A2B AR antagonist inhibited cell MTD induced by transforming growth factor-beta 1. In contrast, an overexpression of A2B AR increased the expression of MTD markers. Immunohistochemical analyses in STZ-induced diabetic rats revealed an increased expression of A2B AR in mesangial cells. To further characterize the pathogenic role of this receptor in progression of DN, we administered MRS1754, a selective A2B

AR antagonist, to diabetic rats at doses of 0.2 and 1.0 mg/Kg for 15 days. Rat treatments began at three weeks post-induction of diabetes. Both treatments blocked MTD of mesangial cells by 85% compared to vehicle-treated rats. In a similar way, overproduction of the vascular endothelial growth factor (VEGF), a marker for podocytopathy, was inhibited in glomeruli of diabetic rats. As a consequence, parameters of renal function recovered to normal values.

Conclusions: The A2B AR is implied in MTD of mesangial cells, a requisite for progression of glomerulosclerosis in DN. The pathogenic effect of the low affinity A2B AR is in accordance with the rising adenosine levels seen in this pathology. We identify A2B AR as a potential new therapeutic target for DN.

C 080 Selective antileukemic effects of new iron(III) and nickel(II) chelates of S-methyl-thiosemicarbazones on K562 and ECV304 cell lines

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Background: Thiosemicarbazones have a wide range of biological activity. Metal complexes of thiosemicarbazones are a class of compounds presenting some biological applications as antiviral, antibacterial and antitumor depending on the parent aldehyde, ketone and metal ion. 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (Triapine) that was shown antitumor effects in preclinical experiments is being evaluated in phase I and II clinical trials.

Observations: We synthesized the iron(III) and nickel(II) chelates of some hdroxy or methoxy-substituted N1,N4-diarylidene-S-methylthiosemicarbazones which are in the [Fe(L)Cl] and [Ni(L)] general formula. Thiosemicarbazones were characterized by elemental analysis and magnetic measurements, 1H-NMR, UV-Vis, IR and mass spectroscopy. Cytotoxicity experiments were done using K562 chronic myeloid leukemia cells and ECV-304 endothelial cells by [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay. The concentration of compounds that provides 50% inhibition cell growth (IC50) were calculated from dose-response curve. The selective effect of thiosemicarbazones were evaluated by comparing the IC50 values of both of cell lines. Caspase-3 ve cythocrome-C activation were determined in cells treated in IC50 of thiosemicarbazones. All of iron(III) chelates of N1,N4-diarylidene-S-methylthiosemicarbazones and some of nickel(II) chelates have selective antileukemic effects in K562 cells while it has no effect in ECV304 cells in the same concentrations. Caspase-3 ve cythocrome-C activity of K562 cells were significantly increase by compared with controls no compounds.

Conclusions: Our findings indicate that selective cytotoxic potential of the chelates depends on metal ion, hydroxy and methoxy substituents, and also substituent locations. We are thinking possessing a drug potential of the new N1N4-diarylidene-thiosemicarbazone chelates which have shown antileukemic effect.

C 081 A repressive EWS-FLI1 subsignature is the result of FOXO1 suppression and nuclear exclusion

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Background: The EWS-FLI1 chimeric protein, characterizing Ewing´s sarcoma (ESFT), is prototypic for an aberrant oncogenic ETS transcription factor. The mechanisms of transcriptional regulation leading to ETS-driven tumorigenesis are poorly understood.

Observations: In-silico analysis of time-resolved expression data revealed enrichment of recognition motifs for forkhead box (FOX) proteins in EWS-FLI1 repressed promoters. Several FOX genes were found to be bound by EWS-FLI1 in ChIP-seq and transcriptionally affected by EWS-FLI1 knockdown. We therefore hypothesized that EWS-FLI1 exerts an important part of its repressive activity via inhibiting FOX proteins. Upon silencing of EWS-FLI1, both FOXO1 and FOXO3 proteins were strongly induced in ESFT cells consistent with this hypothesis, but only FOXO1 translocated to the nucleus. However, in the presence of EWS-FLI1, ectopic FOXO1 is excluded from the nucleus as a consequence of phosphorylation. Nuclear translocation was restored by either inhibition of CDK2, augmented by chemical inhibition of PI3K, or by mutation of CDK2 or AKT phosphorylation sites. Functional restoration of nuclear FOXO1 expression in ESFT cells resulted in impaired proliferation and significantly reduced soft agar colony formation ability. To test the contribution of FOXO1 to the EWS-FLI1 repressive signature on the ESFT transcriptome a significant overlap between EWS-FLI1 repressed and FOXO1 activated genes was identified.

Conclusions: Taken together, these data confirm our hypothesis that a repressive sub-signature of EWS-FLI1 regulated genes is due to suppression of FOXO1 and that FOXO1 plays an important role in ESFT oncogenesis.

C 082 Overcoming the cisplatin resistance in NSCLC cells by 5-aza-2'-deoxycytidine treatment

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Background: Lung cancer is the leading cause of cancer-related mortality around the world. Although chemotherapy has an important role in the treatment of non-small cell lung cancer (NSCLC), clinical response is still unsatisfactory. Cisplatin is widely used in the treatment of NSCLC; however, its efficacy is limited by drug resistance. We aimed to overcome cisplatin resistance by 5-aza-2'-deoxycytidine (Aza) treatment and reveal the gene expression profiles in treated/untreated cell lines.

Observations: Calu1 and A549 cells were treated for six months with cisplatin to obtain 25-times resistant CR-Calu1 and 6.55-times resistant CR-A549 cell series. We observed that, cisplatin

IC50 levels for the A549, CR-A549, Calu1 and CR-Calu1 cell series were 30,26 microM; 198,2 microM; 13,68 microM and 343,5 microM, respectively. A real-time ready custom array was designed for the quantification of 88 different genes that have function in apoptosis, cell cycle, signal transduction, DNA repair and cisplatin influx/eflux. We have found that expression levels of cell cycle genes especially cyclin and cyclin-dependent kinases (CDC2, CDK4, cyclinB1, cyclinB2, CDC25C), DNA repair (MLH1, ERCC8) and apoptotic genes (Caspase 4, Caspase 6, BAD, BID) decreased significantly in both cisplatin resistant cell lines. After treatment with Aza on days 1, 3, 5; cells were returned to regular medium without Aza on day 6. Total RNA preparation was performed and WST assay was set up on day 8. After Aza treatment most of the gene expression levels were upregulated and cisplatin IC50 levels were decreased significantly in A549/Aza (12,96microM; p = 0,0008), CR-A549/Aza (133,8microM; p = 0,0176) and CR-Calu1/Aza (86,19 microM; p = 0,0086) cells.
Conclusions: During the development of cisplatin resistance most of the gene expression levels were decreased significantly and Aza treatment reversed the cisplatin sensitivity via restoring these expressions. Aza could lead to improved therapy for cancer patients with better and more durable clinical responses.

C 083 Evaluation of non-CpG DNA methylation in human neoplasia with respect to biomarker development

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Background: Cytosine methylation (5MeC), an epigenetic event, is a well known DNA modification in vertebrate genomes and occurs exclusively in a CG context. However, a recent study revealed about 17% of 5MeC in the CHG- and 7% in the CHH-context in human embryonic stem cells. Motivated by that finding we investigated the presence of CHG/CHH methylation in DNA derived from tumor cells to examine the relevance in tumorigenesis and to check whether those 5MeC could be used for biomarker development.
Observations: We employed DNA from the MCF-7 breast cancer cell line (8 replicates), DNA from childhood ALL (acute lymphatic leukemia, n = 8) and DNA from blood of healthy people (n = 8) for the methylation analyses. CHG/CHH sensitive digestion was done with the methylation sensitive restriction enzyme PspGI and the methylation-insensitive isoschizomer BstNI (cut site: CCWGG). Those two restriction enzymes allow the detection of methylation patterns in the non-CpG context. Subsequently, the digested DNAs were subjected to multiplex PCR and methylation profiles read out upon hybridization onto targeted microarrays. This approach allows the simultaneous evaluation of 270 genes harboring at least one PspGI/BstNI cut site and thereof the elucidation of DNA-methylation in the CHG/CHH-context. Class comparison of chip-data derived from BstNI and PspGI digests elucidated several significant genes with supposed non-CpG methylation of DNA from the MCF-7 breast cancer cell line as well as in ALL samples. However, qPCR analyses of these marker-candidates did not confirm the microarray results.
Conclusions: CHG/CHH methylation is not suitable for biomarker development. Based on our results it is unlikely that non-CpG methylation of human embryonic stem cell is maintained postnatally as well as there is no retroregression of the methylation pattern to a stem cell like character in neoplastic cells.

C 084 DLX1, a putative downstream target of FOXM1, in regulating ovarian tumorigenesis

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Background: FOXM1 has been reported in various human cancers. Our pervious study has found that FOXM1 is associated with the progression of ovarian cancers. The overexpressed FOXM1 is able to enhance cell growth, cell migration and invasion in ovarian cancer cells. However, the downstream targets of FOXM1 linking to these tumorigenic properties are still largely unknown. Here we report that DLX1 is a novel target of FOXM1 involved in promoting cell growth and migration in ovarian cancer cells.
Observations: By computational analysis on FOXM1 binding site in human genome, we identified DLX1 as one of the putative downstream targets of FOXM1. By biochemical analysis, enforced expression of FOXM1B or FOXM1C elevated DLX1 in both protein and mRNA levels. Conversely, depletion of FOXM1 by either RNAi or Thiostrepton (FOXM1 inhibitor) reduced the expression of DLX1. Additionally, both FOXM1B and FOXM1C were able to upregulate DLX1 promoter activity in a dose-dependent manner by transient luciferase reporter assay. Notably, our quantitative RT-PCR showed that the DLX1 expression was significantly correlated with the FOXM1 expression in ovarian clinical samples (P = 0.001). Functionally, enforced expression of DLX1 could increase cell growth and cell migration rates in SKOV3 and OVCA433 cells by XTT cell proliferation and wound healing assays.
Conclusions: Taken together, our findings suggest that DLX1 acts as a downstream target of FOXM1 transcription factor. The oncogenic capacity of DLX1 indicates DLX1 may be a therapeutic target in ovarian cancer.

C 085 Glycogen storage disease type 2 (Pompe disease) in Germany: wide spectrum of genotypes and phenotypes

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Background: Glycogen storage disease type II is caused by deficiency of lysosomal glucosidase (GAA), resulting in impaired degradation of glycogen. The early-onset form manifests shortly after birth and presents with progressive and generalized muscle weakness. Cardiac and respiratory insufficiency leads untreated to death before 2 years of age. Patient with late-onset (juvenile/adult) forms present with skeletal muscle weakness and respiratory failure and sometimes cardiac problems later in life.
Observations: Mutations at the locus of lysosomal alpha-glucosidase gene were investigated in 55 unrelated patients during past 10 years. Their age at the time of diagnosis ranged from 3 weeks to 77 years. The diagnosis was confirmed by biochemical

and molecular analysis. We were able to define the molecular defect in 97% of mutated alleles. By analysis of 40 German patients, the allele frequency of mutations were as follow: IVS1-13T > G (25%), c.525delT (13%) and delExon18 (10%). 14 mutations were novel: c.719C > T, c.877G > A, c.1564G > A, c.1703A > T, c. 1802C > T, c.1829C > T, c.1859G > A, c.1912G > A, c.2214G > A, c.1050-1051delG, c.1127-1130delG, c.1364-1369delG, IVS3 + 2T > C, IVS6-2A > G, and IVS18-2A > G. The analysis of 15 Turkish patients living in Germany revealed that the most common mutation was the double insertion 2741insC2743insG (39%) and 3 were novel, c.896T > C, 1064T > C and IVS11-2A > G. Two patients compound heterozygous for IVS1-13T > G/c.1050-1051delG and IVS1-13T > G/c.2214G > A respectively showed elevated CK and liver enzymes and a trace of residual enzyme activity but no clinical presentation at infancy. One adult patient homozygous for IVS1-13T > G presented reduced tendon reflexes and myopathic EMG.

Conclusions: The spectrum of mutations can be correlated with the GAA activity, which again reflects the clinical severity. Patients carrying the IVS1-13T mutation present the residual GAA activity and mild phenotypes. Nevertheless the mutation represents a significant involvement in clinical manifestation.

C 086 Protective effects of neutral endopeptidase in mouse model of caerulein-induced acute pancreatitis

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Background: Acute pancreatitis (AP) is characterized by rapid inflammation of the pancreas. Increased substance P (SP) levels and expression in the system have been identified to be important in driving pro-inflammatory processes of AP. However, little is known about mechanisms that regulate SP expression during AP. Neutral endopeptidase (NEP) is well known to degrade SP. Therefore in this study, we investigated the expression of NEP during the course of AP and evaluate its role in caerulein-induced AP.

Observations: Balb/c mice (Male, 20-25g) were subjected to hourly intraperitoneal injections (0-10 hours) of caerulein to induce experimental AP. Injecting caerulein for 3-10 hours decreased NEP enzymatic activity and its protein expression in the pancreas by up to 50%, when compared to their respective saline injected controls. To assess the role of NEP, we treated the mice with a single dose of mouse recombinant NEP (1mg/kg, i.v.) during the second caerulein injection. Both pancreatic and plasma NEP activity was significantly increased after treatment with recombinant NEP, accompanied with decreased SP levels. NEP treatment caused a decrease in plasma amylase activity, indicating a reduction of pancreatic injury. Indeed, histological examination of pancreatic tissue sections showed markedly reduced edema and cellular damage. Moreover, treatment with NEP also reduced myeloperoxidase activity in the pancreas, indicating less neutrophil infiltration. Experiments on pancreatic cytokine/chemokine profile using ELISA also showed marked reductions in IL-1, IL-6, TNF-a, MIP1-a, and MIP-2 levels. Interestingly, NEP treatment also abolished the elevated SP receptor mRNA expression.

Conclusions: This report, for the first time, shows a reduction of NEP activity during experimental AP, which contributes to elevated SP levels and aggravated inflammatory condition. Treatments that increase NEP activity may be a viable option to manage AP and other inflammatory diseases by modulating SP levels.

C 087 Adipokine resistin is a key player to modulate monocytes, endothelial cells, and smooth muscle cells, leading to progression of atherosclerosis in rabbit carotid artery

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Background: Resistin is an adipokine first identified as a mediator of insulin resistance in murine obesity models. But, its role in human pathology is under debate. Although a few recent studies suggested the relationship between resistin and atherosclerosis in humans, the causal relationship and underlying mechanism have not been clarified.

Observations: We cloned rabbit resistin, which showed 78% identity to human resistin, and its expression was examined in 3 different atherosclerotic rabbit models. To evaluate direct role of resistin on atherosclerosis, collared rabbit carotid arteries were used. Histological and cell biologic analyses were performed. Rabbit resistin was expressed by macrophages of the plaque in the 3 different atherosclerotic models. Peri-adventitial resistin gene transfer induced macrophage infiltration and expression of various inflammatory cytokines, resulting in the acceleration of plaque growth and destabilization. In vitro experiments elucidated that resistin increased monocyte-endothelial cell adhesion by upregulating very late antigen-4 on monocytes and their counterpart vascular cell adhesion molecule-1 on endothelial cells. Resistin augmented monocyte infiltration in collagen by direct chemoattractive effect as well as by enhancing migration toward monocyte chemotactic protein-1. Administration of connecting segment-1 peptide, which blocks very late antigen-4 × vascular cell adhesion molecule-1 interaction, ameliorated neointimal growth induced by resistin in vivo.

Conclusions: Our results indicate that resistin aggravates atherosclerosis by stimulating monocytes, endothelial cells, and vascular smooth muscle cells to induce vascular inflammation. These findings provide the first insight on the causal relationship between resistin and atherosclerosis.

C 088 Long lasting enhancement of sensory neuron input strength mediated by olfactory learning

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Background: In mammals, odorant molecules are sensed by odorant receptors expressed by olfactory receptor neurons (ORNs). ORNs select only one receptor out of a large possible repertoire and their axons converge in a receptor specific manner onto specific loci called glomeruli of the main olfactory bulb (OB). Even though, odorants are known to activate different combinations of ORNs and associated glomeruli, the question if these representations can display functional plasticity remains unclear.
Observations: In order to study the physiological impact of olfactory learning at the level of the glomerular maps, we compared the intrinsic optical signals(IOS) of odor-evoked activity from the dorsal OB in trained and untrained awake mice. Mice were trained on a go/no-go operant conditioning paradigm to discriminate different dilutions, ranging from 1% to 10-10, of monomolecular odors, cineol (C) vs. eugenol (E) and Isoamylacetate (IAA) vs. ethylbutyrate (EB). Discrimination accuracy and speed were accessed as the behavioral parameters. Mice discriminated different dilutions of C vs E, from 1% to 10-2 with similar speeds where as they showed the learning from 10-3 to 1%. For IAA vs EB, the concentration for achieving the maximum speed was further optimized to 10-4 where as they started to discriminate from 10-6 towards higher concentrations. The discrimination training caused an enhanced sensitivity, shown by evoked IOS, towards the learned odorants for all odor pairs tested even after several weeks since the last training session. The faster discrimination speed and the maximum accuracy achieved at lower concentrations may have resulted from the enhanced sensitivity at the ORN level.
Conclusions: We conclude that olfactory learning cause long lasting functional plasticity of ORN inputs which helped the mice to achieve fast and accurate odor discrimination with an optimized input strength reaching the OB.

C 089 Activation and silencing screens reveal neurons that direct Drosophila melanogaster walking behavior

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Background: Insects show remarkable adaptability and flexibility in their walking behavior. Although considerable progress has been made in understanding the mechanisms of motor control and central pattern generators, very little is known about how higher order neuronal centers control these motor circuits to determine the walking direction. In the current work we aimed to exploit the power of Drosophila genetics to identify these higher order neuronal centers.
Observations: In order to identify such neuronal elements, we have conducted screens for flies in which walking direction is altered upon activation or silencing of specific neurons. These screens employ either the thermosensitive cation channel dTrpA1 or tetanus toxin light chain (TNT), respectively, which are expressed using a collection of ~3000 GAL4 lines that target sparse populations of neurons. Several dozen lines (hit rate of ~1%) have been identified in each screen, some in both, that result in altered walking direction without a dramatic effect on the coordination or pace of walking. In addition to these specific directional walking phenotypes, with the help of automated tracking software, we have been able to identify and quantify a variety of locomotion phenotypes, ranging from no locomotion or uncoordinated walking to increased walking. Using intersectional genetic and stochastic labeling approaches, we have identified the specific neurons that contribute to these walking abnormalities.
Conclusions: Thus we have identified potential higher order neurons responsible for directional control of walking. These studies provide an entry point into mapping and ultimately characterizing the neuronal circuitry responsible for higher order control of walking behavior in Drosophila melanogaster.

C 090 Uncoupling protein expression in retinal cells

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Background: Several mitochondrial uncoupling proteins (UCP2, UCP4, UCP5) were detected in the central nervous system at the mRNA level. We demonstrated previously that UCP4 is present in brain and spinal cord also at the protein level. Under normal conditions UCP4 is expressed in neurons, astrocytes, hair cells of the Organ of Corti and mechanosensitive Merkel cells of the skin (Smorodchenko et al., 2009; 2011).
Observations: Based on these findings, we hypothesized that UCP4 expression is a common feature of both neuronal and neurosensory cells. To test this we compared UCP4 protein expression in the mature rodent retina and brain at physiological conditions using Western blot (WB) and immunohistochemistry (IH). WB revealed the UCP4-specific bands of 36 kDa in total tissue retina extracts. IH detected strong UCP4 signal in retinal regions abundant in mitochondria: inner segments of rods and cones, outer and inner plexiform layers, inner nuclear layer and ganglion cells. In all cases UCP4 expression was associated with mitochondrial markers COXIV and VDAC.
Conclusions: The hypotheses about a putative common function of UCP4 in neuronal and neurosensory cells are discussed.

C 091 Sensory neuron differentiation in the Drosophila olfactory system

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Background: In the olfactory system, sensory neurons are characterized by the expression of a single type of odorant receptor (OR) and axonal connections to a single synaptic glomerulus, thereby providing the anatomical basis for odor recognition in the brain. In contrast to the mouse olfactory system, the Drosophila odorant receptors are dispensable for the development of synaptic specificity, raising the question of how these two differentiation programs are coordinated.
Observations: In a mosaic screen for genes that control Drosophila olfactory receptor neuron (ORN) differentiation we identi-

fied several transcription factors that regulate distinct aspects of ORN development. Here we describe the functional characterization of Sequoia and Psc in the specification of three lineage-related ORN classes. In Psc mutants these three ORN classes show a mixing of their axonal projection inside their synaptic glomeruli and a switch in OR expression. Interestingly, the level of Psc in the ORN precursors determines their OR class identity. In contrast to the switch in OR expression in psc mutants, loss of Sequoia leads to the co-expression of two ORs in a single sensory neuron.

Conclusions: Further analysis suggests that Psc and Sequoia function in a sequential manner in ORN development, in which Psc determines the OR-sensory type and Sequoia subsequently restrict the OR expression to a single neuron type.

C 092 Neuronal circuits for Drosophila courtship song

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Background: Many animals use acoustic signaling in social interactions. Drosophila melanogaster males produce a courtship song by vibrating one wing. It consists pulse trains with a characteristic interpulse interval (IPI) and number of cycles per pulse (CPP). Singing has been causally linked to the activity of the set of neurons that express sex-specific transcripts of the putative transcription factor fruitless (fru), but the specific neurons involved had not been identified.

Observations: We screened 800 GAL4 lines with expression overlap with fru, driving expression of the neuronal activator dTrpA1 in the intersecting cells. We identified five neuronal classes that trigger or compose song. Acute activation of the central brain neuronal class P1 or the descending interneuron pIP10 elicited song production of a male fly in the absence of a female. Silencing these brain neurons severely reduced song production during courtship. In the ventral nerve cord (VNC), activity of the neuronal classes dPR1 and vPR6 was both necessary and sufficient for pulse song. The strength of experimental activation of vPR6 influenced the IPI. Activation of the neuronal class vMS11 elicited the unilateral wing extension characteristic for singing. Silencing of vMS11, possibly together with two other fru neuronal classes led to an increase of the CPP. Anatomical data suggest that the song neurons are interconnected. Preliminary data indicates that fru positive central pattern generator components connect to fru negative wing motor neurons shared by distinct programs for song and flight.

Conclusions: We conclude that stereotyped fru positive neuronal classes control the generation of Drosophila courtship song. They are likely to constitute a functional circuit composed of a hypothetical action selection center in the brain connected to a central pattern generator in the VNC.

C 093 Characterization of the Piccolo protein variant associated with major depressive disorder

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Background: A recent Genome-wide association study has shown involvement of the Piccolo gene in Major Depressive disorder. Piccolo is an active zone protein involved in active zone assembly, synaptic vesicle trafficking and release. The variant of Piccolo associated with major depression produces serine to alanine substitution in the C2A domain of the protein.

Observations: We have created a knock-in mouse model that carries the risk allele for major depression, to characterize the phenotype associated with this gene variant. Using proteomics tools, we gained some preliminary data on differences in protein binding partners. We are using both native Piccolo and recombinant C2 domains of Piccolo to characterize the properties of the two variants. C2A domain of piccolo is known to exhibit calcium-dependent phospholipid binding. We present results of in vitro liposome binding experiments that compare calcium and lipid binding properties of the two C2A domain variants.

Conclusions: As result of this investigation we hope to gain understanding of the mechanisms underlying contribution of Piccolo to major depression pathogenesis and susceptibility.

C 094 Expression of galanin receptor 2 and galanin receptor 3 in ductal cells of human sweat glands

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Background: Neuropeptide galanin is widely expressed in the brain and the periphery including the skin. Galanin's effects are mediated by three different galanin receptor subtypes. In human dermal sweat glands, galanin-like immunoreactivity and galanin binding sites have been detected suggesting a putative role in sweat gland function. The aim of the present study was to determine the galanin receptor subtype expressed in dermal sweat glands.

Observations: The human sweat gland cell line NCL-SG3 expresses mRNA of GalR2 and GalR3 but not GalR1. Because, for none of the commercially available galanin-receptor antibodies subtype-specificity been demonstrated, we first evaluated several commercially available human galanin-receptor antibodies. Cell lines stably transfected with the respective human galanin receptor subtypes served as positive controls. After antibodies had been validated for galanin-subtyp specificity they were used for immunoblotting and immunohistochemistry. Western blot analysis of whole cell lysates of NCL-SG3 cells confirmed the presence of GalR2 and GalR3 in these cells. Immunohistochemistry performed on paraffin embedded cultured NCL-SG3 also showed presence of GalR2 and GalR3, but not GalR1. Similar results were found in human skin biopsies, where eccrine sweat glands stained positive for GalR2 and GalR3, but not for GalR1.

Conclusions: This is the first study, which has demonstrated subtype specificity for certain galanin-receptor antibodies. Using these antibodies, we could show that GalRs are present on ductal cells of human sweat glands, indicating an involvement of GalR2 and GalR3 in sweat gland function.

C 095 Dissection of the interaction between the memory-related KIBRA protein and dynein light chain LC8

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Background: Expression of the KIBRA protein is crucial for memory formation and the development of neurodegenerative diseases. In neurons, KIBRA is enriched at postsynaptic sites where it interacts with the actin-binding proteins Synaptopodin and Dendrin. Additional KIBRA binding partners include protein kinase M (PKM) zeta as well as the dynein light chain LC8. LC8 was initially discovered as part of the dynein motor complex and was thought to act mainly as a cargo adapter.
Observations: Recent findings suggest that LC8 is also able to stabilize multiprotein complexes by promoting dimerization of its binding partners. Because the intracellular transport of KIBRA is mainly dynein-independent, we examined the functional role of the interaction with LC8 in more detail. Using in vitro binding assays and yeast two-hybrid experiments we were able to map two distinct LC8 binding motifs within the KIBRA molecule. The sequence of the two binding motifs is highly conserved in evolution and is also present in the two KIBRA-related proteins WWC2 and WWC3, respectively. Mutation analysis revealed that only dimeric LC8 is able to associate with KIBRA and that this association is controlled by LC8 phosphorylation.
Conclusions: In summary, our data indicate that LC8 is a molecular hub for KIBRA dimerization. Thus, LC8 could serve as a regulatory element in neurons mediating assembly/disassembly of structural protein networks containing the KIBRA dimer.

C 096 Sensory axon targeting in the olfactory system requires N-acetyltransferase (NatB) subunit Psidin

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Background: Odors elicit specific neural and behavioral responses using a neural network of peripheral receptor neurons that innervate receptor class specific so-called glomeruli in the antennal lobe (AL) in the central brain. Reaching the AL, these olfactory receptor neurons (ORN) segregate in an ORN-specific manner, so that ORNs expressing a certain OR are targeting a unique and stereotyped glomerulus. Psidin is an actin-regulatory protein competing with Tropomyosin-1 for the binding of F-actin.
Observations: We have identified a new hypomorphic allele, psidIG978. Using MARCM analysis in psidIG978 mutants, we identified a subset of ORN classes (e.g. OR47a, 59c and 42a) showing a strong defasciculation targeting phenotype and ectopic glomerular innervation. In the case of Psid-LOF allele, psid1, the defasciculation phenotype is enhanced in OR59c neurons. In addition in psid1 MARCM analysis, we observe a reduction of ORNs of about 40% scoring for OR83b positive cells not seen in the hypomorphic allele. Using forward and reverse MARCM, we find that some classes require the gene cell-autonomously whereas other classes require it non-autonomously. This led us to hypothesize that Psid is involved in several steps of development and targeting of ORN classes and determines their axon growth route together with their ability to recognize the target glomeruli. We show that Psid and CG14222 interact in vivo and in vitro. Using RNAi to reduce the levels of CG14222 in the psidIG978 background, we found a reduction in ORN number similar to the Psid-LOF never observing an axon targeting defect similar to the psidIG978 allele. The psidIG978 protein still interacts strongly with CG14222 in vitro.
Conclusions: We show that mutations in Psid lead to ORN class specific wiring defects in the Drosophila olfactory system (OS). Psid has two independent functions during OS development: Early in neurogenesis, functioning together with CG14222 (NatB-complex), later in axon targeting independent of NatB-activity.

C 097 Neuronal Recognition Identity in Olfactory System Development

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Background: In the Olfactory system, axons from multiple sensory neurons segregate into specific synaptic glomeruli in the brain depending on the type of odorant receptor expressed. The molecular machinery regulating this inter-neuronal recognition is not understood. In a genetic screen to identify molecules affecting olfactory neuron connectivity in Drosophila, the cell-adhesion molecule Dscam was identified.
Observations: Dscam mutant sensory neurons mis-target to form ectopic glomeruli. This indicated that Dscam does not mediate inter-neuronal recognition as such but can regulate it, spatially or temporally. Further experiments have now shown that these ectopic glomeruli have sensory neurons of same class and can form with or independent of cognate post-synaptic partners. These observations added with clonal analysis of Dscam mutant single neuron have shown that Dscam function is cell intrinsic and is independent of neighbouring neurons. Over-expression of a single Dscam isoform does not affect neuronal development but prevents axonal convergence at the target glomeruli, confirming a specific regulation of recognition identity. Developmentally, Dscam regulates the formation of proto-glomeruli by sensory neurons while entering the target area. We are now investigating the role of Dscam isoforms and intra-cellular signalling in regulating inter-neuronal recognition. We also aim to find intra-cellular modulators of Dscam function by doing a genetic modifier screen.
Conclusions: Our experiments have suggested a cell intrinsic role of Dscam in regulating inter-neuronal recognition as opposed to its documented function in mediating repulsion between neurites of a same neuron. We are now investigating the role of Dscam diversity in inter-neuronal recognition.

C 098 Rodent Dynactin p150Glued Mutant Characterization and Novel Dynactin1 Interaction with Tubulin Binding Cofactor B (TBCB)

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Background: Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that affects specifically motor neurons, leading to muscular paralysis and death. About 10% of cases are familial while the dominant 90% are sporadic. A dynactin p150 mutation (G59S) has been shown to cause a variant of ALS, targeting only lower motor neurons. TBCB, one of a group of tubulin chaperones or cofactors, participates in tubulin dynamics with great functional diversity and regulation of microtubules in cells.

Observations: Here, we characterize 5 mutations identified in dynactin p150-mediated ALS patients from generated clones, in view of depicting whether they are causal or risk factors to the disease. We also examine whether p150 mutations were homologous in humans and rodents. We further investigate the role of TBCB in the central nervous system through overexpression and repression. We observed that the D63Y mutation is a risk factor to ALS. The investigated mutations were homologous in both humans and rodents. Based on a yeast-two-hybrid screen, we also show that dynactin p150 is an interacting partner of TBCB. This interaction was validated with colocalization and pull-down experiments. Overexpression and downregulation of TBCB has no effect on synapse and dendrite propagation as well as on dendrite branching in hippocampal cultures.

Conclusions: Mutations in dynactin p150 stand as potential risk factors in developing ALS .The dynactin p150 and TBCB interaction suggests an impact in cellular mechanisms that may involve transport via cytoskeletal proteins.

C 099 Extracellular heparan sulfate oligosaccharides affect cell adhesion and polarization

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Background: Mucopolysaccharidosis III (MPSIII, Sanfilippo syndrome) is a lysosomal storage disease characterized by the accumulation of heparan sufate (HS) oligosaccharides in brain cells and extra-cellular environment, which induces mental retardation and neurodegeneration in children. Mechanisms mediating HS oligosaccharides deleterious effects on brain cells are not well understood. Neuro-inflammation is a consequence rather than the cause of neurodegeneration.

Observations: Heparan sulfate bound to the extra-cellular matrix (ECM) are essential for adequate fibroblast growth factor (FGF) signaling and integrin functions. Our recent observations indicate that FGF-2 signaling is altered in MPSIII cells, integrin clusters are formed at the cell surface that co-localized with HS immuno-reactivity, and focal adhesion components are over-expressed. Adhesion of deficient astrocytes is increased. These results suggested that HS oligosaccharides present in the ECM affect cell sensing of extra-cellular cues. Consistently, cell polarity was defective in oriented migration assays performed in deficient astrocytes and neural stem cells, and neuron polarity was defective in co-cultivation assays with astrocytes. We showed that polarization defects were related to extra-cellular components. Clearance of HS oligosaccharides through enzyme digestion rescued normal phenotype. Addition of HS oligosaccharides purified from patient's urines to unaffected cells induces the MPSIII phenotype.

Conclusions: HS oligosaccharides affect cell-matrix interactions with consequences on cell adhesion and polarity. Accumulation of these compounds in affected newborn brain may have deleterious consequences on central nervous system post-natal development which likely account for the clinical manifestations.

C 100 Ephexin, a Rho specific GEF plays a role in olfactory system development

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Background: The olfactory system of Drosophila offers an excellent opportunity to study the molecular mechanisms of neuronal wiring specificity. A class of molecules involved in axon guidance in different nervous systems is Eph receptor and ephrin ligand. Ephexin acts as a Rho specific GEF downstream of Eph/ephrin signaling for re-arranging the actin cytoskeleton during axon targeting. We addressed whether Eph, ephrin and/or Ephexin play a role in a dis-continuous and non-graded olfactory system.

Observations: ORN axons are sorted by bundling together to target properly. A repulsive cue (like Eph/ephrin signaling) could play a role in the process of sorting. We analyzed previously published mutants of the Eph receptor, ephrin ligands and Ephexin. The requirement of these molecules for wiring specificity of ORNs was analyzed by knocking them down and visualizing the targeting of specific ORN classes. Although the study is still under progress, we found that these molecules are involved in the correct targeting of the ORNs. The requirement of these molecules for wiring specificity is restricted to certain ORN classes. We further observed that the mistargeting phenotype seen with specific ORN classes is different in the background of Eph and Ephexin mutations, respectively, with ephrin mutation showing only a very weak phenotype. MARCM analysis of Ephexin and rescue experiments, suggest that it might not be required cell-autonomously in the ORNs for correct targeting. All these molecules seem to be expressed during crucial stages of development suggesting their potential role.

Conclusions: From the evidences we have until now, our study indicates an involvement of these molecules in olfactory targeting of Drosophila in a very specific manner. Additionally the requirement of Ephexin is non-cell autonomous for correct targeting of ORNs.

C 101 Expression level of the nuclear receptor TLX in the anterior pituitary and pituitary adenomas

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Background: Multipotent adult stem cells in the mature anterior pituitary have been shown to generate all subtypes of endocrine cells of the pituitary gland. A small fraction of these stem cells can also be found inside of pituitary tumors and may represent tumor stem cells. It could be shown that TLX is crucial for the self-renewing potential of stem cells. Transgenic overexpression causes expansion of neuronal stem cells and initiation of brain tumor formation.
Observations: Since TLX is present in different parts of the human brain we investigated the expression of this receptor in the pituitary gland and its neoplasms by quantitative real time PCR and immunofluorescence staining. For the first time, it could be shown that TLX is expressed in the human anterior pituitary. Additionally, ten out of 29 pituitary adenomas (non functioning adenomas n = 15, growth hormone-secreting n = 7, ACTH-secreting n = 4, prolactin-secreting n = 3) exhibited TLX expression of various extent. TLX does not seem to play an important role in the formation of neuroendocrine tumors located at other endocrine glands. Only two (one medullary thyroid carcinoma and one pheochromocytoma, respectively) out of 14 tested endocrine tissues and tumors showed a moderate TLX expression.
Conclusions: Based on previous findings and our initial data it is likely that TLX plays an important role in the maintenance of the stem cell character in the human pituitary gland and might be to some extent involved in adenoma formation.

C 102 Dopamine neurons modulate pheromone responses in Drosophila courtship learning

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Background: Learning through experience allows animals to adapt even their most instinctive behaviours to the local environment. Naive Drosophila melanogaster males, for example, instinctively court both virgin and mated females, even though only virgins are likely to accept them. Through courtship experience, however, males learn to selectively suppress the futile courtship of mated females. The aim of this study is to identify molecular and neuronal factors involved in this courtship learning.
Observations: We show here that courtship learning reflects an enhanced behavioural response to the male pheromone cis-vaccenyl acetate (cVA), which remains on females after mating and distinguishes them from virgins. Dissociation experiments suggest a simple learning rule in which the experience of rejection enhances sensitivity to cVA, without requiring an explicit association of the two. The learning experience can be mimicked by artificial activation of dopaminergic neurons, and we identify a specific class of dopaminergic neuron in the protocerebrum that is critical for courtship learning. These neurons provide input to the mushroom body (MB) gamma lobe. The DopR1 dopamine receptor functions in the intrinsic MB gamma lobe neurons during both natural and artificial courtship learning.
Conclusions: Our work thus reveals critical behavioural, circuit, and molecular components of the learning rule by which Drosophila fine tunes its innate mating strategy.

C 103 The intracellular domain of amyloid precursor protein (APP) alters gene expression and induces neuron-specific apoptosis

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Background: Many membrane proteins, including APP and Notch, are substrates for gamma-secretase. The intracellular domains of these substrates are released from the cell membrane by gamma-secretase, and translocate to the nucleus. These observations have led to the hypothesis that mechanisms similar to Notch signaling may contribute to APP signaling which leads to Alzheimer's disease (AD). To explore APP signaling, we established EC P19 cell lines overexpressing the intracellular domains of APP (AICD).
Observations: Although neurons were differentiated from these cell lines after aggregation culture with retinoic acid treatment, AICD expression induced neuron-specific cell death. Furthermore, DNA fragmentation was detected, and TUNEL-positive cells were also Tuj1-positive neurons. Thus, we concluded that AICD can induce neuron-specific apoptosis. The effects of AICD were restricted to neurons and no effects were observed on non-neural cells. To identify the genes involved in this process of neuron-specific cell death, we employed DNA microarray analysis. The levels of expression of 41256 transcripts and transcript variants of more than 20000 independent genes were monitored by DNA microarray analysis. The expression levels of 277 genes were upregulated by more than 10-fold in the presence of AICD, as estimated from the intensity of hybridization signals. Conversely, 341 genes showed downregulation of expression to less than one tenth of the original level. Furthermore, we evaluated AICD-induced changes in expression of genes thought to be involved in cell death in AD; however, we found no significant changes in expression of these genes.
Conclusions: As mentioned, AICD alters gene expression and induces neuron-specific apoptosis. Based on these results, we will discuss the possibility that APP may play a role in signaling events and that gamma-secretase-regulated APP signaling may be responsible for the onset of AD.

C 104 Selective autophagy in prion diseases?

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Background: Prion diseases belong to the group of conformational disorders and are characterised by the pathogenic conformer of the prion protein, termed PrPSc. The ubiquitin-proteasome system (UPS) and the autophagy-lysosome pathway (ALP) provides a complementary defence mechanism for clearance of unwanted proteins and consequently reducing cell vulnerability in conformational diseases. PrPSc, as an aggregate-prone protein, is expected to stimulate these housekeeping systems in affected neurons.
Observations: Ultrastructural analysis of prion-infected neuronal cells and murine brain sections shows significant endosomal-lysosomal activity and a prominent accumulation of autophagic vacuoles containing especially degenerating mitochondria. We applied imunnohistochemistry, double labeling and immuno-electron microscopy for the subcellular localization of PrPSc, the endosomal marker Rab5, the autophagy marker p62 and ubiquitin in in vitro and in vivo prion models as well as in a human genetic prion disease. Co-localization of Rab5 and the disease-associated PrP in endosomal vesicles, and the presence of PrP in multivesicular bodies support an important role for the endosomal-lysosomal system (ELS) in the prion pathogenesis. In genetic human case, additionally to the ELS relation, we detected PrPSc accumulation in close vicinity of RER and in large cytoplasmic inclusions showed partial ubiquitin and p62 immunoreactivity.
Conclusions: Ubiquitinated PrP can be a common substrate for both UPS and p62-mediated autophagy. However, permanent production of mutant PrP overwhelms the UPS. This shifts the balance towards either selective autophagy and/or to ubiquitinated inclusions and aggresome formation, which might also cytoprotective.

C 105 Investigating the regulation of neurogenesis onset in vivo

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Background: Neural progenitor divisions can be classified according to the type of progeny that is generated. Proliferative divisions occur early in development, whereas neurons are generated later. The timing of the transition from proliferative divisions to neurogenic divisions is important because it determines neuronal output during development and, ultimately, brain size. However, the mechanisms by which a progenitor decides which type of progeny to generate remain incompletely understood.
Observations: We are using Xenopus laevis embryos to investigate neurogenesis onset in the optic tectum. At 6-8 days post fertilisation the optic tectum is a neuroepithelium composed of radial progenitor cells and a layer of recently born neurons. Using in vivo single cell electroporation and live cell imaging techniques to monitor progenitor cells at different positions within the tectum, we have found a clear separation between proliferative divisions in the posterior part of the tectum and neurogenic divisions in the anterior. Furthermore, progenitor cells move in an anterior direction as they undergo a series of divisions, suggesting that each progenitor cell switches from proliferative to neurogenic divisions at a defined point in their life history. It has been suggested that the onset of neurogenesis could be regulated through changes to the cell cycle length. To investigate this possibility in Xenopus, cell cycle length at different positions in the tectum was measured using cumulative BrdU labelling. We found that cell cycle time increases from posterior to anterior parts of the tectum, in a manner that parallels the transition from proliferative to neurogenic divisions.
Conclusions: The optic tectum is well suited for investigating the regulation of neurogenesis onset and will enable us to explore the role of intercellular signalling systems in the regulation of cell cycle length, cell division type and neurogenesis onset in a live and intact embryo.

C 106 Microfluidic systems for the reconstruction of complex oriented neuronal networks

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Background: Experimental models used to study brain development and degeneration range from whole animal models, which preserve the anatomical structures but greatly limit the experimentation at the cellular level, to dissociated cell culture systems, that allow detailed manipulation of cell phenotype but lack the highly ordered and instructive brain environment. Thus new experimental models are needed to facilitate both individual cell manipulation and brain connectivity reconstruction.
Observations: I am developing microfluidic celle culture systems to handle neurons and their neurites in micron size environments and to study in vitro the development and/or degeneration of reconstructed physiological neuronal pathways. Our systems are composed of macro-chambers – that allow the seeding and growing of different neuronal populations – separated by arrays of microchannels with designed geometries – that allow us to control the orientation of axonal outgrowth. Thus the geometries of our macro and micro patterns allow us to precisely control synaptic connections between the different neuronal populations and thus to mimic in vitro some physiological pathways. We have already demonstrated that synaptic transmissions in such neuronal networks are fully functional and we are now using a cortex-hippocampus network to study the effect of acute or chronic stress on the loss of synaptic connections.
Conclusions: Our microfluidic chips allow the reconstruction of neuronal networks in vitro. In addition the spatial orientation of axons and compartimentation of neuronal populations allow us to address independently different cell compartments: the cell body, axons and synapses.

C 107 Real-time automated recording and analysis of feeding behavior in Drosophila melanogaster

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Background: To survive and adapt to environmental changes, animals need to optimize the amount and quality of food consumed. The regulation of food intake is controlled in the

nervous system by evaluating external chemosensory information and internal feeding state of the animal. In the fly, Drosophila melanogaster, feeding state regulates attractiveness to food odours and responsiveness to sugars. After fasting, flies increase their food intake to compensate for the energy deficiency incurred.
Observations: In the last years, development of multiple genetic tools that can control neural activity has made Drosophila melanogaster an important model organism for the study of neural circuits and function. Although these genetic manipulations are very powerful, behavioral assays that measure their effects are limited because of the absence of thorough, quantitative, and automated methods. Recently camera-based methods have become available for the analysis of aggression, sexual, and social behaviors in flies. Currently there is no automated assay to analyze fly feeding behavior in which the amount of food consumed is measured directly. Here we propose a new method to measure liquid food consumed by individual flies in real time. The method works by measuring electrical capacitance in a glass-capillary filled with liquid food. When a fly consumes the liquid food, the decrease in liquid level alters the capacitance of the glass capillary that is picked up by a pair of electrodes attached and then translated into a low voltage DC signal. A data acquisition instrument reads this signal and the change in capacitance is converted to the amount of liquid food consumed in real time.
Conclusions: We are in the process of developing the first prototype and testing it on fly feeding behavior. We expect that our automated system will enhance the temporal analysis of fly feeding behavior and facilitate genetic screens for flies that are defective in regulating food satiety and attraction.

C 108 A Non-apoptotic Role of the Apoptosome in Drosophila Olfactory System Development

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Background: Sensory systems allow animals to receive information of the external world, which requires precise connections of sensory neurons with their target neurons. In the olfactory system of Drosophila more than 50 different functional classes of olfactory receptor neurons (ORNs) project to specific regions in the olfactory brain center. We found that dark, a cell death promoting gene, plays a non-apoptotic role in proper development of the olfactory system of Drosophila.
Observations: The Drosophila Apaf-1-related killer (dark) plays a key role in regulating programmed cell death, where it promotes the activation of caspases. We identified dark to be necessary for mediating proper ORN axon pathfinding and for recognizing the precise synaptic target area. Intriguingly the number of neurons is not increased in dark mutants and we could show that the connectivity phenotype is independent of apoptosis. Dark-mutant ORNs ectopically express the cell-adhesion molecule Connectin, and by ectopic expression of Connectin we were able to phenocopy the dark connectivity defects while the suppression of Connectin in dark mutant ORNs leads to a wild type ORN projection.
Conclusions: From these data we propose that the precise expression of cell surface molecules in projection axons depends on the tight regulation of caspase activity.

C 109 Release and transcellular spread of cytosolic amyloids: Implications for neurodegenerative diseases

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Background: Neurodegenerative diseases including Alzheimer's, Parkinson's and Prion diseases, are characterized by protein aggregation and deposition in specific brain regions. While the exact pathological significance of these aggregates remains to be conclusively resolved, the biology behind their formation is crucial to the understanding of the disease. Recent findings on the spread of amyloid-forming proteins suggest that both the luminal and cytosolic proteins can be released from cells.
Observations: How are these aggregates released from the cells? Where does the aggregation take place? Once released, how do they form plaques and propagate in the aqueous extracellular space to gain access to their host counterparts? We propose that exosomes, endocytically derived nanovesicles, are a major way to shuttle amyloids out of the cell and aid in the plaque formation. We provide evidence that luminal amyloids involved in neurodegeneration such as amyloid beta are generated in early endosomes and then released via exosomes. Using lipidomics, biophysical and cell biological techniques we demonstrate that exosome-associated amyloids act as seeds for further accumulation into plaque-like structures. We also provide fresh evidence that a cytosolic amyloid protein, alpha-synuclein, is secreted into the extracellular milieu both in exosome-dependent and independent manner. Further characterization reveals novel mechanisms by which alpha-synuclein is unconventionally secreted from the cell.
Conclusions: The release of cytosolic proteins challenges our basic cell biological understanding. Studying the cell biology of their release will help in understanding the spread of neurodegenerative diseases and also enable effective therapies to limit their transmission.

C 110 Synaptic pruning by microglia is necessary for normal brain development

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Background: Microglia are highly motile phagocytic cells of the hematopoietic lineage that infiltrate and take up residence in the developing brain where they are thought to provide a surveillance and scavenging function. However, although microglia have been shown to engulf and clear damaged cellular debris following brain insult, it remains unclear what role microglia play in the uninjured brain.
Observations: Here we show that microglia actively engulf synaptic material and play a major role in synaptic pruning during postnatal development in mice. Microglia lacking the chemokine receptor Cx3cr1 were compromised in their capacity to prune synaptic material as evidenced by a surplus of synapses and dendritic spines. Deficits in pruning were associated with increased long-term depression and decreased susceptibility to seizures, hallmarks of immature brain circuits.
Conclusions: These findings provide a direct link between microglia surveillance and synaptic maturation and suggest that deficits in microglia function may contribute to the increased spine density seen in some neurodevelopmental disorders.

C 111 Dissecting the role of Drosophila Orb2 in long-term memory

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Background: Long- term behavioral memory and synaptic plasticity both require new protein synthesis locally at activated synapses. Members of the CPEB protein family have been implicated in this process. They have been shown to regulate translation, localize to synapses and to be required in the long-term facilitation in Aplysia. We previously reported Orb2, a Drosophila homologue of neuronal CPEB proteins, to be required for long- term courtship memory.
Observations: Here we aim to define the molecular and cellular mechanisms of Orb2 function in long- term memory. We have generated an orb2attP allele, in which we replaced most of its reading frame with the target sequence for the site specific recombinase C31. This allowed us to rapidly introduce any desired replacement and modification of the orb2 endogenous locus to characterize its expression pattern and conduct structure function analysis of its role in long- term courtship memory. We have established that Orb2 is specifically enriched in the nervous system through out developmental stages, that can be fully functionally replaced by members of its own CPEB2 family of proteins but not CPEB1 and that Orb2B isoform functions both in the development and long-term memory while Orb2A isoform is exclusively required for long-term memory. The function of Orb2A in long-term memory is critically dependent on the N-terminal Glutamine stretch, a putative prion forming domain.
Conclusions: Using a novel Orb2attP allele we have characterized the expression pattern of Orb2 at the cellular level. We have established that Orb2A isoform is exclusively required for long-term memory while Orb2B isoform seems to function in both, the development and in long-term memory.

C 112 Lack of CIN85 in brain leads to impaired dopamine receptor endocytosis and hyperactivity in mice

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Background: Cbl-interacting protein of 85 kDa (CIN85) is a multi-adaptor protein implicated in the regulation of receptor endocytosis, cell division and the cellular cytoskeleton. Although much is already known about the molecular mechanisms by which CIN85 regulates these processes, its physiological functions in vivo remain unclear. To investigate the function of CIN85 in the central nervous system, we created CIN85 knockout mice (CIN85 KO) deficient of the two major CIN85 isoforms expressed in the brain.
Observations: CIN85 KO displayed a hyperactivity phenotype in a novel environment, characterized by an increase in forward locomotor activity, speed of movement and spontaneous entering and exploration of the unprotected area. The hyperactivity of CIN85 KO was accompanied by increased levels of dopamine and dopamine D2 receptors (D2DR) in the striatum, an important centre for the coordination of animal behavior, an attribute caused by reduced receptor internalization. In wild-type mice, CIN85 formed a complex with activated D2DR and endocytic regulators such as dynamin and endophilin. In CIN85 KO, the recruitment of the endocytic machinery required for D2DR endocytosis was diminished. The lack of CIN85 led to impaired internalization of D2DR, but not D1DR. Moreover, CIN85 localized to the post-synaptic compartment of striatal neurons in which it co-clusters with D2DRs. Taken together, our findings indicate an important role of CIN85 in the regulation of dopamine receptor functions and provide new insight into a molecular explanation for hyperactive behaviour.
Conclusions: CIN85 is a novel regulator of D2DR endocytosis in striatal neurons, involved in controlling locomotor and exploratory behaviour in mice.

C 113 APP phosphorylation at S655 positively regulates its nuclear signalling – implications to neurite outgrowth

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Background: The Alzheimer's Amyloid Precursor Protein (APP), precursor of the Abeta peptide and central to the Alzheimer's disease, is also involved in key physiological processes such as cell adhesion and neuritogenesis. APP is regulated through RIP (regulated-intramembranar protein) cleavage, generating products that include the neuritogenic alpha sAPP (secreted

APP) fragment, and AICD (APP Intracellular Domain), which can be nuclear targeted and form transcriptionally functional protein complexes.

Observations: Various factors may modulate APP RIP signalling, including phosphorylation. APP is a transmembranar receptor-like phosphoprotein, which can be phosphorylated by PKC at the S655 residue within the C-terminal 653YTSI656 basolateral signal. We have previously reported that S655 phosphorylation regulates APP trafficking between the trans-Golgi-network and the cell surface, favouring APP cleavage to alpha sAPP. During the trafficking analysis we noticed that, upon 24h of transfection, a phosphomimicking APP-GFP S655E mutant presented more fluorescing nucleus. Further analyses in various cells confirmed a highly dynamic S655E AICD-GFP nuclear targeting. This was accompanied by a higher APP RIP cleavage to AICD, and was confirmed to correlate with S655 phosphorylation by biochemical assays. Analysis of potentially AICD-induced genes, such as APP itself, the gene coding the signalling kinase GSK-3beta, and the actin remodelling protein Transgelin, revealed a correlation between nuclear targeting and gene transcription, but in a cell-type and gene-type specific manner. Interestingly, the nuclear targeting also appears to correlate with neurite outgrowth in SH-SY5Y cells.

Conclusions: These findings demonstrate that S655 phosphorylation regulates several steps of the APP RIP signalling mechanisms, including AICD generation and nuclear targeting. The observation that it might correlate with neurite outgrowth supports a modulatory role for S655 phosphorylation in APP physiology.

C 114 Sigma-1 receptors are essential for synaptic mitochondrial functions

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Background: The endoplasmic reticular (ER) chaperone sigma-1 receptor (Sig-1R) has been implicated in CNS diseases such as neurodegenration, schizophrenia, depression, amnesia, and drug abuse. It is known that Sig-1Rs are particularly enriched in the mitochondrial associate ER membrane, the MAM; however, little is known about how Sig-1R regulate neuronal mitochondria. We previously showed that Sig-1R promote the process of neuritogenesis and dendritogenesis in hippocampal neurons.

Observations: In this study we examined the effects of Sig-1R siRNA on axon elongation and synaptic mitochondrial activities. Neurons were transfected at DIV 1 and axon lengths were measured on both DIV 3 and DIV 7 by using tau immunostaining. Knocking down of Sig-1Rs impaired axon elongation significantly. The lengths of axon of the Sig-1R-knockdown neurons were as short as 40% of the controls at DIV 3. The difference in axon lengths became more noteworthy as neuron culture extended. Although Sig-1R siRNA-transfected neurons were able to extend axon gradually in time, they showed a slower extension rate; the axonal growth rate is 72 micrometer/day in control neurons whereas 34 micrometer/day in Sig-1R siRNA-transfected neurons during DIV3-7. Knocking down of Sig-1R causing aberrant axon extension was found to associate with hyperphosphoylation of tau as indicated by MC1 antibody that recognized an early conformational change in tau. We further examined the synaptosomal mitochondrial mass and membrane potentials by using flow cytometry analysis. Rhodamine 123 and 10-N-nonyl acridine orange (NAO) were used as membrane potential- or mass- dependent fluorescent dyes, respectively.

Conclusions: Sig-1R siRNA neurons possessed significantly less mitochondrial mass and lower membrane potentials in the synaptosome. Mitochondrial movement in the growth cones was also less active in Sig-1R siRNA neurons. These data indicted the impact of Sig-1R in maintaining synaptic functions and CNS diseases.

C 115 NMDA-mediated translocation of SPIN90 induced by tyrosine dephosphorylation in cultured hippocampal neurons

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Background: The activity-dependent reorganization of postsynaptic density (PSD) is a critical process underlying learning and memory. We have previously shown that SPIN90 is located in dendritic spines, but its significance in synaptic functions is not well understood. Here we demonstrate that SPIN90 moves from dendritic spine to shafts in an activity-dependent manner.

Observations: In the basal level state, SPIN90 has high level of dynamics comparing to PSD95, which is anchored to core of postsynaptic density. These observations led us to investigate the translocation of SPIN90 by local signals in a synapse. Of many local signals, we assumed that glutamate might affect SPIN90 dynamics since several actin-binding molecules were redistributed in spines responding to glutamate. Via immunocytochemistry and live cellular imaging, our data indicate that SPIN90 is also seems to be in hippocampal neurons. SPIN90 is tyrosine-phosphorylated in the basal state by Src kinase, but NMDA receptor activation alters the SPIN90 phosphorylation level. We assume that SPIN90 is dephosphorylated by STEP (Striatal Enriched Phosphatase), which is a downstream signaling molecule of NMDA receptor located in dendritic spines, resulting in SPIN90 translocation to dendritic shaft. In addition, SPIN90 knockout mice seem to show different patterns of LTD but normal basal synaptic activity and LTP comparing to wild-type mice.

Conclusions: SPIN90 is involved in NMDA receptor-linked synaptic plasticity, especially LTD.

C 116 LUX ARRHYTHMO, a transcription factor involved in circadian oscillations and direct control of growth in Arabidopsis

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Background: Circadian clocks allow anticipation of daily and seasonal environmental changes. They regulate fundamental processes and impaired clock function confers a striking loss

of fitness. Eukaryotic oscillators rely on complex transcriptional and post-translational regulatory circuits. Current Arabidopsis clock models comprise three or four interlocked feedback loops. Components of different parts of the network have been described, but the molecular connections between loops are poorly understood.

Observations: LUX ARRHYTHMO (LUX) was identified by genetic screens as an evening-expressed putative transcription factor essential for circadian rhythmicity in Arabidopsis. We determined the in vitro DNA-binding specificity for LUX by using protein binding microarrays. We then demonstrated that LUX directly represses the expression of PSEUDO RESPONSE REGULATOR 9 (PRR9), a major component of the morning transcriptional feedback circuit, through association with the newly discovered binding site. In addition, LUX binds to its own promoter, defining a new autoregulatory feedback loop within the core clock. In Arabidopsis, light and the circadian clock interact to consolidate the phase of hypocotyl cell elongation to dawn under diurnal cycles. We established the role of LUX in a protein complex that controls this phasing. The Evening Complex (EC), comprising LUX, EARLY FLOWERING 3 (ELF3), and EARLY FLOWERING 4 (ELF4), is regulated to peak at dusk and required for proper expression of the growth-promoting transcription factors PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) and PHYTOCHROME-INTERACTING FACTOR 5 (PIF5). LUX targets the complex to the promoters of PIF4 and PIF5 in vivo.

Conclusions: We identified novel connections between the archetypal loops of the Arabidopsis clock, significantly advancing towards defining the molecular dynamics that underlie circadian networks in plants. Additionally, we elucidated a direct molecular link between the circadian clock and plant growth.

C 117 Analysis of Thionin-related Genes from Arabidopsis thaliana

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Background: Thionins are a group of cysteine-rich peptides with a compact 5kDa amphipathic structure stabilized by three or four disulphide bridges. These small proteins have toxic and antimicrobial activities and are involved in the resistance of plants against fungal and bacterial pathogens.

Observations: It was recently shown that the Arabidopsis genome contains, in addition to the previously known thionin genes, approximately 50 other genes encoding thionin-like peptides. We selected 11 genes from this group for further study and produced promoter::GUS lines and over-expression lines. Only four of these genes are included on the Affymetrix GeneChip and thus nothing is known about their expression.

Conclusions: Here we report the expression analysis of these genes using promoter::GUS lines. For the four genes our analysis confirmed the data derived from GeneChip analysis. The majority of genes showed moderate or strong expression in seedlings and weak expression in flowers and siliques.

C 118 Connection between morphological traits of drought stress acclimation and RNA regulation is exemplified by the cbp20 Arabidopsis thaliana mutant

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Background: The nuclear cap binding complex (nCBC) mutants of Arabidopsis thaliana; cap binding protein 20 (cbp20) and cbp80/abh1 are impaired in steps of miRNA maturation and splicing of a specific set of mRNAs. These mutants are abscisic acid hypersensitive and show elevated tolerance towards drought stress. Thus far faster stomatal closure mechanisms were implicated as the basis of elevated drought hardiness in this class of mutants.

Observations: We performed a detailed phenotypic analysis of the cbp20 mutant and found thicker cuticle as well as more frequent occurrence of trichomes on shoot surfaces. Besides, we also found abnormal spatial patterning of stomatal pore complexes on various organs of the mutant. All these observations indicate profoundly disturbed development of the epidermal tissue in the cbp20 mutant, which has not been previously reported for nCBC mutants.

Conclusions: Cuticle thickening has also been shown as an acclimation process in response to water deprivation in Arabidopsis and other species. Our results suggest a connection between disturbed miRNA/RNA metabolism and morphological traits supporting increased drought tolerance in Arabidopsis.

C 119 A multigenic NB-LRR cluster as the source of multiple hybrid incompatibilities in Arabidopsis thaliana

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Background: Genetic incompatibilities between independently evolving genomes of the same species reduce gene flow, which can lead to speciation. Hybrid necrosis is a type of postzygotic genetic incompatibility prevalent in plants, characterized by autoimmune responses triggered by two or three epistatically interacting loci. In several hybrid necrosis cases, immune genes have been identified as causal, but it has not been clear whether all immune genes are equally likely to contribute to hybrid necrosis.

Observations: Here, we present a multigenic cluster of RECOGNITION OF PERONOSPORA PARASITICA1 (RPP1) homologs, DM2 (DANGEROUS MIX2), is responsible for at least two inde-

pendent hybrid necrosis cases in Arabidopsis thaliana. The DM2 cluster of nucleotide binding site-leucine rich repeat (NB-LRR) genes is characterized by complex expansion and contraction events in different lineages, which have spawned multiple independent incompatibilities. The two incompatibility genes we identified show distinct pattern of evolution and different epistatic interactors: the DM2d gene in the Uk-1 strain likely arose through a recent within-cluster duplication and interacts with the Uk-3 allele of the unlinked NB-LRR locus DM1. DM2h is apparently much older than DM2dUk-1 and has clear orthologs in many accessions. The Bla-1 allele of DM2h interacts specifically with the Hh-0 allele of DM3, which encodes a peptidase. In all four genes, the causal changes are located in the coding region, with the DM2h causal polymorphisms showing signature of selection.

Conclusions: Our results suggest both functional and structural features interact to make specific regions of the genome, such as the DM2 cluster, particularly likely to generate genetic incompatibilities. We propose that the danger of autoimmunity acts as a coevolutionary constraint between the interactors.

C 120 Identification of CYP90A1/CPD function supports the dominance of campestanol-independent brassinosteroid synthesis in Arabidopsis

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Background: Brassinosteroids (BR) are steroidal plant hormones regulating essential functions of growth and development. In Arabidopsis the network-like routes of BR biosynthesis have been elucidated in considerable detail, but the roles of some important enzymes and the functional hierarchy of the various sub-pathways remained to be clarified.

Observations: Mutational inactivation of the cytochrome P450 monooxygenase CYP90A1/CPD causes the strongest BR-deficient phenotype in Arabidopsis. We studied the function of this enzyme, which earlier had been proposed to act as BR C-23 hydroxylase. Our GC-MS and genetic analyses demonstrated that the cpd mutation blocks BR synthesis upstream of the DET2-mediated 5-alpha reduction step, and that CPD promoter-driven overexpression of the BR C-23 hydroxylase-encoding CYP90C1 gene does not alleviate BR deficiency in the cpd mutant. In agreement with these results, we found that CYP90A1/CPD heterologously expressed in baculovirus-insect cell system catalyzes C-3 oxidation of the early BR intermediates (22S)-22-hydroxycampesterol and (22R,23R)-22,23-dihydroxycampesterol to their respective 3-oxo forms, which are the preferred substrates of DET2. In addition, CYP90A1/CPD also catalyzes C-3 oxidation of 6-deoxocathasterone and 6-deoxoteasterone, but does not participate in the reaction route leading from campesterol to campestanol. Considering the severe BR deficiency of the cpd mutant, this latter result suggests a subordinate role for the campestanol-dependent sub-pathway in BR synthesis.

Conclusions: CYP90A1/CPD is a multifunctional BR C-3 oxidase showing highest activity with 22-hydroxycampesterol substrate. This, and substrate preferences of all other early BR biosynthetic enzymes (DET2, CYP90B1, CYP90C1 and CYP90D1) indicate the dominance of the campestanol-independent routes in BR synthesis.

C 121 Regulatory networks integrating cell division with plant development

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Background: Cell division and cell differentiation controls respond to intra and extra-cellular cues, which will guide the plant to particular developmental programs. Both events must act in a coordinated and balanced way to promote proper organogenesis in plants. The transition from a dividing to a differentiating cellular state is gradual, when cells must tightly integrate transcription regulation with cell cycle progression controls.

Observations: Our group identified a protein that connects DNA replication and transcription machineries, called ABAP1 (Armadillo BTB Arabidopsis Protein 1). We have demonstrated that ABAP1 acts as a negative regulator of mitotic cell division in leaves, through its interaction with members of the pre-replicative complex (preRC) and the transcription factor AtTCP24. ABAP1 associates with different partners during development, possibly taking up diverse functions depending on the developmental context. In addition to leaves, high expression of ABAP1 in reproductive tissues suggests a role on the reproductive development of Arabidopsis. In this developmental phase, many specialized cell divisions take place, such as the ones that participate in the gametes formation. Ectopic expression of ABAP1 results in the formation of embryo sacs without polar nuclei fusion (an embryo lethal phenotype). These plants also develop defective pollens, unable to perform the mitotic divisions necessary for their maturation process. The mechanism of action of ABAP1 in the gametes involves association with different transcription factors that integrate developmental signals with ABAP1 role on cell division control.

Conclusions: Altogether our data indicates that, in addition to the role on the control of proliferative mitotic cell divisions during leaf development, ABAP1 also participates in mechanisms controlling specialized cell divisions and differentiation processes during gamete formation.

C 122 rps16 gene in tobacco chloroplasts is well transcribed and its transcript is correctly spliced, but mRNA is not translated

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Background: The chloroplast is the plant specific organelle and has specific expression system. The expression of chloroplast genes are generally controlled at translation level. To analyze the translation regulation in chloroplasts, we developed an in vitro translation system from tobacco chloroplasts.
Observations: In many flowering plants, the gene for chloroplast 30S ribosome subunit S16 (rps16) is encoded by the chloroplast genome and consists of two exons and one intron. RNA analyses showed that rps16 is well transcribed and its transcripts spliced correctly. Using an in vitro translation system, we analyzed the translation activity of rps16 mRNA, and found that rps16 mRNA has no or little translation activity. Further analysis revealed that the translation activity of 5' UTR from rps16 has over 500 times less than that of rps2. Furthermore, we suggested S16 of 30S ribosome in tobacco chloroplasts is derived from a nuclear-encoded rps16 gene.
Conclusions: We demonstrated that rps16 in chloroplasts is a silent gene at the translation level. Thus, translation inactivation may be one of the triggers to become a pseudogene in the chloroplast genome. Alternatively, chloroplast rps16 mRNAs are likely to have unknown functions as RNA molecules.

C 123 Co-expression of ARP and KNOX1 genes in simple leaved plants; an example from the Gesneriaceae, Streptocapus

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Background: As Streptocarpus species (Gesneriaceae) show a great diversity in shoot morphology, including one leaf plants, the molecular basis for this diversity was followed from expressions of two types of genes of ARP and KNOX1, in cosidering their expression in model plants.
Observations: The caulescent Streptocarpus glandulosissimus possesses a shoot apical meristem (SAM) and opposite phyllotaxis, while the acaulescent Streptocarpus rexii lacks a standard SAM and produces an irregular rosette from a groove meristem. Our results show an unusually extended basal meristem activity for lamina expansion in both species. We attempted to explain the unique leaf morphogenesis in terms of developmental genetics by studying ARP and BP-KNOX1 gene expression patterns. In Arabidopsis, a plant with simple leaves, KNOX1 genes are expressed in the SAM, and ARP genes in differentiated organ primordia. Plants with compound leaves, such as tomato, show a co-expression of ARP and KNOX1 in the SAM and leaves. We found that, irrespective of their simple leaves, both Streptocarpus species co-express ARP and BP-KNOX1 in close spatio-temporal correlation with the activities of the SAM, the groove meristem and the basal leaf meristem.
Conclusions: Our results suggest that two genes of ARP and KNOX1 are retained in Streptocarpus, but their interactions are modified in this genus. While these interactions are not responsible for the diversity in shoot morphogenesis in Streptocarpus, this may have allowed a unique leaf development to evolve.

C 124 A marker-enrichment method for mapping mutations in Arabidopsis thaliana

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Background: Forward genetic screens have been widely used for many years to identify new mutants affecting different biological processes in Arabidopsis. Even though the identification of the causal mutations has been greatly improved over the past years it still represents a long and laborious procedure. Recently, whole genome sequencing using of next-generation platforms have drastically reduced the efforts for mapping causal mutations in reference and non-reference Arabidopsis accessions.
Observations: Although efficient for mapping, most reads obtained during whole genome sequencing do not contain informative polymorphisms. In order to sequence marker-specific regions, we developed a marker-enrichment strategy associated with next-generation sequencing. Genome-wide RNA biotinylated probes were designed in order to hybridize in a specific position sharing a polymorphism between Col-0/Ler and C24 ecotypes. Bulked segregant analysis of the F2 population (Ler/Col-0) was used to map a mutant candidate isolated in a suppressor screen designed to identify components of photoperiodic pathway that act downstream of FLOWERING LOCUS T (FT). Col-0 and Ler alleles were efficiently captured being nearly equally represented in our F1 control sample. In addition, the genomic DNA was efficiently captured, with very low plastid DNA contamination. Each marker position was highly covered and about 12 Mb genomic DNA represented in our sample. Furthermore, the region of the causal mutation was efficiently mapped to the top of chromosome 2. The causal mutation was further confirmed by using standard whole genome sequencing, genetic crosses and complementation using the whole genomic fragment.
Conclusions: We demonstrate a proof-of-principle method to map mutants in Arabidopsis. The high coverage and efficient capture of informative regions in the genome represents an advance compared to the standard whole genome sequencing and could be expanded to non-model organisms or organisms with larger genomes.

C 125 Transcriptome Analysis of Giant Embryo Mutants in Rice

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Background: Plant embryogenesis is the first developmental phase during which morphogenetic events occur to establish fundamental body plan. The regulatory process is complicated during embryogenesis which includes pattern formation, organ determination, positional regulation, size regulation and so on. Recent studies using various embryo mutants in rice, maize and Arabidopsis have shown the processes and the mechanisms of embryogenesis gradually, but in rice the mechanisms are almost unknown.

Observations: To explain the mechanism of embryogenesis in rice, we analyzed three giant embryo mutants (EM-39, EM-40 and EM-131) and Kinmaze which is the original cultivar of the mutants. These mutants were derived from Kinmaze mutagenized with N-metyl-N-nitrosourea. We collected young rice seeds at various developmental stages during two weeks after fertilization. Collected samples were frozen in liquid nitrogen and kept -80 degrees Celsius. Total RNA was extracted with TRIzol reagent (Invitrogen). cRNA was amplified with Low Input Quick Amp Labeling Kit (Agilent Technologies) using 200ng of total RNA. We used Agilent microarray (Rice 60mer oligo DNA microarray 4x44K RAP-DB) for transcriptome analysis. After obtaining all array data, we applied a K-means clustering to them. In early stages, some transcripts in three EM strains were less than those in Kinmaze. Then in late stages, some transcripts in three EM strains were more than those in Kinmaze, in which several genes concerned with lipid transfer and storage were involved. Now we advance detailed analysis of these genes, and to get more data from the transcriptome analysis of the tissues excised with LMD (laser microdissection).

Conclusions: From the transcriptome analysis of rice giant embryo mutants, the genes having the changes of their expression specifically in mutant strains were observed. Further, it is possible to get more information for rice embryogenesis by examining their expression and function in detail.

C 126 Analysis of coronin function in Dictyostelium discoideum

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Background: The coronin protein family is characterized by a central WD repeat domain linked to a C-terminal coiled coil region. For most of the coronin molecules, the exact in vivo function remains unknown. In mammals, coronin 1, the most conserved coronin isoform, was recently shown to modulate Ca2+ signalling, thereby regulating the Ca2+/calcineurin pathway. Whereas mammalian cells express up to seven isoforms, the lower eukaryote Dictyostelium discoideum expresses a single coronin protein.

Observations: In this work, we analyzed a role for coronin in the unicellular amoeba Dictyostelium discoideum, which is a well known model for many essential biological processes. This haploid organism is especially useful for the study of immune cell functions, including chemotaxis and phagocytosis. Under conditions of starvation, Dictyostelium cells aggregate by secreting cAMP, and form a motile slug, which ultimately forms a fruiting body allowing spore dispersal. Our experiments showed that coronin-deficient Dictyostelium cells are defective in chemotaxis toward a cAMP gradient. As a consequence, the subsequent stages of the multicellular formation, i.e. aggregation and development in fruiting bodies, were also affected. Nevertheless, folic acid chemotaxis was not impaired. Furthermore, a phagocytosis defect was observed but turned out to be dependent on the particle type. These results lead us to hypothesize that Dictyostelium coronin is also involved in specific signaling pathways. Interestingly, we observed a hyper-phosphorylation on tyrosine-53 of actin in coronin-deficient cells, which could explain at least part of the observed phenotypes.

Conclusions: The co-expression of several coronin isoforms in mammalian cells may prevent a uniquivocal elucidation of their function; Definition of the function of the single coronin molecule in Dictyostelium may therefore help to better understand the role of the coronin proteins in the eukaryotic kingdom.

C 127 Functional dissection of the TBK1 molecular network

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Background: TANK-binding kinase 1 (TBK1) and inducible IkB-kinase (IKK-i) are central regulators of type-I interferon induction. They are associated with three adaptor proteins called TANK, Sintbad (or TBKBP1) and NAP1 (or TBKBP2, AZI2) whose functional relationship to TBK1 and IKK-i is poorly understood.

Observations: We performed a systematic affinity purification–mass spectrometry approach to derive a comprehensive TBK1/IKK-i molecular network. The most salient feature of the network is the mutual exclusive interaction of the adaptors with the kinases, suggesting distinct alternative complexes. Immunofluorescence data indicated that the individual adaptors reside in different subcellular locations. TANK, Sintbad and NAP1 competed for binding of TBK1. The binding site for all three adaptors was mapped to the C-terminal coiled-coil 2 region of TBK1. Point mutants that affect binding of individual adaptors were used to reconstitute TBK1/IKK-i-deficient cells and dissect the functional relevance of the individual kinase-adaptor edges within the network. Using a microarray-derived gene expression signature of TBK1 in response virus infection or poly(I:C) stimulation, we found that TBK1 activation was strictly dependent on the integrity of the TBK1/TANK interaction.

Conclusions: We conclude that TANK, Sintbad and NAP1 form alternative complexes with TBK1 that are localized to distinct subcellular compartments and that the TBK1 adaptors are required for TBK1 activation under physiological conditions.

C 128 Interplay between C-Raf kinase and Rassf1a tumour suppressor in liver cancer

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Background: The Mst signalling cascade regulates cell survival and proliferation in metazoans and numerous reports indicate its further role in tumour suppression, especially in the liver. C-Raf, indirect activator of Erk in MAPK pathway, can interact with, and inhibit, Mst2. In addition, C-Raf is implicated in the protection from apoptosis in mouse liver, and found overexpressed in human liver tumours. We investigate the possible interaction between the Mst pathway and C-Raf in liver tumorigenesis.
Observations: We have crossed animals with hepatocyte-specific deletion of c-raf-1 with animals harbouring a germline disruption of the gene encoding for Rassf1A, an upstream regulator of MST signalling, with tumour suppressor functions lost in liver cancer through methylation of the isoform-specific promoter. Surprisingly, the livers of c-raf-1 knock-out animals were significantly more prone to chemically-induced liver carcinogenesis than organs of wild-type animals. This phenotype was absent in a rassf1a knock-out background, indicating genetic interaction between c-raf-1 and rassf1a. In most of the c-raf-1 knock-out livers we could detect progression to carcinoma stage, whereas in wild-type animals we detected almost exclusively benign nodules. On the other hand, double knock-out animals had similar numbers of carcinomas in comparison to those with sole rassf1a deletion. Efficient c-raf-1 deletion was confirmed both in non-affected and liver tumour tissue but the analysis of signalling pathways revealed no changes in Erk activation.
Conclusions: We propose a tumour suppressive function of C-Raf in the liver and an interaction between c-raf-1 and rassf1a in chemically-induced mouse liver cancer model.

C 129 Evolutionarily Conserved Residues the GLP-1 Receptor Core Confer Selective Interaction with GLP-1 and Receptor Activation

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Background: Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) play important roles in insulin secretion through GLP1R and GIPR. Although GLP-1 and GIP are attractive candidates for treatment of type 2 diabetes and obesity, little is known regarding the molecular interaction of these peptides with their receptors. Here, we determined specific amino acid residues in the heptahelical core domain of GLP1R responsible for interaction with ligand and receptor activation.
Observations: We found that transmembrane helix 2 (TMH2), extracellular loop 1 (ECL1), and ECL2 within the core of GLP1R are necessary for specific ligand binding and receptor activation. Based on amino acid sequence comparison of these regions of vertebrate GLP1Rs and GIPRs, we further constructed site-directed mutated receptors, revealing that Ile196 of TMH2, Leu232 and Met233 of ECL1, and Asn302 of ECL2 in GLP1R confer specific GLP-1 binding and receptor activation. Using GLP-1/GIP chimeric peptides, we found that His1 of GLP-1 interacts with Asn302 of GLP1R, and Thr7 of GLP-1 has close contact with a binding pocket formed by Ile196, Leu232, and Met233of GLP1R.
Conclusions: This study is the first to demonstrate molecular determinants within the core of the receptors conferring ligand binding and receptor activation, which may provide critical clues for development of nonpeptide agonists for treatment of diabetes mellitus and obesity.

C 130 The RhoGDIalpha-dependent balance between RhoA and RhoC is a key regulator of cancer cell tumorigenesis

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Background: RhoGTPases are key signaling molecules regulating main cellular functions such as migration, proliferation, survival and gene expression through interactions with various effectors. Within the RhoA-related sub-class, RhoA and RhoC exert distinct functions in several key steps of cancer progression. Our aim was to investigate the respective contribution of these RhoGTPases to the tumorigenic phenotype by using models of reduced or forced expression.
Observations: The silencing of RhoC, but not of RhoA, increased the expression of genes encoding tumor suppressors, such as NSAID-activated gene 1 (NAG-1), and decreased the migration and the anchorage-independent growth of prostate adenocarcinoma cells (PC-3) in vitro. In vivo, siRhoC impaired tumor growth. Interestingly, co-transfecting PC-3 cells with siRhoC and siNAG-1 suppressed NAG-1 expression, as expected, but also repressed most of the siRhoC-related effects, demonstrating the central role of NAG-1. These regulations are not restricted to one cell type as they were also observed in LnCaP and MCF7 cells. Surprisingly, overexpression of RhoA induced the same phenotype as silencing RhoC. These seemingly contrasting results are explained by the RhoGDIalpha-dependent balance existing between RhoA and RhoC. The silencing of RhoGDIalpha and the overexpression of a RhoA mutant (RhoAR68E) unable to bind RhoGDIalpha revealed that the effect of RhoC silencing is indirect and results from the up-regulation of RhoA level through competition for RhoGDIalpha. Finally, the use of small inhibitors demonstrated that ROCK and p38MAPK are required downstream of RhoA for the induction of NAG-1.
Conclusions: Our study highlights the dynamic balance existing between two closely related members of the RhoGTPase network and illustrates its biological relevance in cancer progression.

C 131 Characterization of gene regulatory network formed by unfolded protein response modulators

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Background: One third of proteins are translated in the endoplasmic reticulum (ER). Once ER stress is evoked by massive unfolded proteins, BIP (GRP78/HSPA5) promotes unfolded protein response to maintain cellular homeostasis and avoid apoptosis. It has well been documented that perturbation of unfolded protein response can be a cause for diabetes and neurodegenerative disease; however, regulatory mechanism that underlies the significance of connection between ER stress and apoptosis is still obscure.

Observations: To investigate the transcriptional network that governs a bifurcation between ER stress and apoptosis, we prepared the pGL3-based promoter-luciferase reporters for BIP (-761/+120), XBP1 (-410/+49), CHOP (DDIT3/GADD153) (-863/+23), PERK (EIF2AK3) (-874/+26), IRE1 (ERN1) (-977/+23), ATF6 (-910/+60) and ATF4 (-723/+188). Numbers in the parenthesis is based on the transcriptional start site as +1 nucleotide. These reporters were transfected into HeLa cells exposed to ER stress inducer thapsigargin (TG). The activities of pGL3-BIP, pGL3-XBP1 and pGL3-CHOP were significantly induced by TG. In contrast, luciferase activity of pGL3-XBP1 was upregulated, whereas those of pGL3-BIP and pGL3-CHOP were downregulated, in etoposide-induced apoptotic cells. Subsequently, pGL3-BIP, pGL3-XBP1 or pGL3-CHOP was co-transfected with the expression vectors encoding BIP, XBP1, CHOP, PERK, ATF6+YY1 and ATF4. XBP1 activated all of the three reporters. BIP, CHOP and ATF4 activated pGL3-BIP and pGL3-CHOP but not pGL3-XBP1. ATF6+YY1 activated pGL3-BIP and pGL3-XBP1 but not pGL3-CHOP. PERK failed to induce the activity of three reporters.

Conclusions: In line with the established function of CHOP in apoptosis, XBP1 could also participate in the regulation of apoptosis as well as ER stress, based on an increase in XBP1 reporter activity. Of noted, BIP, XBP1 and CHOP upregulated their own promoter as well as BIP promoter.

C 132 Fasudil, a specific inhibitor of ROCK, stimulates secretion of CXCL14/BRAK and suppresses tumor formation in fibrosarcoma cells

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Background: RhoA – ROCK pathway is key regulators of the trafficking of molecules in cells. Activation of this pathway would stimulate tumor invasion and metastasis. It was well known that fasudil has been approved for the treatment of cerebral vasospasm. However, effects of fasudil on tumor growth have not been investigated. We previously reported that CXCL14/BRAK has anti-tumor activity in several carcinoma cells. Thus, we investigated the effects of Fasudil both on BRAK secretion and tumor growth.

Observations: Murine fibrosarcoma (MC57) cells were injected subcutaneously into both sides of dorso-lateral region of 4 BRAK Transgenic mice and 4 wild type mice. The size of the tumors formed in the allografted BRAK Tg mice was significantly smaller than those of in Wild type mice, irrespective of number of MC57 cells. BRAK that is one of the secretory protein, is secreted in the extracellular and displays the antitumor effect in fibrosarcoma cells. We developed stable cell lines, mouse fibrosarcoma, expressing BRAK (MC57-BRAK) and mock vector introduced (MC-57-MOCK), and examined the effects of fasudil on the secretion of BRAK by using ELISA in MC57-BRAK cells. The secretion of BRAK was significantly increased by treatment with fasudil in MC57-BRAK cells. In order to determine the effect of fasudil on tumor growth, MC57-BRAK and MC-57 MOCK cells were inoculated subcutaneously into both sides of the dorsolateral regions of 28 C57BL/6 mice. These mice were daily-administered fasudil, i.p. (50mg/kg/day). Fasudil suppressed the growth of tumors in mice which was received transplants of MC57-BRAK.

Conclusions: Fasudil would inhibits tumor growth in the fibrosarcoma cells, and they suggest that drug therapy using ROCK specific inhibitor such as a fasudil for tumor may have clinical efficacy through restoration of BRAK secretion.

C 133 Simvastatin influence on IL-6 signalling in human melanoma cells

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Background: High plasma levels of Interleukin-6 (IL-6) are associated with bad prognosis and reduction in overall survival in melanoma patients. Statins are HMG-CoA reductase inhibitors, and well-tolerated therapeutics for hypercholesterolemia. We have recently shown that simvastatin triggers apoptosis in 518A2 human melanoma cells which is paralleled by concentration dependent changes in IL-6 secretion. Here, we investigated the regulation of the IL-6 pathway by simvastatin.

Observations: Our FACS analysis of the heteromeric IL-6-receptor (IL-6-R/gp130) showed an increase in the surface expression under simvastatin treatment mainly in the apoptotic cells. This was confirmed by Western blot analyses. The precursor level of the less glycosylated gp130 was enriched in simvastatin probes as well as the mature receptor. On mRNA level IL-6-R and gp130 were in a similar range independent of statin treatment. Downstream of the in IL-6-receptor we traced the fluorescent fusion protein STAT3-YFP in the transfected melanoma cells. Stimulation with IL-6 resulted in the formation of cytosolic density spots, which co-localized with lysosomal markers. Simvastatin significantly delayed the accumulation of STAT3, whereas the activation and phosphorylation of STAT3 was unaffected by simvastatin exposure. A typical biphasic phosphorylation pattern was observed in Western blots. The suppressor of cytokine signalling (SOCS) proteins, which may play a crucial role in the IL-6 pathway, were not regulated by simvastatin on protein or mRNA level.

Conclusions: We here show that statins, like simvastatin are capable to interfere with the IL-6 pathway on the heteromeric IL6-receptor but not on the level of STAT3 activation or negative feed back protein SOCS 3.

C 134 Effects of Resveratrol with Combined Retinoic Acid on Matrix Metalloproteinases (MMPs) in Human HT1080 Fibrosarcoma and HTB94 Chondrosarcoma Cells

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Background: Resveratrol, a grape poly phenol, is thought to have anti-inflammatory, anti-carcinogenic, anti-viral and anti-aging. However, the mechanisms by which resveratrol might produce these effects are not clearly defined. Retinoic acid is a metabolite of vitamin A and has been investigated in cancer differentiation and anti-metastatic. Matrix metalloproteinases (MMPs) are key enzymes in the degradation of extracellular matrix, and their expression may be regulated in cancer metastasis.
Observations: In this study, we aimed to evaluate the effect of resveratrol on MMPs and to understand the mechanism of action in HT1080 fibrosarcoma and HTB94 chondrosarcoma cells. Here the influence of resveratrol on expressions of MMPs was examined by gelatin zymography assay or Western blot analysis in human HT1080 and HTB94 cells. It is shown that resveratrol significantly up or down regulated the expression of MMP-2 and MMP-9 in dose-dependent manner by gelatin zymography assay and Western blot analysis in HT1080 cells. But, HTB94 is reduced expression of both MMP-2 and MMP-9 in dose-dependent manner by gelatin zymography assay. Also, we found that resveratrol inhibited the expression of type collagen and SOX-9 by Western blot analysis in HTB94 cells. Moreover, we treated resveratrol with retinoic acid in HT1080 and HTB94 cells. The results showed that retinoic acid regulated resveratrol-mediated on expression of MMP-2, MMP-9, type collagen and SOX-9.
Conclusions: Our results suggest that resveratrol regulates expressions of MMPs in HT1080 fibrosarcoma and HTB94 chondrosarcoma cells. In addition, retinoic acid regulated resveratrol-mediated on expressions of MMPs. Furthermore, it involved metastasis in various cancer including HT1080 and HTB94 cells.

C 135 Alterations in purinergic receptor-mediated signaling as a consequence of epidermal growth factor-induced epithelial mesenchymal transition (EMT) in breast cancer cells

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Background: Epithelial-mesenchymal transition (EMT) is a pathway implicated in cancer metastasis, involving a change in cell shape to a spindle-like morphology, the loss of cell-cell contacts and the acquisition of mesenchymal markers including vimentin. Although the role of the tumor microenvironment in cell migration and EMT is well established, relatively little is known regarding alterations in cell surface receptors to external stimuli that may occur during EMT and facilitate tumor cell dissemination.
Observations: We assessed changes in intracellular calcium signaling as a consequence of EGF-induced EMT in MDA-MB-468 breast cancer cells loaded with Fluo-4 AM calcium indicator and observed a 10-fold shift in the potency of ATP in addition to an alteration in the nature of the ATP-induced calcium transient. No change was observed in the sensitivity to protease-activated receptor (PAR2)-mediated calcium signaling. To determine whether changes in ATP-mediated calcium signaling are preceded by alterations in the transcriptional profile of purinergic receptors, we analyzed the expression of a panel of 15 ionotropic (P2X) and metabotropic (P2Y) purinergic receptors. We report significant and specific alterations in the suite of ATP-activated purinergic receptors as a consequence of EGF-induced EMT in breast cancer cells. P2X5 ionotropic receptors were enriched in the mesenchymal phenotype of breast cancer cells. P2X5 levels were also increased in the basal-like subtype of human breast cancers where the mesenchymal phenotype is over-represented. Silencing of P2X5 in MDA-MB-468 cells led to a significant (25%) reduction in EGF-induced vimentin protein expression, suggesting a functional role.
Conclusions: A remodeling of some cell surface receptors, including an increase in the P2X5 ATP-receptor, may impart advantageous phenotypic traits during EMT in breast cancer cells. Such receptors may represent novel targets for the treatment of breast cancer metastasis.

C 136 Does the von Hippel Lindau gene act through the insulin pathway to extend lifespan in C.elegans?

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Background: Von Hippel–Lindau disease, a complex multisystem hereditary neoplastic syndrome whose most common clinical features are hemangioblastomas and renal cell carcinoma. The tumor suppressor protein pVHL functions as a substrate- recognition subunit of an E3 ubiquitin ligase, mediating ubiquitination of hypoxia-inducible factor (HIF), leading to its proteasomal degradation. Here, we demonstrate that pVHL is a key factor in lifespan in C.elegans independent from insulin-signaling.
Observations: We compared VHL -/- worms to WT N2 worms and we found that the loss of pVHL significantly increases lifespan. We performed RNAi-experiments knocking down daf-2 – the nematode insulin-receptor in WT N2 and VHL -/-. Daf-2 mediated insulin-signaling exerts a negative effect on C.elegans lifespan through inactivation of daf-16. Daf-16 activity is strongly controlled by its subcellular localization. Activated daf-16 is translocated into the nucleus where it can exert its functions as transcription factor. In WT N2 the vast majority of daf-16 protein is dispersed throughout the cytoplasm. In order to check daf-16 nuclear translocation and thus functioning, we employed a transgenic strain expressing a sod-3::GFP fusion protein, with sod-3 being a target gene of daf-16. Knockdown of daf-2 induces transport of daf-16 to the nucleus and expression of sod-3, whereas RNAi against VHL did not show this effect, confirming the finding that pVHL does not exert its effect on lifespan

through modulation of insulin- signaling. We finally confirmed this finding at the protein level comparing GFP levels in vhl-1, daf-2 knockdowns versus empty vector.

Conclusions: vhl1 extends lifespan in C.elegans through a pathway independent from insulin-signaling.

C 137 Neurofibromin, a GTPase activating protein (GAP) of Ras connects Ras to the actin cytoskeleton dynamics by inhibiting LIMK2

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Background: Actin cytoskeleton rearrangement controls many cell functions and is regulated by diverse actin-binding proteins. Among these, cofilin, an actin-depolymerizing factor (ADF), leads to rapid turnover of actin filaments. Its activity is repressed by phosphorylation by LIM kinase proteins (LIMK1 and LIMK2). Neurofibromin (Nf1), a Ras GAP responsible for neurofibromatosis type I disease, has previously been shown to favor actin depolymerisation but the molecular mechanism involved was unknown.

Observations: We have discovered this molecular mechanism and shown that Nf1 directly interacts with LIMK2 but not LIMK1 and inhibits it by preventing its activation by ROCK. Using two-hybrid and co-immunoprecipitation experiments, we have shown that it is the SecPH domain of Nf1 (adjacent to its GAP domain and composed of two protein-protein interaction modules: a Sec14 homologous domain ans a Pleckstrin homologous domain) which specifically interacts with LIMK2. The LIMK2 domains involved in the interaction are the PDZ/SP (Ser and Pro rich) and the kinase domains. Using in vitro kinase assays, we have shown that SecPH inhibits specifically and in a dose-dependent manner, LIMK2 kinase activity on cofilin. Furthermore, we have validated this inhibitory effect of SecPH in vivo by showing that its overproduction into HeLa cells inhibits the formation of actin stress fibers induced by the overproduction of LIMK2. We have further demonstrated that SecPH does not inhibit a constitutively active LIMK2 mutant (LIMK2 T505EE) and elucidated the molecular mechanism of this inhibition by showing that SecPH prevents LIMK2 kinase domain phosphorylation by ROCK.

Conclusions: Our data reveal for the first time a precise molecular mechanism explaining how Nf1 favors actin depolymerisation. Furthermore, it appears that Nf1 constitutes a novel direct molecular link between Ras and actin cytoskeleton by coupling Ras inhibition to actin depolymerisation via LIMK2 inhibition.

C 138 Sulforaphane Induces Inhibition of Rabbit Articular Chondrocytes Cells Proliferation, Differentiation and Inflammation via MAP Kinase Signaling Pathway

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Background: Sulforaphane, a well-characterised dietary isothiocyanate, has been demonstrated to be a potent anti carcinogenic agent in numerous cancer models. The association between proliferation, differentiation and inflammation of the chondrocytes remains obscure. This study we investigated the effect of Sulforaphane on cells proliferation, dedifferentiation and anti-inflammation in rabbit articular chondrocytes.

Observations: Sulforaphane decreased cells proliferation, Type collagen and COX-2 expression, as indicated by the Western blot analysis, RT-PCR, PGE2 assay, Alcian blue staining, Trypan-blue dye exclusion assay, MTT assay and immunofluorescence staining. FACS showed that Sulforaphane induced cell cycle G2/M arrest in dose-and time dependent manner. Also, Sulforaphane increased expression of p21 and p53, while reducing expression of cyclin B, cdc2 and cdc25C in chondrocytes as detected by Western blot analysis. Moreover, Sulforaphane treatment stimulated activation of ERK-1/2 and p38 kinase. Inhibition of ERK-1/2 with PD98059 reinforced Sulforaphane-induced differentiation. whereas inhibition of p38 kinase with SB203580 caused Sulforaphane-induced COX-2 expression.

Conclusions: In summary, we found that Sulforaphane regulates dedifferention, inflammation and cell cycle arrest at G2/M phase through MAP kinase signaling pathway in rabbit articular chondrocytes.

C 139 Drug screening on signal transduction proteins via micro-patterned surfaces

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Background: The plasma-membrane of living cells is the major organelle for cellular signaling cascades. To warrant the diverse functions of a cell, communication between the cytoplasm and its organelles and the extracellular space is crucial. Thus, membrane-localized protein receptors activated by various messengers are key to transmit signals into the cell. Defective regulation of these signaling cascades may lead to cell death or uncontrolled proliferation.

Observations: An important point is the interaction of these receptors with cytosolic proteins. To analyze such interactions we use micro-structured surfaces in combination with fluorescent microscopy ('micro-patterning assay'). This technique was developed to detect protein-protein interactions (Schwarzenbacher et al., 2008; Weghuber et al., 2010) and offers the possibility to measure and quantify also weak or short-lived interactions in-vivo. We visualized the interaction of the following membrane-receptors with their respective intracellular binding

partners: EGF-receptor (Grb2), Insulin-receptor (IRS1-4), and ß1- and ß2 adrenergic receptors (Arrestin, G-proteins). Additionally, we analyzed the insulin-dependent transfer of Glucose-transporter 4 (Glut4) to the plasma-membrane. In a next step we started to determine the effects of various messengers (EGF, Insulin, Epinephrine,…) on the described interactions. By doing so we characterized variations in the interaction-properties of these interaction pairs upon application of messenger molecules.

Conclusions: Since the micro-patterning assay is a robust technique to analyze a large number of cells within a short time, it is our endeavor to investigate the effects of further, medically relevant messenger molecules (secondary plant metabolites) on the interaction of the aforementioned signaling proteins.

C 140 Detection of phosphorylated MAP kinases in developing spinal cord of mouse embryo

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Background: Spatio-temporal detection of biological molecules might be one of the major research topics. MAP kinases easily change its phosphorylated states even by the generally used experimental manipulations including anesthetics, formalin and nerve injury. Therefore, it is very challenging task to detect physiologically phosphorylated MAP kinases in vivo with any precision. We tried to detect in vivo physiologically phosphorylated MAP kinases in developing spinal cord of mouse by using improved method.

Observations: Fetuses were immediately removed by cesarean section, euthanized by decapitation, and immediately frozen. Fresh-frozen sections were cut using a cryostat. The sections were then put on silanized slides, which were immediately fixed and then dried. Tissue sections were stained with anti-phospho-MAP kinase antibodies. On E13 to E17, the entire marginal layer was intensely stained with anti-phospho-ERK1/2 antibody throughout the level of the spinal cord. Higher immunoreactivities of p-ERK1/2 were observed as dots in marginal layer. The p-ERK1/2 immunoreactivities were investigated to be spotted or to be fibrous in the dorsal horn and dorsal side of mantle layer. The p-ERK1/2 immunoreactivities were scarcely observed in the posterior median septum and were lower in the area around anterior median septum throughout the level of the spinal cord. Ependymal layer was not stained with anti-phospho-ERK1/2 antibody throughout the level of the spinal cord. We also detected phospho-JNK1/2 and obtained similar patterns with p-ERK1/2.

Conclusions: Phosphorylated MAP kinases in the spinal cord of the mouse from E13 to E17 were detected with the improved method. Our results suggest that p-ERK1/2 and p-JNK1/2 might play any critical roles in developing spinal cord. It might be possible to construct phospho-protein atlas in future.

C 141 The ciliary protein NPHP4 translocates canonical Wnt-regulator Jade-1 to the nucleus

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Background: Nephronophthisis (NPH) is a genetic cystic kidney disease caused by mutation of genes encoding one or more nephrocystin proteins (NPHP). NPH is characterized by renal fibrosis and cysts, resulting in end-stage renal failure necessitating kidney transplantation or dialysis by an average age of 14.

Observations: Cystogenesis is thought to involve over-activation of canonical Wnt signaling, but the mechanism remains unclear. Jade-1 has recently been identified as a novel ubiquitin ligase targeting beta-catenin for proteasomal degradation. Jade-1 was further shown to be regulated by the cystic kidney disease-causing ciliary protein, pVHL. As the NPH proteins also localize to the primary cilia, this work addressed the question of whether Jade-1 could similarly be a route through which nephrocystin proteins influence Wnt signaling. This work demonstrates that Jade-1 localizes to the ciliary base, and interacts with NPHP4. Furthermore, NPHP4 stabilizes protein levels of Jade-1 and is involved in the translocation of Jade-1 to the nucleus. Finally, NPHP4 and Jade-1 were demonstrated to additively inhibit Wnt signaling, and this interaction was shown to exist genetically in zebrafish.

Conclusions: The stabilization and nuclear translocation of Jade-1 by NPHP4 enhances the ability of Jade-1 to negatively regulate canonical Wnt signaling. Mutation of NPHP4 in Nephronophthisis could disrupt this mechanism, leading to increased Wnt activation and contributing to cyst formation.

C 142 Plaque-like A3 Adenosine Receptor Microdomains Are Associated with Bacteria-Tethering Nanotubes in Human Neutrophils

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Background: G protein-coupled adenosine receptors, which are activated by extracellular adenosine, have been shown to regulate neutrophil function; however, the precise role of these receptors in cell migration and the phagocytosis of pathogens remains controversial.

Observations: Using the fluorescent A3 adenosine receptor (A3AR) ligand CA200645, in conjunction with confocal microscopy and flow cytometry, we show here that endogenous adenosine receptors accumulate in plaque-like microdomains on the surface of human neutrophils and neutrophil-like HL60 cells. Studies with HL60 cells expressing an RFP-tagged actin-binding protein that does not interfere with actin polymerization (LifeAct) revealed that the adenosine receptor plaques are strongly co-localized with actin-rich leading-edge protrusions. In human neutrophils, pre-incubation with the selective A3AR antagonist MRS1334 blocked the binding of the fluorescent ligand, indicating that these plaques are composed predominantly of

A3ARs. MRS1334 also significantly inhibited the chemotaxis of human neutrophils in the presence of the chemoattractant fMLP. In addition to their involvement in chemotaxis, A3AR plaques were found at the base of nanotubes that extend from the plasma membranes of neutrophils in the presence of bacteria. These structures can reach in excess of 80 micrometer in length and are capable of tethering and 'reeling in' bacteria for subsequent phagocytosis.

Conclusions: Taken together, these results suggest that A3AR plaques act as chemosensory domains in human neutrophils, which facilitate cell migration and are associated with nanotubes, thereby facilitating the ability of neutrophils to phagocytose pathogens in a rapid, targeted fashion.

C 143 Regulation of Arp2/3 dependent actin polymerisation by Nck and N-WASP

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Background: Actin polymerisation is critical for many cellular processes including migration and endocytosis. Analysis of the signalling networks regulating actin polymerisation is often difficult due to their transient and dispersed nature. In contrast, many pathogens, including vaccinia, induce actin polymerisation by recruiting cellular signalling complexes in a robust and sustained manner. This makes them useful model systems to understand regulation of actin polymerisation in a quantitative manner.

Observations: During the vaccinia replication cycle, as newly assembled virus particles fuse with the plasma membrane, they activate Src and Abl family kinases. This leads to phosphorylation of a vaccinia protein, A36, and recruitment of a complex of Nck, Grb2, WIP and N-WASP, which activates the Arp2/3 complex to induce actin 'tails'. Nck is the most upstream member of the complex and is recruited independently of Grb2, WIP and N-WASP. To understand the role of Nck in this complex; we have examined the consequences of mutating its three SH3 domains, alone or in combination. We find that multiple Nck SH3 domains, together with those of Grb2 are required for efficient actin polymerisation. We have also identified putative Nck binding sites in N-WASP. We are investigating the effect of mutating these sites on actin tail formation by expressing N-WASP mutants in N-WASP null cells. Preliminary data reveals that mutation of these binding sites results in the formation of fewer actin tails, which are also shorter. Combining these mutations with one that abrogates the interaction of N-WASP with WIP results in a more severe phenotype.

Conclusions: Our data reveals that vaccinia uses a highly cooperative signalling network to induce actin polymerisation. Recruitment and activation of N-WASP requires multiple inputs from WIP, Nck and Grb2. Future work will aim to clarify the specific role of each of these interactions in actin tail formation.

C 144 Pulmonary surfactant protein A-modulated subcellular TLR4 localization in primary alveolar macrophages involves beta-arrestin2

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Background: The soluble C-type lectin surfactant protein (SP)-A modulates innate immune responses of the lung partially via its direct effects on alveolar macrophages (AM), the most predominant intraalveolar cells under basal conditions. Signaling by lipopolysaccharide (LPS) from Gram-negative bacteria is regulated through Rab GTPase-mediated subcellular localization of TLR4/TLR4 adaptor proteins. Both SP-A and the scaffold protein beta-arrestin2 act as negative regulators of LPS-induced TLR4 activation.

Observations: Confocal microscopy studies on the kinetics of TLR4 trafficking in freshly isolated AM from rats, wild-type and beta-arrestin2 deficient (bArr2-KO) mice show that LPS induces a rapid recruitment of TLR4 to the plasma membrane and enhances the colocalization of TLR4 with the early endosome antigen (EEA) 1 after 5 min. Pretreatment of rat AM with SP-A reduces the LPS-induced colocalization of TLR4 and EEA1 by promoting the colocalization of TLR4 with the post-Golgi compartment marker vesicle transport through interaction with t-SNAREs homologue 1B (Vti1b) and, assuming that the receptor complex is unable to signal from this compartment, thereby limits TLR4 activation. The small GTPase Rab10 upregulates LPS-induced TLR4 signaling by promoting the continuous replenishment of TLR4 from the Golgi to the plasma membrane. Investigations on the dynamic subcellular localization of Rab10 in primary rat AM reveal that treatment of the cells with LPS enhances the colocalization of Rab10 with TLR4 which is decelerated by pretreatment of the cells with SP-A. In bArr2-KO AM SP-A fails to inhibit LPS-induced colocalization of TLR4 with EEA1 at the plasma membrane compared to AM from wild-type mice.

Conclusions: The data demonstrate that SP-A modulated LPS-induced subcellular positioning of TLR4 in primary rat and mice AM critically involves beta-arrestin2 and directly affects Rab10 kinetics.

C 145 WFA-caused reactive oxygen species (ROS) regulates dedifferentiation, apoptosis and inflammation in rabbit articular chondrocytes

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Background: Withaferin A (WFA) is a steroidal lactone purified from the medicinal plant Withania somnifera. Many recent reports have provided evidence for its anti-tumor, anti-inflammation, anti-oxidant, and immuno-modulatory activity. Although it is suggested to have a large of effects, the defined mechanisms of action have not yet been determined.

Observations: We investigated the effects of WFA on dedifferentiation, inflammation, and apoptosis in rabbit articular chondrocytes. WFA resulted in the production of reactive oxygen species (ROS). We found that WFA dramatically reduced differentiation as determined by the expression of type II collagen and the synthesis of sulfated proteoglycan, and significantly induced inflammation as detected by the expression of cyclooxygenase-2 (COX-2) and apoptosis as analyzed by the expression of p53 and p21, and the fragmentation of DNA.
Conclusions: These results demonstrated that WFA-induced ROS regulates dedifferentiation, inflammation and apoptosis in rabbit articular chondrocytes.

C 146 Myosin-dependent coordination of cell movements underlies epithelial cyst morphogenesis

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Background: The formation of epithelial cysts, where a layer of epithelial cells surrounds a lumen, is a key step in the formation of most organs. While the molecular and cellular mechanisms mediating cyst formation have been extensively studied, comparable little is known about the cellular rearrangements underlying this process. To gain insight into cyst morphogenesis, we are analyzing the formation of Kupffer's Vesicle (KV).
Observations: KV is the first lumen-containing organ formed during zebrafish development and represents a unique assay system to analyze cyst formation as it amendable for both live imaging and functional studies. Using high-resolution 2-photon microscopy, we found that KV is formed from a compact mesenchymal cluster of around 60 progenitors cells. Within 2 hours, these progenitors cells rearrange to form an epithelial layer surrounding a single fluid-filled lumen. KV formation is initiated by the appearance of apical foci within the mesenchymal progenitor cell cluster, which subsequently begin accumulating fluid and coalescing into a single foci/lumen. Often, fluid accumulation precedes the completion of foci coalescence leading to a transient state with multiple primary lumens, which eventually will fuse into a single lumen. We propose that the cellular rearrangements underlying foci/lumen fusion are driven by a contractile actomyosin network spanning several cell diameters and connecting the different foci. Consistent with this notion, we found that inhibiting myosin2 activity leads to delayed foci coalescence and lumen fusion.
Conclusions: Our observation suggest a role of myosin dependant contractility in coordinating the cell movement leading to cyst formation. Currently, we are analyzing how the actomyosin network is formed and how it directs the cellular rearrangements underlying KV formation in zebrafish.

C 147 KRAB/KAP1 epigenetic regulation in hematopoietic stem cells homeostasis and lineage commitment

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Background: Temporal and spatial control of gene expression by epigenetic means is of fundamental importance to the morphogenesis and homeostasis of organs and organisms. The fate of a given precursor cell is dictated by a delicate balance of activating and repressing factors present at developmental crossroads.
Observations: The tetrapod-restricted krüppel-associated box domain-containing zinc finger proteins (KRAB-ZFPs), with some four hundred genes in both human and mouse, represent the largest group of transcription factors encoded by higher vertebrates. They function as transcriptional repressors through binding to KAP1/Trim28 which forms a bridge between DNA sequences specifically recognized by KRAB-ZFPs and chromatin-modifying complexes. Although only little is known about their physiological roles and specific targets, emerging evidence implicates KAP1, and in rare cases specific KRAB-ZFPs, in DNA repair, embryonic stem (ES) cells pluripotency, genomic imprinting, control of endogenous retroelements and in behavioral stress. Our analysis of conditional KAP1 knockout in the murine hematopoietic system hints at a crucial role of KRAB/KAP1 in the renewal and repopulation ability of hematopoietic stem cells (HSC). Our ongoing studies focus on HSC-specific KRAB-ZFPs that are candidate mediators of these effects. Our approach encompasses targeting the in vivo function of those genes by means of lentiviral knockdown and overexpression of KAP1 binding-deficient mutants in hematopoietic precursors.
Conclusions: Our long-term goal is to identify the mechanisms by which KRAB/KAP1-mediated regulation impacts on HSC homeostasis and differentiation.

C 148 Specifically different microRNAs profiles provide new insights on the molecular mechanisms activated by cytotoxic proinflammatory cytokines in mammalian pancreatic alpha- and beta-cells

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Background: Knowledge on the molecular events leading to pancreatic beta cells death in Diabetes Mellitus (DM) has increased significantly in recent years: it is now generally accepted that proinflammatory cytokines induce apoptosis of beta cells after altering insulin secretion. On the other hand, the knowledge on the fate of pancreatic alpha cells, subjected to the same cues, and their role in DM pathogenesis is still scanty. A similar statement may also be applied to the involvement of microRNAs in DM.
Observations: By applying a molecular systems approach and exploiting a TaqMan Low Density Array real-time PCR system, we sought to detect microRNAome alterations in both aTC1 and bTC1 cells, treated with cytokines (IFN-gamma, IL-1beta, TNF-alpha), for a time-course of 24 and 48 hours. All real-time PCR reactions were performed in biological triplicates. After identifying differentially expressed (DE) microRNAs (FDR < 10 %), we

filtered them to obtain a list of DE microRNAs whose expression varied at least 2 folds respect to non-treated controls. We checked for validated and predicted targets of DE microRNAs and enriched them for these Gene Ontology categories ($p < 0.01$): (i) apoptosis; (ii) regulation of cell differentiation; (iii) zinc ion binding. Expression alteration of target genes was investigated through real-time PCR and western analysis. We identified a total of 42 DE microRNAs: 3 of them were specifically dysregulated in aTC1, 7 in bTC1, both at 24 and 48 hours post-treatment; 2 microRNAs were dysregulated in both cell lines, at both time points. Real-time PCR and western analysis of target genes confirmed that their expression was anticorrelated respect that of candidate microRNAs.

Conclusions: The DE microRNAs, identified through our approach, are valuable candidates for involvement in important biological functions as apoptosis, cell differentiation, hormone secretion in alpha and beta pancreas cells.

C 149 Release of post-abscission midbodies from stem and cancer cells

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Background: The midbody is a cytoplasmic bridge formed between the nascent daughter cells at the end of cell division. Following its asymmetric inheritance by one of the daughter cells after abscission, the central portion of the midbody was generally thought to be retained by the cell and to be eventually degraded by autophagy. However, recent studies have revealed an alternative fate of the post-abscission midbody, that is, to be released into the extracellular space.

Observations: Here, we quantitatively analysed midbody fate by means of differential centrifugation of conditioned cell culture media, by immunofluorescence and by time-lapse video microscopy of various cell lines. We show that the extent of midbody release is greater in stem cells than cancer-derived cells, and greater if cancer cells are capable of differentiation. Moreover, induction of differentiation in neural and haematopoietic stem cells is accompanied by an increase in midbody release. To investigate the mechanism of midbody release, we performed a fluorescence image-based siRNA screen in neuroblastoma cells. Knockdown of the ESCRT family member Alix was found to result in increased midbody retention. Strikingly, the differentiation potential of neuroblastoma cells, as assessed by quantification of neurite outgrowth, was increased after Alix knockdown.

Conclusions: Our results reveal a link between midbody release and cell differentiation and suggest mechanistic similarities between midbody release and ESCRT-mediated abscission.

C 150 Cell adhesion contact up-regulates the expression of chemokine CXCL14/BRAK with concomitant increase in cell differentiation markers for squamous epithelium

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Background: The epithelial basal cell layer is comprised of immature keratinocytes. Cell-cell contact is known to be a critical regulator of cellular differentiation and motility. CXCL14/BRAK is a chemokine that is expressed in various normal tissues but is absent from cancerous tissues. In this connection, BRAK is rarely expressed in immature cells, we determined whether cell adhesion signaling up-regulates the expression of BRAK in squamous epithelium.

Observations: We reported that BRAK was not expressed or existed in negligible level in head and neck squamous cell carcinoma cells. BRAK protein was immunohistochemically detected in upper layer of the epithelium, which was considered to be differentiated keratinocytes. We determined efficacy of cell adhesion signaling in differentiation of keratinocytes. BRAK mRNA was up-regulated when the number of cells in culture was increased, and this accompanied by increase in involucrin, TGM1, TGM3 and TGM5, which considered as cell differentiation markers for squamous epithelium. We previously reported that GC box, which was important for the expression of BRAK mRNA, bind SP-1 transcription factor located on 14 bp upstream of the BRAK gene. Interestingly, BRAK mRNA up-regulation by cell adhesion signaling was inhibited by sh-RNA for SP-1.

Conclusions: In conclusion, SP-1 up-regulates the expression of BRAK mRNA induced by cell contact signaling in epithelial cells. Furthermore, we proposed the possibility that BRAK would be a new differentiation marker for epithelial cells.

C 151 Application of DNA methylation analyses for clinical cancer diagnostics

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Background: Aberrant DNA methylation is an early event during neoplastic transformation. Thus DNA methylation changes might be potent tumor-markers for diagnosis and specific methylation patterns might also be used for classification and prediction of disease outcome.

Observations: For efficient elucidation of DNA methylation changes we established several strategies using methylation sensitive restriction enzymes. Along with optimizing methods for genome-wide methylation profiling we established a methylation test targeting 360 DNA regions by combining multiplex-PCR and microarray hybridization. Although the Tm of CpG rich DNA is very high, primer and probe-design as well hybridization conditions have been optimized, thus this assay enables

multiplexed methylation testing from limited amounts of clinical samples. Using this AIT-CpG360 test we analysed malignancies of the breast, lung, thyroid, and others starting with 500ng DNA. We successfully applied this method also for elucidation of methylation changes in Down syndrome and for elucidation of epigenetic changes during epithelial to mesenchymal transition. Bioinformatical analyses of the microarray-data deduced many significant methylation changes in all of our settings. For confirming results we found that quantitative methylation analyses by qPCR is inevitable for validation and confirmed that this approach is reliable and correlates with bisulfite deamination based quantification of DNA methylation.

Conclusions: In all situations methylation profiling was capable for defining biomarkers for diagnostics. Here we present an efficient technical workflow for methylation analyses and illustrate the very high potential for using DNA methylation changes for improving diagnostics.

C 152 FAM124B is an interacting partner of CHD7 and CHD8

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Background: Mutations in the CHD7 gene are the underlying cause of CHARGE syndrome (coloboma, heart defects, atresia of the choanae, retarded growth and development, genital hypoplasia and ear anomalies) in approximately 2/3 of cases. To identify candidate genes involved in the pathogenesis of this disease, we searched for CHD7 interacting partners using the SILAC (stable isotope labelling with amino acids in cell culture) method in combination with mass spectrometry.

Observations: We identified FAM124B as a potential interacting partner of CHD7 and another CHD member CHD8, which also interacts with CHD7. We confirmed the result by direct yeast two hybrid studies and co-immunoprecipitation. Furthermore, we characterized the CHD7-CHD8-FAM124B binding site and studied the effect of four CHD7 missense mutations (p.His2096Arg, p.Val2102Ile and p.Gly2108Arg and p.Trp2091Arg) on the binding capacity between CHD7 and CHD8 in the absence or in the presence of FAM124B in a Yeast three hybrid experiment. In the absence of FAM124B the direct CHD7-CHD8 interaction is disrupted by the CHD7 mutations p.Trp2091Arg, p.His2096Arg and p.Gly2108Arg. The presence of FAM124B has no negative effect on the direct CHD7-CHD8 binding.

Conclusions: The characterisation of CHD7 containing complexes and the influence of CHD7 containing missense mutations on the binding capacity of complex partners will help to understand the pathogenesis of CHARGE syndrome.

C 153 DNA methyltransferase 1, 3a, 3b and 3L are transiently upregulated during the development of renal fibrosis

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Background: The cell biological process underlying all chronic renal disorders is chronic fibrosis. Crucial in this process is the DNA-methylation mediated terminal activation of fibroblasts. To gain insight into which DNA methyltransferases are involved, this study aimed at a time course evaluation of the expression of Dnmt1, 3a and 3b and their non-functional homologue Dnmt3L, in two different mice models of fibrosis: aristolochic acid nephropathy (AAN) and ischemia/reperfusion (I/R) injury.

Observations: Mice (20-25g) received a single intraperitoneal injection of aristolochic acid I (AAI, 3.5 mg/kg) or phosphate buffered saline (control) or underwent unilateral left ischemia during 30 minutes. In case of I/R, the contralateral kidney was used as a control. Mice were sacrificed after 1, 3, 6 and 12 weeks. The expression level of Dnmt1, 3a, 3b and 3L was assessed by quantitative real-time PCR on total renal tissue mRNA. All Dnmt's were significantly upregulated (3-6 fold) at week 1 after I/R, whereas at week 1 after AAI injection only Dnmt1 showed a slight 2-fold increase. In the I/R model, expression of Dnmt1 and 3L remained high (resp. 3-4 and 5-6 fold) until week 6 after reperfusion, whereas at that time Dnmt3a and 3b reached their maximum (5-8 fold). In the AAI model, maximum expression levels (3-5 fold increase) for all Dnmt's were reached 3 weeks after AAI injection. At week 12, when fibrosis was most prominent, the expression of the Dnmt's clearly decreased in the I/R model or even decreased towards control levels in the AAI model. Furthermore, fibrosis at week 12 was far more pronounced in the I/R model than in the AAI model.

Conclusions: Dnmt 1, 3a, 3b and 3L show a different expression pattern during the development of fibrosis after either an acute toxic or ischemic insult. Dnmt upregulation appears to be only transient, suggesting that their continued expression is not a prerequisite for progression of fibrosis.

C 154 Detection of pre-leukemic cell clone in ETV6/RUNX1 positive childhood acute lymphoblastic leukemia

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Background: Acute lymphoblastic leukemia (ALL) is the most frequent malignancy in childhood. The amount and dynamics of residual tumor load presented during induction therapy is an independent prognostic factor in pediatric ALL. It is therefore used increasingly in modern treatment protocols to define at-risk groups. In this study, we examined the tumor dilution of bone marrow of patients with ETV6/RUNX1+ ALL following therapy using a combined cell-, RNA- and DNA based methodology.

Observations: 55 bone marrow samples from 14 children with ETV6/RUNX1+ ALL were analyzed. Samples were collected according to the applied ALL IC-BFM 2002 protocol. Combined CD10 immunophenotyping and ETV6/RUNX1 fluorescent in situ hybridization analysis was performed on cytospin preparations using automated microscopy. Results were compared to data obtained using reverse transcription PCR targeting the

ETV6/RUNX1 fusion gene. If significant discrepancy between the results of cell- and RNA-based techniques was observed the ETV6/RUNX1 gene fusion was examined at DNA level as well by multiplex-PCR. Cell population positive for both CD10 and ETV6/RUNX1 fusion was detected in 89%, 30%, 13% and 36% of cases at day 15, day 33, week 12 and month 5, respectively. Additional chromosomal aberrations also detected at the time of diagnosis were found in cells positive for ETV6/RUNX1 fusion at days 15 and 33. At week 12 and month 5, these secondary changes were not observed suggesting the presence of pre-leukemic cells instead of residual tumor load. Significant ETV6/RUNX1 expression were not detected after day 15. The DNA-based multiplex-PCR analysis successfully validated the results of cell based method.

Conclusions: Application of methods able to detect pre-leukemic clones has a great importance in the monitoring of therapy of children with ALL because using these techniques an impending late relapse typical to this entity can be indicated in early phase.

C 155 Automated evaluation of a multicolor, multiprobe FISH assay for the detection of bladder cancer

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Background: Signal pattern enumeration of Urovysion Fluorescence in Situ Hybridization (FISH) test is tedious and requires great experience. Our aim was to minimize human interaction by automating the process, using a user-trainable, automated image acquisition and analysis system.

Observations: For extensive analytical analysis control cell populations were used, while preliminary clinical study was performed on 21 patients with clinical suspicion for bladder cancer. All investigations were carried out using an automated user-trainable workstation (Metafer4-Metacyte). The system identified nuclei with a specificity and sensitivity of 92.7% and 96.6%, respectively, while signal detection accuracy was 81.1% on average. Both analytical and diagnostic accuracy of automated analysis was comparable to manual approach (94.8% and 71% vs 97.9% and 76%, resp.), but classification accuracy increased with degree of polysomy, thus diagnostic sensitivity in low grade, low stage cases was poor.

Conclusions: It is possible to automate evaluation of Urovysion using a user-trainable system, and achieve efficiency comparable to manual analysis. However, the problem of reduced detection accuracy in cases featured with low polysomy is likely to remain a great challenge of automated signal enumeration.

C 156 Osteoporosis and Dyslipidemia – Does the lipid profile improve the power of Bone Mass Density correlation with Body Mass Index, 25OHVitamin D, Alkaline Phosphatase or Beta Cross Laps?

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Background: Bone Mass Density and Beta CrossLaps have been recognized as valid tests for the diagnostic and treatment control, as well as fracture prediction for osteoporotic patients. Statin treatment has been reported to be associated with a reduced risk fracture in these pacients also. However, no studies, as of our knowledge, have tried to see if adding the lipid profile to the analysis of Body Mass Density would improve the statistical power of prediction for any of the afore mentioned events.

Observations: In our study 613 osteoporotic (T score mean -3.16, SD 0.81) postmenopausal (mean 15.79 yrs, SD 8.88yrs.) women were enrolled and they were grouped according to age (under 50 yrs., 51-60 yrs., 61-70yrs., over 70 yrs.) and the presence/absence of a history of bone fracture. Bone Mass Density was not correlated with Body Mass Index ($p > 0.05$, $r2 < 0.02$). However, when Bone Mass Density was correlated with both Body Mass Index and the lipid profile (represented by Triglycerides, Cholesterol, LDL-C, HDL-C,) we obtained very good correlations in 9 of the 12 groups, with 5 of them having a great statistical power: under 51 yrs. ($n = 72$, $r2 = 0.27$, $p = 0.11$) with fractures ($n = 56$, $r2 = 0.38$, $p = 0.37$), 51-60 yrs. with fractures ($n = 61$, $r2 = 0.14$, $p < 0.01$), 61-70 yrs. ($n = 201$, $r2 = 0.09$, $p < 0.01$) with fractures ($n = 88$, $r2 = 0.14$, $p < 0.01$) or without fractures ($n = 113$, $r2 = 0.24$, $p = 0.02$) and over 70 yrs. ($n = 247$, $r2 = 0.11$, $p < 0.01$) with fractures ($n = 67$, $r2 = 0.35$, $p = 0.11$) or without fractures ($n = 51$, $r2 = 0.35$, $p = 0.08$). Similar results were obtained when Bone Mass Density was correlated with Alkaline Phosphatase or 25OHVitamin D or Beta Cross Laps and the lipid profile.

Conclusions: Although we recognize the limitations of this study (the lack of complete data for some our pacients and of statistical power in a part of the correlations), we consider that this pilot study opens a new perspective on the management of osteoporosis.

C 157 A new noncoding risk factor for Acute Lymphoblastic Leukemia: 8q24 region preliminary data

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Background: Genome-wide association studies revealed that numerous variations in chromosomal region 8q24 strongly associated with several cancers. One of these variants, rs6983267, was found to be strongly associated with colorectal cancer with the G allele conferring increased risk. The rs6983267 variant is predicted to be located in the consensus binding sequence

of TCF4 and it physically interacts with the c-MYC proto-oncogene.

Observations: In this study, we aimed to determine the frequency of the rs6983267 variation in pediatric acute lymphoblastic leukemia (ALL) patients and to detect effects of this variation on the c-MYC and TCF4 genes mRNA levels. We obtained bone marrow/peripheral blood samples from pediatric ALL patient (n = 64) and healthy controls (n = 62). We used real time PCR (RT-PCR) for genotyping and quantitative gene expression analysis (QRT-PCR). The samples were analyzed in order to determine their rs6983267 genotypes for GG, TT or GT. TCF4 and c-MYC mRNA levels were identified by QRT-PCR in between GG and GT,TT genotypes. Genotype (GG; 26% vs 17%, GT; 50% vs 58% and TT; 24% vs 25%, cases vs control, respectively) and G risk allele frequency (cases 51% vs controls 46%) of rs6983267 were not significantly different between cases and controls. QRT-PCR analysis showed increased c-MYC and TCF4 gene expression pattern for GG allele carriers. In this study we have compared the relative expressions of the TCF4 and c-MYC genes. According to previous studies, the risk allele rs6983267 is located in the binding sequence of the transcription factor TCF4 and it is thought to regulate c-MYC.

Conclusions: In our preliminary data, we determined increased expression levels of c-MYC and TCF4 genes in pediatric ALLs and controls that carried GG allele and our data provide a support for a biological mechanism underlying this non-protein-coding risk variant.

C 158 Insights into the mechanism of action of nonclassical platinum–acridine anticancer agents from comprehensive chemogenomic fitness screens

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Background: The prevalence of platinum resistance in cancers limits the use of the highly effective platinum-based antitumor agent, cisplatin. To overcome this resistance, a novel class of platinum-acridine conjugates was designed to act by a hybrid of platinating and intercalating into DNA, a mechanism unlike current platinum therapeutics. To examine the effectiveness of these compounds as potential chemotherapeutics, we performed unbiased genome-wide screens to examine their mechanisms of action in vivo.

Observations: Platinum–acridines were derived from the prototype PT-ACRAMTU(EN) to produce a library with structural and functional diversity. We profiled these 11 compounds using a full genome sensitivity screen of ~6000 Saccharomyces cerevisiae gene deletion mutants to dissect their mechanisms of action. Four compounds, PT-ACRAMTU(EN), PT-ACRAMTU(PN), PT-ATUCA and PT-AMIDINE, showed unambiguous DNA-damaging profiles and required distinct modules of DNA repair for resistance compared to cisplatin. The profiles revealed the relative importance of each type of DNA repair module for resistance to the individual compounds. In addition, our global analysis revealed insight into the structural modifications that led to these DNA-damaging properties. FACs analysis of S. cerevisiae treated with these compounds identified five conjugates that caused cell cycle disruptions. Interestingly, three of these compounds are ones that did not produce DNA-damaging profiles. This can be important as components of the cell cycle are now viewed as potential anticancer targets. Although the remaining four compounds interfered with cellular fitness, we were unable to discern any clues towards a possible mechanism.

Conclusions: Our genome-wide study of a novel set of platinum-based hybrid agents identified distinct DNA repair modules required for survival. These findings are valuable as promising leads to cancer therapeutics, possibly side-stepping the problem of cisplatin-resistant tumors.

C 159 Proteomic analysis of seminal plasma by 2-D DIGE in men with different spermatogenic impairment

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Background: Seminal plasma is a potential source of biomarkers for many disorders of the male reproductive system including male infertility. Proteomic techniques such as 2-D DIGE and MS techniques have the potential to discover links between certain proteins and conditions. The identification and characterization of differentially expressed proteins in seminal plasma can serve as a basis for estimating male infertility.

Observations: Using 2-D DIGE, we compared protein expression profiles of seminal plasma from four different groups of men: normozoospermic men (group 1) and men with different spermatogenic impairment, asthenozoospermia (group 2), oligozoospermia (group 3) and azoospermia (group 4). The 2-D DIGE analysis was performed on IPG strips pH 4-7, with 4 biological replicates per group. Statistically significant, differentially expressed proteins were selected based on two criteria: Annova < 0.05 and Ratio > 1.8. Our results showed that there are no statistically significant differences in protein expression between normozoospermic group and oligozoospermic and asthenozoospermic groups. However, when comparing azoospermia with other studied groups, we found total of 8 distinct proteins with statistically significant increased expression from 2.0 to 11.3-fold. Among them, one protein (pI ~ 6 and Mw ~55 kDa) was found to be exclusively increased in azoospermic patients compared with the three other studied groups, with mean fold change of 2.63 + 0.65. This protein represents a very good candidate biomarker for azoospermia.

Conclusions: The present data suggested several possible candidate markers for azoospermia. However, further investigations will be necessary to identify these markers, to test their suitability in clinical diagnosis, and to clarify their role in male infertility.

C 160 Proteomic analysis of infiltrating ductal carcinoma tissues by coupled 2-D DIGE/MS analysis

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Background: There is growing interest in protein expression profiling aiming to identify novel diagnostic markers in breast cancer. Proteomic approaches such as 2-D DIGE/MS/MS have been used successfully for the identification of candidate biomarkers for screening, diagnosis, prognosis and monitoring of treatment response in various types of cancer. Identifying previously unknown proteins of potential clinical relevance will ultimately help in reaching effective ways to manage the disease.
Observations: We analyzed five tumor and five normal tissue samples from ten breast cancer subjects with infiltrating ductal carcinoma (IDC) by 2-D DIGE using two types of IPG strips: pH 3–10 and pH 4–7. From all the spots detected, differentially expressed ($p < 0.05$ and ratio > 2) were 50 spots. Of these, 39 proteins were successfully identified by MS, representing 29 different proteins. Ten proteins were overexpressed in the tumor samples. The 2-D DIGE/MS/MS analysis revealed an increased expression in tumor samples of few proteins not previously associated with breast cancer, such as: macrophage-capping protein (CAPG), phosphomannomutase 2 (PMM2), ATPase ASN1, methylthioribose-1-phosphate isomerase (MRI1), peptidyl-prolyl cis-trans isomerase FKBP4 (FKBP4), cellular retinoic acid-binding protein 2 (CRABP2), lamin B1 and keratin, type II cytoskeletal 8 (KRT8). Ingenuity Pathway Analysis (IPA) revealed highly significant (p = 10-26) interaction between identified proteins and association with cancer.
Conclusions: Proteomic approach used in this study has revealed several candidate biomarkers for IDC. These proteins are involved in multiple and diverse pathways in cellular metabolism. It remains goal of future work to test the suitability of the identified proteins in samples of larger patient cohorts.

C 161 Towards improving sensitivity and specificity of p53 diagnosis in human breast cancer

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Background: Mutation of the TP53 gene is the most frequent genetic alteration in human cancer and occurs in approximately 20-40% of all human breast tumors. P53 is routinely diagnosed in clinical breast cancer care by immuno histochemistry (IHC). However, the correlation between p53 protein accumulation detected by IHC and TP53 mutation detected by sequencing is less than 75% in breast tumors. Thus, the clinical and prognostic value of p53 diagnosis by IHC is still debated.
Observations: In this study, p53 status was examined in 114 Austrian breast cancer patients by IHC and sequencing. Moreover, IHC and/or qRT-PCR for the p53 target genes p21 and MDM2, and qPCR to detect amplifications of negative regulators such as MDM2/4 and overexpression of miRNA125b was performed to further refine the functional status of p53. Using a different scoring method of IHC diagnosis of p53 mutation in the patient collective we could show better correlations with sequencing results as the current standard scoring method (cutoff 10%). We found functional TP53 mutations in 31 out of 114 breast cancer patients (27.2%). Mutant p53-status significantly correlated with estrogen and progesterone receptor negativity, younger age of onset, high tumor grade, and a ductal tumor type. Approximately 8% of the tumor samples showed amplifications of MDM2 and/or MDM4. In tumors harboring MDM2/4 amplifications and/or TP53 mutations the mRNA expression of p53 targets was significantly reduced. Identification of new markers correlating with p53 activity status and the development of new IHC-based assays should improve the accuracy of p53 diagnosis in breast cancer.
Conclusions: We could assign each patient to either a 'p53 inactive' or 'p53 active' status based on the sequencing, IHC, qRT-PCR, MDM2/4 copy number, and miRNA 125b expression analyses. This study should eventually improve the prognostic power of p53 in human breast cancer.

C 162 A miRNA signature differentiates Ph+ ALL children according to the first response to therapy

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Background: In the past two decades, childhood ALL cure rate has reached over 80% due to treatment advances, but some resistant ALL subtypes still don't respond to therapy. The presence of BCR-ABL in ALL children is correlated to a very poor prognosis, nevertheless several long-scale studies have shown that Ph + ALL is heterogeneous in terms of clinical parameters and patients respond differently to the therapy, what suggests the presence of additional mechanisms of leukemogenesis.
Observations: Different studies with large series of patients have shown that an earlier remission after induction with glucocorticoids and intrathecal methotrexate (IT MTX) is correlated to a better outcome. In the present study we looked for secondary genetic lesions in a group of 78 consecutive Ph + ALL children diagnosed in Italy between 2000 and 2010, specifically studying the miRNA profile according to the first therapy response. The miRNA signature was analyzed by miRNA array comparing two groups of patients differentiated according to MRD/prednisone response, and later confirmed by single assays. A particular miRNA profile was found in the group of poor responder patients. In vitro studies using ALL Ph + cell lines showed that these miRNA are direct up-regulated by BCR-ABL. On the other hand, in vivo studies using NUDE mice demonstrated they can act as oncogenes, increasing leukemia aggressiveness.
Conclusions: Further studies will be necessary to understand how miRNA act in cooperation with BCR-ABL to induce

leukemia. In the future, novel target therapies can emerge as pro-apoptotic approaches to be added to the anti-tyrosine kinase drugs in order to improve response and survivor in Ph + ALL.

C 163 The Expression of Novel Gene URGCP in Prostate Cancer Cell Lines: Correlation with Rapamycin

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Background: Molecular targets in prostate cancer are continually being explored, for which there are currently few therapeutic options. Rapamycin (RPM) is an antifungal macrolide antibiotic isolated from Streptomyces hygroscopicus which can inhibit the G1 to S transition. URGCP (upregulator of cell proliferation) is a novel gene located on chromosome 7p13. We aimed to investigate of the role of URGCP gene expression changes in PC3, DU145, and LNCAP cell lines with/out RPM.
Observations: Average cell viability were period of 0, 24, 48, 72 and 96 hours determined by trypan blue dye exclusion test and MTT assay. Cytotoxic effects of rapamycin in DU145, PC3 and LNCAP were detected in time and dose dependent manner with the IC50 doses of 5, 10, 25, 50 and 100nM, respectively. As the results were evaluated, IC50 doses in the PC3, LNCaP and DU145 are detected as 25, 50 and 10 nM, respectively. The mean relative ratios of URGCP gene expression in DU145, LNCAP and PC3 were found as -1.48, 6.59 and -13.00, respectively, when compared to rapamycin-free cells. The False Discovery Rate adjusted p-value in DU145, LNCAP and PC3 were 1.25´10-5, 2.20´10-8 and 6.20´10-9, respectively. The URGCP gene expression level is compared between the dose and control group, we found that URGCP gene expression was significantly decreased in dose groups of DU145 and PC3 cells.
Conclusions: In conclusion, prolonged rapamycin treatment inhibits the proliferation of DU145 and PC3 cells. This may be caused by rapamycin-induced cell cycle arrest at the G1 phase and inhibition of URGCP gene expression.

C 164 Prediction of radiotherapy and chemoradiotherapy efficacy in patients with breast or bladder cancer

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Background: Efficacy of chemoradiotherapy (CRT) for patients with solid tumour may be evaluated by a span of disease free survival (DFS). Possibly, it may be predicted by means of determination of individual DNA sensitivity of tumour cells or leukocytes and serum to test-damage ex vivo, for example after their irradiation. The results of such translational studies would be enable to improve a selection of patients for the effective treatment.
Observations: 113 patients were studied. 58 female (36-77 yrs) with the breast cancer (BrC) (adenocarcinoma IIIB-IV stages) were treated by radiation therapy (RT) or CRT including standard schedule CMF-regimen. The RT was performed with 60 Gy total dose. The 55 patients (32-79 yrs, 45 men and 10 women) with the bladder cancer (BlC) (transitional cell carcinoma, T2-3N0M0) were studied as well. These patients were treated by standard CRT with the MVAC scheme and by a local irradiation of the bladder with 62-68 Gy total dose. The DNA radiosen-sitivity of blood leukocytes (Sb-index) and urinary sediment cells was performed by means of fluorescent determination of a nucleoid DNA decay rate by 3 hrs after test-irradiation ex vivo [Ivanov S.D. et al., Pat. No 2319963, 2008]. The radiosensitivity of the bladder urinary epithelium cells (Su-index) was calculated. A significant positive linear dependence ($p < 0.05$) was observed between Sb-index sizes and the DFS-spans in case of RT or CRT for patients with BrC. The duration of DFS-period (Tdfs) in BlC patients after the treatment related significantly with the Sb-index [Tdfs (months) = -7.58 + 20.87 x Sb; $p < 0.01$] as well, but no with the Su-index.
Conclusions: Thus the simple short-term inexpensive test for routine determination of apoptotic index after blood irradiation ex vivo enabled to evaluate DNA radiosensitivity, to predict the DFS-spans for patients with BlC or BrC, and so to help doctors to select the patients for adequate treatment.

C 165 High Throughput Screen for Inhibitors of the Canonical Wnt Signaling Pathway in Colorectal Cancer

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Background: The Wnt/b-catenin pathway is one of the most important signaling mechanisms involved in cell differentiation and proliferation. The pathway is crucial in the embryonic development and in renewal of adult tissues including the intestine. The intestinal epithelium is the fastest self-renewing tissue in a human body. Aberrant activation of the Wnt signaling pathway in the intestinal stem cells leads to development of colorectal cancer, one of the most common types of cancer in the Western world.
Observations: We tested using HEK 293 cells harboring the integrated TCF/b-catenin-dependent luciferase reporter SuperTOPFLASH a large panel of 'small molecules' as potential antagonists of the Wnt-responsive transcription. Subsequently, the selectivity of the primary 'hits' was validated in human colorectal cancer cells with aberrant activation of the Wnt pathway. Moreover, the ability of different chemical compounds to inhibit the accumulation of b-catenin in the nucleus was visualized in mouse L cells stimulated with recombinant Wnt3a ligand. In addition, the

study included two in vivo tests. At first, the compounds were used to inhibit the formation of the XWnt8-mediated secondary axis in Xenopus embryos. Next, we employed APC-CKO/CK-OxLGR5-creERT2 mice to test the ability of the compounds to restrain tumor growth initiated upon the loss of the Apc tumor suppressor gene.

Conclusions: In summary, from total 13 000 molecules tested three compounds performed well in the assays. Currently, we are defining the molecular mechanisms of action of these Wnt signaling inhibitors.

C 166 Biochemical characterization of novel isomere of roscovitine, an anticancer drug in clinical trials

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Background: The deregulation of cell cycle components in cancer cells has provided a rationale for the development of inhibitors as novel anticancer drugs that could act as cyclin-dependent kinases (CDK) activity modulators, restore normal cell cycle regulation or induce cell death. Roscovitine is a synthetic inhibitor of CDKs that undergoes evaluation in clinical trials, but its efficiency is not high.

Observations: We have prepared bioisostere of roscovitine with the pyrazolo[4,3-d]pyrimidine core and evaluated it in diverse biochemical and cellular assays in comparison with roscovitine as a reference molecule. Both compounds were tested on a panel of cancer cell lines and recombinant protein kinases. Effects on cell cycle progression and induction of apoptosis were analyzed by means of flow cytometry, immunoblotting, and immunofluorescence. Moreover, an X-ray crystal structure of its complex with CDK2 has been determined to demonstrate the binding mode.

Conclusions: Our results clearly demonstrate that a change in a position of a single nitrogen atom significantly improves CDK inhibitory properties of roscovitine. Moreover, all biological effects of the new CDK inhibitor are consistent with CDK inhibition as a primary mode of action.

C 167 Memory vulnerabilities and stress reactivity following exposure to lipopolysaccharide in a 6-OHDA-lesion model of Parkinson's disease

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Background: A number of deleterious effects on behavior and cognition in laboratory animals have been observed following immune system activation with lipopolysaccharide. The purpose of the present study is to provide additional support for memory processes vulnerabilities and stress reactivity in a 6-OHDA-lesion model of Parkinson's disease associated with bacterial endotoxin infection. Also, we searched for a possible correlation between behavioral responses and oxidative stress.

Observations: In the present study, male Wistar rats received saline, lipopolysaccharide (LPS, 250 microg/kg in saline, 7 consecutive days), intranigral 6-hydroxydopamine (6-OHDA, 2microg/microliter saline; 5microl/site) and intranigral 6-OHDA plus 7 consecutive days of LPS injections and then tested in two cognitive tasks (Y-maze and radial arm-maze). Altered behavioral responses in Y-maze and radial arm-maze tasks were observed in LPS- and LPS + 6-OHDA-treated rats compared to control group. Notably, positive correlations were detected among LPS and LPS + 6-OHDA-treated rats when behavioral deficits were correlated with indicators of oxidative stress.

Conclusions: Taken together, we demonstrated that activation of the immune system with LPS administration induced memory impairment and brain oxidative stress, significantly correlated with nigral lesion promoted by 6-OHDA.

C 168 Lower sensitivity to amphetamine in Lsamp gene deficient mice is related to lower level of DAT and increased serotonergic tone

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Background: Limbic system associated membrane protein (LAMP) is a cell adhesion molecule of the IgLON family, structurally characterized by three immunoglobulin (Ig) domains. During development this protein has been shown to guide the development of specific patterns of neuronal connections. In mice studies, the Lsamp gene has been implicated in locomotion, anxiety, fear reaction, fear conditioning, learning and social behavior. Human data links the LSAMP gene to several psychiatric disorders.

Observations: Here, by means of Lsamp gene deficient (–/–) mice, we studied the effect of amphetamine and cocaine on the locomotor behavior in the motility box in both young and older animals. Second, we studied the rewarding effects of amphetamine and cocaine in the place preference test. Third, we measured monoamine levels in major brain regions in response to saline and amphetamine administration. Finally, we measured the expression levels of dopamine system-related genes in the brain. Young Lsamp–/– mice displayed significantly lower sensitivity to amphetamine, but not cocaine in the motility box. Older Lsamp–/– mice were still less sensitive to amphetamine, but the difference was less prominent, however, they displayed no age-related loss of sensitivity to cocaine. In all brain regions measured, Lsamp–/– mice displayed lower 5-HT baseline levels, but a greater 5-HT turnover rate, and amphetamine increased the level of 5-HT and lowered 5-HT turnover to a greater extent in Lsamp–/– mice. Gene expression analysis revealed that Lsamp–/– mice had lower level of DAT mRNA in the mesencephalon.

Conclusions: Altered response to psychostimulants in Lsamp−/− mice can be explained by the combined effect of lower level of DAT, increased 5-HT-ergic tone and enhanced 5-HT release in response to amphetamine. Lsamp−/− mice are a promising model to study deviant social behavior observed in psychiatric disorders.

C 169 Sumoylation of MuSK: A regulatory mechanism during the development of the neuromuscular junction

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Background: The neuromuscular junction (NMJ) is the synapse between a neuron and a muscle fiber. The muscle specific kinase MuSK represents the key regulator during NMJ formation. MuSK activity is tightly regulated via post-translational modifications like phosphorylation. Another reversible post-translational modification implicated in many different cellular mechanisms is sumoylation, which results in the covalent attachment of a protein called small ubiquitin-related modifier (SUMO) to the target.
Observations: MuSK is a receptor tyrosine kinase with an intracellularly exposed site containing the sequence LKEE, which conforms to a consensus motif for sumoylation on the lysine residue. Thus, the questions were raised whether MuSK can be sumoylated and whether sumoylation affects MuSK function. This could happen via interference of MuSK signalling or MuSK endocytosis or MuSK stability. We have been addressing these issues by studying MuSK sumoylation in vitro and by examining downstream effects of MuSK function in muscle cells expressing a mutant MuSK protein that cannot be sumoylated. Preliminary data suggest that MuSK is sumoylated in vitro at the proposed sumoylation site. In addition, the formation of AChR clusters, one of the hallmarks of MuSK downstream signalling, is impaired in muscle cells expressing the sumoylation-deficient mutant MuSK. In a biochemical approach we are currently studying ligand-dependent activation of sumoylation-deficient MuSK.
Conclusions: Taken together, we propose that MuSK is sumoylated and that sumoylation modulates MuSK function. Future studies will therefore focus on the effects of sumoylation on MuSK signalling and MuSK trafficking and their downstream role during the development of the NMJ.

C 170 Polyglutamine Atrophin provokes autophagic neurodegeneration by repressing fat

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Background: Large alterations in transcription accompany neurodegeneration in polyglutamine diseases. These pathologies manifest both general polyglutamine toxicity and mutant protein specific effects.
Observations: Here we report that the fat tumour suppressor gene mediates neurodegeneration induced by the polyglutamine protein Atrophin. In a Drosophila model of Dentatorubral-pallidoluysian Atrophy (DRPLA), polyglutamine Atrophins induce autophagic neurodegeneration charcterised by lysosomal blockage. We have monitored early transcriptional alterations in this Drosophila model and found that polyglutamine Atrophins downregulate fat. Fat protects from neurodegeneration and Atrophin toxicity through the Hippo kinase cascade. The Fat/Hippo signalling alters the autophagic flux in photoreceptor neurons, thereby affecting cell homeostasis.
Conclusions: Our data thus provide a crucial insight into the specific mechanism of a polyglutamine disease and reveal an unexpected neuroprotective role of the Fat/Hippo pathway.

C 171 Enhanced anxiety-reducing effect of environmental enrichment and insensitivity to isolation stress in Lsamp gene deficient mice

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Background: Lsamp gene-deficient mice have been shown to be slightly hyperactive in novel environments and less anxious, and they display alterations in fear reaction, fear conditioning and social behaviour. This study was undertaken to determine to role of environment in these phenotypic differences. We assumed that phenotypic differences directly caused by the deletion of the Lsamp gene will be observable regardless of the environment in which the mice are raised.
Observations: In this study, we exposed male Lsamp gene-deficient mice to 5-week environmental enrichment (EE), a technique that has often been shown to abolish phenotypic deviations in knockout mice, and 1-week social isolation, a stressful manipulation, after which all mice were tested in a behavioural battery. EE had a much stronger anxiolytic effect on Lsamp gene-deficient mice than on their wild-type littermates both in the plus maze and open field tests, however, the knockouts were insensitive to isolation stress as confinement to single housing induced no loss temporary weight loss, typical to wild-type mice on the first week of isolation. Isolation even seemed to have an anxiolytic-like effect on Lsamp gene-deficient mice as it induced a significant drop in swimming speed, another parameter of anxiety. Isolation and EE lowered blood corticosterone concentrations in wild-type, but not Lsamp gene-deficient mice. Overall, Lsamp gene-deficient mice were remarkably insensitive to the stressful aspects of environmental manipulations, but EE augmented their anxiolytic-like phenotype.
Conclusions: The study underlines the importance of testing knockout animals in several environments as social isolation and EE abolished some phenotypic differences observed in Lsamp gene-deficient mice in standard housing conditions, such as different body weight, while augmented others, such as lower anxiety.

C 172 The autophagy-related scaffold protein GOPC reduces the level of intracellular huntingtin aggregates

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Background: Autophagy has been implicated in degradation of mutant huntingtin, a protein responsible for a lethal neurodegenerative disorder known as Huntington's disease. GOPC is a scaffold protein that interacts with several autophagy-related proteins, including beclin 1, a known regulator of huntingtin degradation. As the involvement of GOPC in clearing protein aggregates has never been investigated, we tested the impact of this scaffold protein on the intracellular aggregation of mutant huntingtin.

Observations: The over-expression of GOPC in human HeLa or mouse neuronal HT-22 cells significantly reduced the aggregation level of N-terminal mutant huntingtin fragments of different length (N64, N171 and N588). Unlike beclin 1, GOPC never co-localized with aggregating huntingtin species, thus suggesting that it was not directly involved in the autophagy-mediated process of degradation of huntingtin aggregates, but likely contributed to the early steps of autophagy regulation. Interestingly, GOPC showed stronger anti-aggregational potential towards the very short N-terminal huntingtin fragments (N64 and N171) that are generally regarded as more pathogenic, as they are able to form both cytoplasmic and nuclear inclusions. This was in contrast to two other regulators of autophagy-mediated huntingtin degradation, beclin 1 and p62, that were more efficient against the exclusively cytoplasmic aggregates built by longer huntingtin fragments (N588).

Conclusions: Our data indicate that GOPC is engaged in the autophagy-mediated process of huntingtin degradation and can be employed in future therapeutic strategies against the Huntington's disease.

C 173 Cell culture modeling of tau aggregation and intercellular spread

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Background: During the development of Alzheimer's disease, tau pathology arises in a progressive, reproducible manner transiting from the locus coeruleus, to the neocortex. Recent data suggests this stereotypical course may proceed in a prion-like manner whereby aggregated tau is transferred between cells and acts as a seed to begin homotypic protein misfolding. To gain a better understanding of the mechanisms underlying this phenomenon we sought to model tau aggregation and transfer in cell culture.

Observations: Because protein misfolding is believed to progress in a templated manner, we reasoned that initiating aggregation in cells using tau from diseased brain would provide a model system in which the conformation and biological activity of disease-associated species would be maintained with high fidelity. We found wild type and mutant P301S tau expressed in HEK293T cells to be readily converted from a soluble to an aggregated state upon exposure to brain extracts from transgenic mice expressing human P301S tau. Moreover, the aggregated species generated were characterized by the presence of phosphoepitopes closely linked to human disease. We were also interested in determining whether tau might spread between cells, and if so, whether it was capable of seeding aggregation in neighboring cells. We began by generating a YFP fusion protein (Tau-YFP) and monitored transfer using flow cytometry. We found efficient intercellular transfer of Tau-YFP that required direct cell-to-cell contact. Unexpectedly, however, when aggregate producing cells were co-cultured with a fresh population of acceptor cells we were unable to detect transmission of tau misfolding.

Conclusions: We have developed a new model to study tau aggregation using brain extracts to induce misfolding. Despite intercellular transfer of Tau-YFP, aggregated tau does not induce robust misfolding in neighboring cells. This may be consistent with the lengthy clinical course observed in Alzheimer's disease.

C 174 ATP-sensitive P2X3 receptors of trigeminal ganglion neurons are constitutively upregulated in a genetic model of familial migraine

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Background: The role of Calcitonin Gene Related Peptide (CGRP) in migraine pain has been argued in the recent past. We studied the role of CGRP on purinergic P2X3 receptors of trigeminal ganglia (TG) of a Familial Hemiplegic Migraine-1 mouse model. P2X3 receptors from the trigeminal ganglia of this KnockIn (KI) model show increased activity compared to WT because of the R192Q mutation of P/Q type calcium channel conferring gain of function with consequent enhancement in intracellular calcium.

Observations: With whole cell patch-clamp recording, we investigated the effect by CGRP or BDNF on P2X3 receptors of KI TG ganglia in culture. We found that exogenous CGRP (1microM) failed to further potentiate the P2X3 receptor currents that were significantly larger than WT ones, and it actually returned them to WT level. A similar effect was also seen with RT-PCR and immunoreactivity of P2X3 receptors. We next investigated the role of BDNF which is an intermediate in the CGRP mediated signaling pathway. By overnight pretreatment with an anti-BDNF antibody in culture, we found no significant change in P2X3 receptor function of KI neurons. Thus, these results suggest that the activity of P2X3 receptors in sensory neurons of KI TG was constitutively very large and insensitive to additional increase via the intracellular signalling pathways mediated by CGRP.

Conclusions: A migraine genetic mouse model showed constitutive upregulation of the pain-sensing P2X3 receptors in trigeminal sensory neurons in culture. Such a high level of basal activity was not further potentiated by CGRP.

C 175 Localization and secretion study of Neurturin

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Background: Neurturin (NRTN), a member of GDNF-family ligands (GFLs), regulates the development and maintenance of neuronal populations in the brain and in the periphery. Outside the nervous system NRTN involves in kidney development and spermatogeneses. Because of its neurotrophic effects, NRTN has been widely studied as a therapeutic agent for neurodegenerative diseases, particularly Parkinson´s disease. Very little is known about the basic cell biology of NRTN and its precursor form pre-pro-NRTN.

Observations: The aim of this work was to study intracellular localization and secretion of NRTN and pro-NRTN proteins. Human pre-pro-NRTN cDNA was overexpressed in non-neuronal cells. After 48 hours, media and cell samples were collected and analyzed in western blot. NRTN was detected with goat anti-mouse-NRTN antibody. Although NRTN was strongly detected from the cell samples, only little was detected from the media samples indicating a poor protein secretion to the media. Immunocytochemistry was used to characterize intracellular localization of NRTN. Double-staining with cellular organelle markers revealed co-localization of NRNT with Golgi marker and secretory vesicles. In order to study intracellular targeting mechanisms of pro-NRTN, we deleted the pro-sequence and cloned a new construct, pre-NRTN. We overexpressed pre-pro-NRTN and pre-NRTN cDNAs in neuronal cells and compared the localization of wt and mutated proteins using immunocytochemistry. The mutated pre-NRTN co-localized primarily with endoplasmic reticulum marker and not with Golgi or secretory vesicle markers.

Conclusions: Our analyses indicate a poor secretion of NRTN and that the secretion may require a cell specific stimulation. After deleting the pro-sequence, the localization of NRTN was significantly altered. This indicates that the pro-sequence may be required for targeting the protein to secretory pathway.

C 176 Determinants of PIN transcription in Arabidopsis thaliana

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Background: Substantially less is known however, about molecular determinants that control the transcription of PIN genes.AUXIN RESPONSE FACTOR (ARF) genes were identified as a gene family that controls expression of auxin-inducible gene via binding to canonical AuxRE elements found in a range of promoters including those of PINs. However, so far only circumstantial evidence suggested a role for ARF proteins in modulating hormone responsiveness of PIN transcription.

Observations: In a systematic approach we aimed at a characterization of cis- and trans-acting determinants that control auxin responsiveness of PIN transcription. Evidence for a direct interaction between PIN promoters and ARFs, is provided by EMSA demonstrating specific binding of GST-ARF fusions to AuxREs in PIN promoters. To further address the biological relevance of these sites we mutagenized more than 10 predicted AuxREs in the PIN2 promoter region. Single site and combinatorial AuxRE site mutant combinations have been introduced into wild type, pin2 as well as a range of arf mutant combinations. Besides, altered response to exogenous auxin was observed by the ARF double mutants in real time analysis.

Conclusions: EMSA demonstrating specific binding of GST-ARF fusions to AuxREs in PIN promoters. Mutation in 10 predicted AuxREs in the PIN2 promoter region was not fully able to recover the pin2 mutant phenotype. No effect has been observed by the AuxRE site mutant combinations in arf mutant combinations.

C 177 Identification of novel genes in Chlamydomonas reinhardtii important for transcriptional regulation of chlorophyll biosynthesis in the dark

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Background: Unlike angiosperms, the green unicellular alga Chlamydomonas reinhardtii (Chlamy) can grow heterotrophically, using acetate as an organic carbon source and continues to synthesize chlorophyll in the dark. The genetic control of light-independent chlorophyll biosynthesis (dCB) has been investigated using the Chlamy mutant brc-1, defective in dCB on the stage before protochlorophyllide to chlorophyllide conversion, its revertant and secondary mutant.

Observations: The brc-1 cells can't synthesize chlorophyll, accumulate protoporphyrins and form orange colonies in the dark. After illumination they turn green. The lesion in the mutant were mapped to the nuclear gene LTS3 encoded a protein with a domain structure specific for a GATA1 transcriptional factors (TF). The mutant displayed the dark repression of of Mg-chelatase enzyme activity (CHLa) needed for protoporphyrin IX convertion in CB. The mRNA levels of of the genes encoding this enzyme subunits in mutant were same as in wild type on the light and hardly reduced in the dark, indicating that the mutation in LTS3 gene affects dark CHL on transcriptional level. The green in the dark revertant brc-8 of the mutant brc-1 were used for obtaining the orange secondary insertion mutant T8-3. The brc-8 displayed the recovering of dCB and two-fold increased dark CHLa. In the T8-3 high level of CHL were saved, but dCB were blocked and transcription of the LTS3 gene were strongly reduced compare to Brc-8. Genetic analysis revealed that (1) reversion was caused by extragenic suppressor SUP3, mapping near the gene LTS3; (2) the insertion in T8-3 destroyed the gene SUPI located on the chromosome XI.

Conclusions: The study of Chlamy mutants revealed the role of transcriptional regulation of CHLa in dCB. Possible, the expression of the genes encoded Mg-chelatase subunits in the dark activated by the LTS3-encoded GATA-TF as well as SUP3, and the product of the gene SUPI controls the expression of LTS3 gene.

C 178 Analysis of Arabidopsis XH/XS-domain proteins

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Background: XS domain proteins (SGS3 and rice protein X) have previously been suggested to function in post-transcriptional gene silencing. In addition, SGS3 was shown to act as dsRNA binding protein, an activity that seemingly depends on its XS domain. More recently, a member of the Arabidopsis XH/XS-domain family was identified as IDN2 involved in de novo DNA methylation and in siRNA-mediated maintenance methylation.

Observations: In a systematic approach, we have identified and confirmed loss-of-function insertion mutant lines in all 9 members of this gene family. Furthermore, mutant combinations were generated, since none of the identified single mutant lines exhibit pronounced phenotypes. In contrast, overexpression of selected members of this gene family results in distinct developmental growth defects, suggestive of only limited functional redundancy. This hypothesis is supported further by expression/localization studies of reporter lines. Some of the translational fusion proteins analyzed so far, localize exclusively to the nucleus, whilst further members of the XH/XS protein family appear to accumulate preferentially in the cytoplasm. In ongoing experiments we now analyse/compare the epigenetic status in single and high order mutants as well as in overexpression lines, by using established read-outs and reporter lines. Aspects of these analyses will be discussed.

Conclusions: Phenotypes exhibited by overexpression lines are indicative of a role of the corresponding proteins in various aspects of development and organ formation. Remarkably, whilst analyses of loss-of-function lines is suggestive of a high degree of functional redundancy and involvement in DNA methylation.

C 179 MAP kinase-linear motif interactions restrain signalling specificity in paralogous pathways

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Background: Cellular signaling is an orchestrated process of signaling transitions where a limited number of signaling enzymes match myriad of extracellular inputs with a similarly vast number of outputs. In the last decade, linear motifs directly binding (LMDB) to signaling enzyme surface grooves have been shown to contribute to the specificity of signaling circuits.

Observations: MAP kinases (MAPKs) do not possess dedicated protein-protein interaction domains that could enhance the specificity of their promiscuous active site, and it is their docking groove that determines their partner profile. This surface groove, however, is highly conserved in all MAPK paralogues (e.g. ERK, p38 and JNK). Moreover, most LMDBs located in MAPK partners are thought to share the same consensus. What factors are then to determine the ligand binding space of a given MAPK paralogue? The answer to this question is likely to be the key for understanding how MAPK paralogues may be involved in fundamentally so different physiological processes such as in apoptosis or in cell division. In our present study we have probed the specificity of fifteen linear motifs binding to MAPK (ERK2, p38alpha and JNK1) docking grooves in vitro so that to compare their biochemical specificity with the logic of established MAPK sub-networks observed in vivo. Furthermore, information from X-ray structure of key MAPK-LMDB protein-peptide complexes allowed us to change the binding specificity of LMDBs or to design artificial peptides with tailored MAPK interaction profiles.

Conclusions: Finally, we propose that short (< 10-20 aa) LMDBs are simple tools to govern specificity in paralogous MAPK networks as well as to make new connections in signaling networks during their evolution.

C 180 Biochemical characterization of scaffolded MAPK modules

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Background: Mitogen activated protein kinase (MAPK) signalling systems employ scaffold proteins to organize kinase cascade components into functional modules. Despite of the tremendous progress in identifying individual proteins building up signal transduction pathways, how signalling components are assembled into specific and efficient pathways are still poorly understood.

Observations: Currently, we are investigating how scaffold proteins influence the biochemical signalling properties of MAPK modules comprised of three sequentially acting kinases – MAPKs, MAP2Ks (MAPK kinases), and MAP3Ks (MAPK kinase kinases) by attempting to reconstruct whole and/or parts of scaffolded MAPK modules in vitro. This biochemical approach gives insight into what aspects of kinase cascade activity scaffolds may modulate. This approach, in contrast to using solely genetic based in vivo sudies, provides mechanistic insight into the function of protein scaffolds, which is a new and exciting field of cellular signalling. Earlier, we have demonstrated that a classical signalling scaffold, Ste5, plays a complex role in the yeast mating pathway: (a) it facilitates the MAP2K-MAPK transition allosterically and (b) it is involved in a negative feed-back loop that keeps pathway output low under non-stimulating conditions.

Conclusions: Similar studies on human MAPK scaffolds with JIP1, OSM and beta-arrestin2, which all have been shown to play an important role in different MAPK pathways in vivo, also demonstrate that these scaffolds modify the biochemical signalling properties of their MAPK cascades in vitro.

C 181 LPA binding to PH domains: new aspects of lysophospholipid-protein interactions

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Background: The aim of this study is to understand the mechanistic details of some protein-lipid interactions in signaling. Several cell membrane-associating proteins contain domains which directly interact with lipids, such as plectsrin homology

(PH) domains. Our attention turned to physiologically important lysophospholipid mediators, especially lysophosphatidic acid (LPA), as it previously has been reported that LPA binds PH domain of gelsolin, but the molecular details of the interaction is not known.

Observations: In the course of our work we aimed at characterizing the interaction between several PH domains and LPA using biochemical and biophysical methods in vitro. After expression of the PH domain and the entire protein of gelsolin, fluorescence and CD spectroscopy as well as isothermal titration calorimetry measurements indicated that LPA can bind to the PH domain of gelsolin over its critical micelle concentration. Thus the protein interacts with the lipid in an associated form instead of the individual lipid molecule. This interaction is specific to LPA with a nanomolar Kd value. Based on our findings we have looked for other PH domain containing proteins, namely Akt1 and Grp1, seeking for similar interactons. We expressed the PH domains of both proteins and characterized their interaction with LPA. We also carried out live-cell confocal microscopy with GFP-tagged PH domains of Akt1 and Grp1. We studied the changes in spatiotemporal localization of these GFP-PH domains by modifying the intracellular level of LPA, either by stimulating or inhibiting its metabolizing enzymes.

Conclusions: Our results show that LPA can act a second messenger-like fashion on membrane surfaces via recruiting protein domains. LPA, in a clustered form mimicked by micelles in vitro, can bind to at least some PH domains with high affinity, revealing new aspects of lysophospholipid-protein interactions.

C 182 Insulin receptor-related receptor as an extracellular alkali sensor

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Background: Insulin receptor-related receptor (IRR) is a member of the family of three structurally related receptor tyrosine kinases that includes insulin receptor (IR) and insulin-like growth factor receptor (IGF-IR). None of the known IR or IGF-IR ligands can activate IRR and no endogenous ligands for IRR have been identified since the discovery of IRR in 1989. The physiological role of IRR also has remained enigmatic. Main goal of our study was established agonist and physiologocal function for IRR.

Observations: We identified the membrane receptor that is directly activated by mildly alkaline media (pH > 7.9) and this receptor appeared to be the insulin receptor-related receptor (IRR). Treatments of cell lines with transfected IRR cDNA or with endogenous IRR by alkaline pH shows strong phosphorylation of IRR. The activation of IRR by alkali is dose-dependent, with a characteristic saturation curve, and reversible. Also it is specific; two closely homologous receptors IR and IGF-IR, as well as another receptor EGFR, are not activated by alkaline media. IRR activation by alkali triggers a conformational change in the receptor molecule and stimulates intracellular signaling. We found that, in contrast with IR activation, IRR shows strong positive cooperativity in response to pH. Using multiply chimeras IRR with IR we show that sites for alkali-sensing is primarily located in the first two domains of IRR ectodomain. To get an insight into the function of IRR we found disruption of IRR in mice impairs the renal response to alkali loading attested by development of metabolic alkalosis and decreased urinary bicarbonate excretion in response to this challenge.

Conclusions: We found that IRR activated by alkaline media at pH > 7.9. This property of IRR is conserved in frog, mouse and human. IRR activation is specific and triggers intracellular signaling. Disruption of IRR in mice impairs the renal response to alkali loading and decreased urinary bicarbonate excretion.

C 183 Identification in breast cancer cells of a novel mechanism of CSF-1R signalling which involves nuclear localization and binding to the promoter of genes involved in cell proliferation

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Background: Breast cancer is the most prevalent cancer in non-smoking women and the second leading cause of cancer-related deaths in western countries. The Colony-Stimulating Factor 1 (CSF-1) and its receptor CSF-1R physiologically regulate the monocyte/macrophage system, trophoblast implantation and breast development. An abnormal expression of CSF-1R, associated or not whit that of CSF-1, has been also documented in several human epithelial tumors, including breast carcinomas.

Observations: We found that CSF-1 and CSF-1R are coexpressed, although to different levels, in 17 breast cancer cell lines belonging to different molecular subtypes. Exogenous CSF-1 was able to activate the ERK1/2 proliferative pathway in 9/17 breast cancer cell lines. Treatment of SKBR3 cells, autocrine for CSF-1, with siRNA targeting CSF-1R or an anti-serum blocking CSF-1 inhibited cell growth by 50%, while the proliferation of MDAMB468 cells increased by 40% in response to exogenous CSF-1. In both cell lines, which belong to different molecular subtypes, CSF-1R signaling sustained the expression of cyclin D1 and activation of c-Jun. Treatment with Imatinib partially inhibited the proliferation of SKBR3, MDAMB468 in the presence of CSF-1. Further, we found that CSF-1R is localized in the nucleus and nucleolus of breast cancer cells, both in cell lines and tissue samples and irrespectively of the molecular class of breast cancer. By chromatin immunoprecipitation we found that CSF-1R binds to the promoters of genes involved in cell proliferation and other cellular functions relevant to cancer progression.

Conclusions: This study demonstrated that CSF-1R and CSF-1 are important for the proliferation of breast cancer cells, thus sustaining their targeting for the tretment in this neoplasia, and identified a new mechanism of CSF-1R signalling which involves its nuclear localization.

C 184 Cooperation between the PB1 domain and an adjacent MAP kinase binding linear motif in MKK5 enables its specific interaction to its cognate ERK5 substrate

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Background: MAP kinases (MAPKs) are activated through phosphorylation by MAPK kinases (MAP2K.) Mammals possess seven MAP2Ks, where MKK1/2, MKK3/6, MKK7 activates ERK, p38 and JNK, respectively, while MKK4 phosphorylates both p38 and JNK MAPKs. MKK5 is the specific activator of ERK5, which fulfils non-redundant physiological roles compared to its ERK1/2 paralogues.
Observations: While ERK1/2 is generally involved in mediating mitogenic signals, ERK5 is a more specific MAPK involved in cardiovascular development for example. Both MAPKs are expressed ubiquitously and their activation patterns in different tissues often determine physiological outcomes in a combinatorial manner. ERK1/2 and ERK5 share a highly similar kinase domain. Indeed, both MAPKs have some overlapping substrates, however they are clearly differentially activated by MKK1/2 and MKK5. All MAP2K-MAPK transitions require intact docking interactions between a linear motif located in MAP2Ks and the docking groove on MAPKs. The MKK5-ERK5 transition is unique, as in addition to a linear docking motif it also requires an intact MKK5 PB1 domain. In our study we have biochemically characterized the contribution of the docking motif and the PB1 domain towards the specificity of this MAP2K-MAPK interaction. Direct comparison with the MKK1/2-ERK1/2 system suggests that the short linear motif (8-10 amino acids), conferring loosely to the consensus sequence of motifs found in other MAP2Ks, cooperates with a structured domain so that to be able to discriminate between the ERK2 and the ERK5 docking grooves.
Conclusions: These MAP2K-MAPK signalling transitions serve as an excellent paradigm how existing protein-protein interaction elements, structurally and functionally unrelated, may come together during the evolution of paralogous signalling enzymes.

C 185 A novel linear ubiquitin ligase complex regulating TNFalpha-induced NF-kappaB activity and apoptosis

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Background: SHARPIN is a ubiquitin-binding and ubiquitin-like domain-containing protein which, when mutated in mice, results in immune system disorders and multiorgan inflammation.
Observations: We found that SHARPIN functions as a novel component of the Linear Ubiquitin Chain Assembly Complex (LUBAC) and that the absence of SHARPIN causes disregulation of NF-kappaB and apoptotic signalling pathways, explaining the severe phenotypes displayed by chronic proliferative dermatitis in SHARPIN deficient mice. Upon binding to the LUBAC subunit HOIP, SHARPIN stimulates the formation of linear ubiquitin chains in vitro and in vivo. Co-expression of SHARPIN and HOIP promotes linear ubiquitylation of NEMO, an adaptor of the IkappaB kinases (IKKs) and subsequent activation of NF-kappaB signalling, while SHARPIN deficiency in mice causes an impaired activation of the IKK complex and NF-kappaB in B cells, macrophages, and mouse embryonic fibroblasts (MEFs) by CD40L, LPS and TNFalpha, respectively. This effect is further enhanced upon concurrent downregulation of HOIL-1L, another HOIP-binding component of LUBAC. In addition, SHARPIN deficiency leads to rapid cell death upon TNF-alpha stimulation via FADD- and Caspase-8-dependent TNFR complex II pathway, which was not induced by HOIL-1L deficiency.
Conclusions: SHARPIN thus activates NF-kappaB by linearly ubiquitinating NEMO and inhibits apoptosis via TNFR complex II pathways in vivo.

C 186 PIPKII alpha regulates alpha and gamma globin expression in hematopoietic-derived cells

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Background: Phosphatidylinositol phosphate kinases belong to a family of enzymes that generate various lipid messengers. In a recent study in our laboratory, the PIPKII alpha gene was differentially expressed in reticulocytes from two siblings with hemoglobin (Hb) H disease. Expressions of both the PIPKII alpha and beta globin genes were higher in the patient with the higher Hb H level, suggesting a possible relationship between PIPKII alpha and the production of globins.
Observations: A specific siRNA expression vector targeting the PIPKII alpha gene was electroporated into the K562 cell line. Quantitative PCR (qPCR) and Western blot (WB) analysis were performed to determine the expression of PIPKII alpha, gamma and alpha globin. After 48 hours of culture, proliferation was assessed by MTT assays; apoptosis was assessed by Annexin-V and propidium iodide staining; and cell cycle analysis was carried out by flow cytometry. After 15 days of silencing, differentiation was evaluated by flow cytometry with FITC-conjugated anti-glycophorin and anti-transferrin. Imatinib was used as a positive control for the arrest of proliferation and as an inducer of apoptosis in K562 cells. The levels of PIPKII alpha mRNA and PIPKII alpha protein in knockdown K562 cells were reduced by 80% and 75%, respectively. MTT assays showed that the proliferation was slight reduced by 10% in PIPKII alpha knockdown cells. Cell cycle, apoptosis and differentiation analysis showed no difference in PIPKII alpha knockdown. Interestingly, PIPKII alpha silencing resulted in a significant increase in alpha and gamma globin expression compared to control cells, as observed by qPCR and WB.
Conclusions: We have demonstrated that PIPKII alpha silencing results in an increase in alpha and gamma globin expression. In K562 cells there may be, at least in vitro, a regulatory mechanism

that acts on alpha and gamma globin genes in response to the reduction in PIPKII alpha gene expression.

C 187 Subcellular localization of S6K1 and S6K2 is related to functional activity of rat thyrocytes in vitro

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Background: The kinase of ribosomal protein S6 is an important member of PI3K signal transduction pathway involved in control of protein synthesis and G1/S transition of the cell cycle. There are two forms of this kinase S6K1 and S6K2. Previously we have shown that in normal thyroid tissue they are detected predominantly in the cytoplasm of thyrocytes whereas in monolayer culture in course of follicle outspreading S6K1 and S6K2 appeared in nuclei as well.
Observations: The goal of presented work was to detect which processes involved in follicle transformation from 3D structure to monolayer colony (migration, proliferation, loss of follicle organization) is related to S6K subcellular redistribution. The cultivation of thyrocytes resulted in activation of cell proliferation. But there was not the correlation between S6K1/2 subcellular relocalization and Ki-67 appearance in proliferating cells. While, there was an increase of total S6K1/2 content in proliferating cells.To study possible effect of migration on subcellular localization of S6K1 and S6K2, cultured thyrocytes were stimulated to penetrate a porous membrane of Transwell. But immunocytochemical analysis revealed predominantly cytoplasmic localization S6K1/2 as well. Detection of the content of these kinases in thyrocytes in 3 D culture with retention of follicle structure shown that in thyroglobulin-positive cells from follicle like structures S6K1/2 localized in cytoplasm, but in thyroglobulin-negative cells from solid areas of cultured aggregates of follicles S6K1/2 were observed in nuclei as well (like in monolayer cultures).
Conclusions: Thus, the change of subcellular localization of S6K1/2 in cultured thyrocytes is directly related to change of level of functional activity, unlike the processes of proliferation and migration.

C 188 Real-time integration of beta2-adrenergic receptor signalling reveals additional ligand signalling texture and novel pathway engagement

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Background: The discovery that drugs targeting a single G protein-coupled receptor (GPCR) can differentially modulate distinct subsets of the signalling repertoire engaged by the receptor has created a formidable and exciting challenge for drug discovery and development at this important class of therapeutic targets. We sought to determine how such differences in pathway engagement among ligands are reflected in the overall cellular responses generated upon GPCR stimulation.
Observations: Here, we demonstrate that a label-free assay based on cellular impedance provides a real-time integration of multiple signalling events engaged upon GPCR stimulation. Stimulation of the beta2-adrenergic receptor (beta2AR) in living cells generated a complex, multi-featured impedance response over time. Selective pharmacological inhibition of specific arms of the beta2AR signalling apparatus revealed the differential contribution of Gs-, Gi- and Gbetagamma-dependent signalling events, including activation of the canonical cAMP and ERK1/2 pathways, to specific components of the impedance response. Further dissection of the impedance response led to the discovery of a novel $Ca2+$ mobilization event to the overall beta2AR-promoted cellular response. Recognizing that impedance responses provide an integrative assessment of ligand activity, we screened a collection of beta-adrenergic ligands to determine if differences in the signalling repertoire engaged by compounds would lead to distinct impedance signatures. An unsupervised clustering analysis of the impedance responses revealed the existence of 5 distinct compound classes, revealing a richer signalling texture than previously recognized for this receptor.
Conclusions: Impedance responses provide an unbiased, integrative assessment of the complex signalling dynamics generated upon GPCR stimulation. As a holistic readout of ligand activity, such analytical integrative methodologies should be transformative in future drug discovery strategies.

C 189 Chicken embryonic stem cells

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Background: In avian species, chicken embryonic stem cells (cESCs) have been isolated from the in vitro culture of early chicken blastodermal cells (cBCs) taken from stage X embryo (EG&K) These cESCs can be maintained under specific culture conditions and have been characterized on the basis of their morphology, biochemical features, in vitro differentiation potentialities and in vivo morphogenetic properties.
Observations: The relationship between these cESCs and some of the chicken germ cells identified and grown under specific culture conditions are still under debate, in particular with the identification of the Cvh gene as a key factor for germ cell determination. More- over, by cloning the avian homologue of the Oct4 mammalian gene, we have demonstrated that this gene, as well as the chicken Nanog gene, was involved in the characterization and maintenance of the chicken pluripo- tency.
Conclusions: These first steps toward the understanding development and characterization of putative new cell types such as chicken EpiSC and raises the question of the existence of reprogramming in avian species. These different points are discussed.

C 190 ARX contributes to the differentiation of human pancreas endocrine and exocrine cells

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Background: Aristaless-related homeobox gene (ARX) mutation leads to X-linked lissencephaly with abnormal genitalia (XLAG). The crucial extra-brain symptoms of XLAG are severe growth

retardation, transient hyperglycemia and intractable diarrhea. Since ARX expresses in the islets of Langerhans during the embryonic stage, these visceral phenotypes may be related to a loss of ARX function, which develops endocrine cells in the pancreas.

Observations: We investigated the abnormal pancreatic development of XLAG patients with ARX-null mutation. We performed immunohistochemistry of XLAG pancreases, using the antibodies against glucagon, insulin, somatostatin, pancreatic polypeptide, ghrelin, Brn4, Nkx2.2, Mash1, amylase and pancreatic lipase. As the results, the glucagon- and pancreatic polypeptide-producing cells were found to be completely deficient in the islets of Langerhans. We also discovered marked interstitial fibrosis, small exocrine cells with loss of amylase-producing cells and an enlargement of the central lumen of the glandular acini.

Conclusions: These pathological findings indicate that ARX contributes not only to endocrine development, but also to exocrine development of the human pancreas, and its deficiency may lead to the severe phenotypes of XLAG patients.

C 191 Vestigial-like (Vgl)-4, an inhibitor of apoptosis protein (IAP)-interacting protein, counteracts apoptosis-inhibitory function of IAPs by nuclear sequestration

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Background: The inhibitors of apoptosis proteins (IAP), which include cIAP1, cIAP2 and XIAP, suppress apoptosis through the inhibition of caspases. These IAPs are also involved in various signal transduction pathways. In particular, cIAP1 and cIAP2 are critical for activation of the TNFalpha-induced NF-kappaB activation pathway, in which direct interaction with TRAF2 is required for their recruitment to TNFR1 receptor complex. The activity of IAPs is regulated by a variety of IAP-binding proteins.

Observations: We took a proteomic approach to identify regulatory proteins that might interact with IAP proteins. From this screen, we isolated Vgl-4 as a novel IAP interacting factor. Vgl-4 is known to function as a transcription cofactor through interaction with transcriptional enhancer factor-1 and myocyte enhancer factor 2. Here, we showed that Vgl-4 is expressed predominantly in the nucleus and its overexpression triggers a relocalization of IAPs from the cytoplasm to the nucleus. cIAP1/2-interacting protein TRAF2 prevented the Vgl-4-driven nuclear localization of cIAP2. Accordingly, the forced relocation of IAPs to the nucleus by Vgl-4 significantly reduced their ability to prevent Bax- and TNFalpha-induced apoptosis, which can be recovered by co-expression with TRAF2.

Conclusions: Our results suggest that Vgl-4 may play a role in the apoptotic pathways by regulating translocation of IAPs between different cell compartments.

C 192 Role of CYP2C9 and VKORC1 polymorphism in dose dependent Warfarin therapy management

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Background: Warfarin as vitamin K antagonists is the most commonly prescribed anticoagulant worldwide, to prevent the risk of a thrombotic event. Two genes; CYP2C9 and VKORC1 encode enzymes involved in Warfarin metabolism. Polymorphism in either CYP2C9 or VKORC1 affect Warfarin sensitivity. This study aimed to investigate the frequency of CYP2C9 and VKORC1 polymorphism among patients under Warfarin therapy to determine the variability in response to Warfarin and its influence on the dose requirement.

Observations: The study was conducted on forty male patients 40-60 years old receiving stable Warfarin dose that remain constant for three previous clinic visits over a minimum period of three months and with INR within the range of 2-3. Patients with liver disease, and/or on antiplatlets or NSAIDS therapy were excluded. After their consent, these patients were genotyped for the VKORC1 −1639G > A and 3730G > A polymorphisms as well as for the CYP2C9 *2 and *3 polymorphisms using a reverse hybridization-based test strip assay. (ViennaLab Diagnostics,Vienna, Austria). VKORC1 homozygous AA, hetrozygous AG, and homozygous GG had a frequency of 7.5%, 65% and 27.5 respectively. For CYP2C9gene homozygous*1/*1(wild type) made up the majority of the studied sample 65%, while all other variants (*1/*2, *1/*3, *2/*2, and *3/*3) made 10%, 15%, 5%, 5% respectively. Thirteen Patients were categorized into low dose responders (4mg/day), 21 cases were intermediate dose responders (4-9mg/day) and 6 cases were high dose responders (> 9 mg/day). All low dose responders were homozygous AA at position -1639 for VKORC1 gene. While genotype CYP2C9 *1/*1 showed the highest dose response for Warfarin therapy.

Conclusions: Polymorphism in CYP2C9 and VKORC1 genes significantly affect individual response to Warfarin therapy; CYP2C9 variant alleles CYP2C9*2 and CYP2C9*3 require lower Warfarin dose than CYP2C9*1 allele, while VKORC1 variant allele A require lower Warfarin dose than G allele.

C 193 Generation of an Enriched Pool of DNA Aptamers for a HER2 Overexpressing Cell Line selected by Cell–SELEX

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Background: Overexpression of HER2 occurs in a large percentage of breast cancers. Monoclonal antibodies targeting HER2 are vastly used for both diagnostic and therapeutic aims. However, identifying new molecular probe against HER2 with improved diagnostic and therapeutic features is of great importance.
Observations: In this report, we have applied cell SELEX strategy for 16 selection rounds to generate an enriched pool of aptamers that specifically recognize HER2 positive cell line. During cell SELEX procedure, a human HER2 overexpressing breast cancer cell line, and a human HER2 negative breast cancer cell line were used. Our results reveals that PCR amplification of random DNA libraries and selected ss DNA pool in different Cell SELEX rounds are different from what we expect from PCR amplification of homologous DNA. Our results also confirmed previous studies describing positive HER2 status of SK-BR3 and the absence of the HER2 expression in the MDA-MB468. We also developed a new method, Cell ELA, to monitor the enrichment of aptamers in a given round of cell SELEX. This method would also be useful in other experiments using live cell ELISA on adherent cells.
Conclusions: Our results indicate that the generated pool is enriched of aptamer candidates for HER2 overexpressing cell line. These results also suggest that cell ELA can be confidently used as an alternative method over flow cytometry to monitor SELEX progress.

C 194 Viability screens in cancer cells with pooled shRNA libraries identify potential therapeutic targets and synergistic lethal interactions

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Background: One strategy to circumvent problems with conventional chemotherapy is to develop drugs against more specific cancer targets. Another is to use molecularly targeted agent (MTA) combinations to circumvent tumor resistance and increase the therapeutic index. Such synergistically lethal (SL) MTA combinations, however, are not easily predicted based on our rudimentary knowledge of cancer biology and drug action mechanisms.
Observations: In the first strategy, genes modulating proliferation and survival in oncogenic cells have been identified using pooled lentiviral-based libraries expressing many thousands of shRNAs. In the second strategy, we have adapted the approach to combinatorially screen shRNA sequences targeting hundreds of genes to discover additive and synergistic combinations that generate a synthetic-lethal phenotype. A viability assay with leukemic cell lines transduced with pooled shRNA libraries targeting thousands of genes identified a few hundred essential genes for each panel of cells. Subsequent validation using single shRNA-expressing constructs showed that in each screen, about 80% of shRNAs identified did indeed lead to cell death when transduced in cells. Analysis of the identified essential genes for known biological interactions revealed several non-random clusters of interacting proteins that provide some insight into signaling pathways and protein networks specific to these cancers. Analysis of the lethal combinations from the SL screen indicated redundant, complementary, and compensatory responses in cancer cells.
Conclusions: We believe that newly discovered hematopoietic-specific genes represent potentially novel drug targets. Based on SL screen results, we believe we can predict the most promising synergistic lethal combinations and develop highly effective anti-cancer therapeutics with unique mechanisms of action.

C 195 Prevalence and distribution of genetic diseases in Tunisia: Impact of the past on the present

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Background: Tunisia is a North African country at the cross road between Europe and Africa. The indigenous background population is Berber. Throughout its history, Tunisia witnessed successive invasions and migratory waves of allogenic populations. As in other Arab countries, consanguinity and endogamy are culturally favoured. Consequently, recessive genetic disorders are encountered at relatively high rates.
Observations: We report on the spectrum of genetic diseases and on founder mutations in Tunisia by a review of the literature. More than 300 genetic disorders have been reported. Among these, 63% are autosomal recessive, 23% autosomal dominant, 6% X-linked and the remaining are of Y-linked, mitochondrial and unknown mode of transmission. Nearly half of these conditions are caused by at least one mutation. For autosomal recessive diseases, most of the mutations were identified at homozygous state among patients. For 68 inherited diseases the underlying mutations were the result of a founder effect. Several heavily disabilitating diseases are caused by founder mutations. Two classes of founder mutations have been identified in Tunisian patients. The first includes founder mutations so far reported only among Tunisians that were responsible for 26 genetic diseases. The second group represents founder haplotypes described in 42 inherited conditions that occur among Tunisians and are also shared with other North African and Middle Eastern countries.
Conclusions: The large spectrum of diseases could be explained by the high degree of inbreeding and allelic heterogeneity. Part of the described mutations is founder and has captured historical events. They are useful for designing measures for carrier screening and setting up of preventive plans in the region.

C 196 The impact of joint possession of single nucleotide gene polymorphisms on susceptibility to gastric cancer development

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Background: Gastric cancer is a multi-factorial disease resulting from interaction between host, pathogen and environmental factors. It has been speculated that geographic variations in gastric cancer risk may be partly due to single nucleotide gene polymorphisms in genes causing susceptibility to cancer. The aim of this study was to assess the impact of a number of gene polymorphisms and gastric cancer risk in an Iranian population.
Observations: We evaluated the joint effects of several single nucleotide polymorphisms on gastric cancer development and its subcategories. Ten gene polymorphisms involved in inflammation (interleukins: IL-1beta, IL-1RN, IL-8, IL-10), detoxification of carcinogens (glutathione s-transferases M1, T1: GSTM1, GSTT1), folate metabolism (methylenetetrahyrofolate reductase: MTHFR), and intercellular adhesion (E-cadherin: CDH-1) were evaluated by CTPP-PCR and PCR-RFLP on genomic DNA extracted from white blood cells. In this study, 98 patients diagnosed with gastric cancer (GC) were compared with 140 healthy individuals using logistic regression analysis, calculating odds ratios (ORs) and 95% confidence intervals (95% CI) by SPSS 18.0 statistics software. We observed that individuals with 5 or more SNPs were at more than two fold increased risk of gastric cancer (OR: 2.255, 95% CI: 1.006-5.055, P = 0.048) which was further enhanced in the older (> 50 years) age stratum (OR: 3.632, 95% CI (1.324-9.965), P = 0.012). In addition, stratification based on GC subsites demonstrated that the increased risk was mostly owed to the cardia GC subcategory (OR: 3.255, 95% CI: 1.009-10.503, P = 0.048) in this age stratum.
Conclusions: In conclusion, due to the high incidence and mortality of GC in Iran, non-invasive screening for joint possession of single nucleotide polymorphism in genes causing susceptibility to GC, is recommended for early detection of gastric cancer and timely preventive measures may increase survival rates.

C 197 miRNA profiling in EGFR antibody treatment-sensitive and resistant KRAS and BRAF wild type colorectal carcinoma

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Background: Although anti-EGFR antibodies have improved treatment results in metastatic CRC, almost half of the patients with the negative mutation of KRAS and BRAF still receive no benefit from the antibody treatment. MicroRNAs (miRNAs), known as gene regulators at the transcriptional or post-transcriptional level and have played important roles in the control of many biological processes. Our aim was to identify miRNAs that could serve as predictive markers related to therapeutic response.
Observations: To find whether any differences exist among CRC patients in miRNA expression to anti-EGFR treatment between responders and non-responders, we carried out miRNA microarrays on 47 non-mutated KRAS and BRAF samples including 12 partial response (PR), 14 stable disease (SD), and 21 progressive disease (PD) samples. Preliminary analyses showed exclusively expression of four miRNAs (miR-632, miR-200c*, miR-297, miR-32) only in PD and six miRNAs (miR-217, miR-223*, miR-506, miR-514, miR-516b, miR-942) only in PR samples. MiR-9 and miR-127-5p were expressed in both SD and PR but in no PD samples. More detailed results will be presented and further plans discussed in the meeting.
Conclusions: The results suggest that the miRNAs identified may serve as potential markers for resistance or response to anti-EGFR treatment.

C 198 A Urokinase Plasminogen Activator (uPA)-Activated Anthrax Lethal Toxin (PrAgU2/LF) is a potent and selective dual-specific Toxin for the Targeting of Acute Myeloid Leukemia (AML)

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Background: We tested efficacy and safety of a urokinase-activated anthrax lethal toxin (PrAgU2/LF). In this molecule the furin activation site of anthrax lethal toxin is replaced by a uPA activation site. PrAgU2 binds cells, is cleaved by uPA, heptamerizes, binds LF, undergoes endocytosis and releases LF in the cytosol. LF is a metalloprotease that cleaves all MEKs. Introduction of the uPA activation site targets tumor cells expressing active uPA in addition to the LF-mediated targeting of the MAPK pathway.
Observations: A panel of 11 human AML cell lines was tested and 7 were sensitive to the furin-activated anthrax lethal toxin PrAg/LF (IC50 = 13.3-94 pM), indicating sensitivity to the LF-mediated inhibition of the MAPK pathway. 5 of the 7 PrAg/LF-sensitive cell lines were also sensitive to the urokinase-activated toxin PrAgU2/LF (IC50 = 12.3-151 pM) demonstrating efficacy of the dual targeting of the urokinase system and the MAPK pathway in AML cells. Addition of a neutralizing anti-uPA antibody significantly decreased toxicity of PrAgU2/LF to AML cells, increasing the IC50 by an average of 77 fold and demonstrating requirement for expression of active uPA for selective activation of PrAgU2/LF. When injected intraperitoneally (i.p.) into Balb/c mice, every other day for a total of 3 injections, PrAgU2/LF did not induce mortality or show signs of toxicity at doses up to 75microgram PrAgU2/15microgram LF (per injection) while PrAg/LF caused more than 20% mortality at doses equal to or higher than 25microgram PrAg/5microgram LF (per injection). This demonstrates that introduction of the urokinase-activation site decreased the toxicity of PrAU2/LF by at least 3-fold compared to the furin-activated toxin PrAg/LF.
Conclusions: The dual-specific, urokinase-activated anthrax lethal toxin PrAgU2/LF is potent against AML cell lines in vitro and shows significantly improved safety when injected systemically in mice. PrAgU2/LF is a promising dual-specific therapeutic for the selective treatment of AML.

C 199 Assessment of Aurora-A kinase expression in breast cancer for early diagnosis

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Background: The Aurora-A is a kinase that plays a crucial role during mitosis. Overexpression of this protein alters centrosome number and function leading to aberrant mitotic spindle, miss-segregation of chromosomes and cellular transformation. Aurora-A protein is frequently overexpressed in several human cancers. Breast cancer is the most common type of cancer in women, it's a hormone related tumor with various grade and stage presentations.
Observations: Aurora-A status was evaluated in 107 patients with confirmed breast cancer using immunohistochemistry. The experimental findings showed that high expression of this protein was correlated with an elevated nuclear grade, a low expression of progesterone receptor and a positive nodal status. The experimental results showed also that the localization of this kinase shift from cytoplasm in non malignant adjacent tissue to both cytoplasmic and nuclear compartment in tumoral tissue, suggesting an oncogenic role of the nuclear accumulation. We have additionally detected the overexpression of this protein in non malignant adjacent tissue; cells with healthy appearance could begin early to overexpress this protein before the malignant processing is histopathologically observable.
Conclusions: The expression of the Aurora-A kinase in non malignant tissue may represent an earlier diagnosis tool for breast cancer.

C 200 Involvement of ERK and Akt/CREB and Nrf2 signaling pathway in FGF9-induced upregulation of heme oxygenase-1 and gamma-glutamylcysteine synthetase

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Background: Our previous studies had demonstrated that fibroblast growth factor 9 (FGF9) induced gamma-glutamylcysteine synthetase (gamma-GCS) and heme oxygenase-1 (HO-1) up-regulation to protect cortical and dopaminergic neurons from 1-methyl-4-phenylpyridinium (MPP+)-induced oxidative insult. In the present study, we investigate the signaling pathways of that FGF9 upregulates HO-1 and gamma-GCS expression and reduces hydrogen peroxide production.
Observations: We found that FGF9-induced upregulation of HO-1 and gamma-GCS and neuroprotection was prevented by PD173014, an inhibitor of FGF receptor (FGFR). FGF9 treatment induced the phosphorylation of FGFR downstream signals of extracellular signal-regulated kinase 1/2 (ERK1/2) and Akt in a dose and time-dependent manner. The inhibition of ERK1/2 or PI3K/Akt activity by U0126 or wortmannin, but not the inhibition of phospholipase Cgamma by U73122, prevented FGF9-induced responses, including gamma-GCS and HO-1 up-regulation, hydrogen peroxide reduction and glutathione elevation, and the neuroprotection against MPP+ toxicity in primary cortical and dopaminergic neurons. Furthermore, FGF9 treatment enhanced the promoter activity of cAMP respond element binding protein (CREB) and nuclear factor erythroid-derived 2-like 2 (Nrf2). Knockdown of CREB and Nrf2 by shRNA prevented FGF9-induced all responses, but did not affect the ERK and Akt phosphorylation. Consistently, in vivo study showed that FGF9 overexpression using lentivirus delivery system induced ERK1/2 phosphorylation and HO-1 upregulation and prevented MPP+-induced dopaminergic neuron loss in rat substantia nigra.
Conclusions: The results indicate that FGF9 binds to FGF receptor and activates ERK and Akt/CREB and Nrf2 signaling pathway to enhance antioxidant enzymes of HO-1 and gamma-GCS expression against MPP+-induced oxidative stress and neuron death.

C 201 Bimodal regulation of circadian glucocorticoid rhythm: Roles of the SCN central and the adrenal peripheral clocks

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Background: Mammalian circadian timing system consists of a hierarchical organization of the master clock in the hypothalamic suprachiasmatic nucleus (SCN) and subsidiary peripheral clocks in other tissues and organs. Glucocorticoid (GC) is a multi-functional adrenal steroid hormone following daily oscillations in circulation. We have recently shown that adrenal clock plays a key role in the rhythm by producing a circadian GC biosynthesis.
Observations: Here we demonstrate that a robust daily rhythm in circulating GC levels is controlled by bimodal actions of the central and adrenal clockworks. When mice are subjected to daytime restricted feeding (RF) regimen, which can dissociate peripheral rhythms from the SCN central pacemaker, adrenal GC contents as well as steroidogenic acute regulatory protein expression (StAR) show daily rhythms with their peaks around CT/ZT00 in accordance with shifted adrenal clock gene expression. Interestingly, plasma GC levels in RF mice exhibit two distinct peaks in a day: a peak around the time of adrenal peak and another around CT/ZT12, which requires the intact SCN. Along with the evidence obtained by the daytime RF regimen, light-induced activation of the SCN increases circulating GC levels without marked StAR gene induction both in wild-type and adrenal clock-disrupted mutant mice.
Conclusions: Taken together, these findings strongly suggest that the adrenal clock-dependent rhythmic steroidogenesis and the SCN-driven central mechanism cooperate to produce a robust daily GC rhythm in circulation.

C 202 Acupuncture treatment at SP6 alleviates depression-like behavior induced by Bacillus Calmette-Guérin inoculation in mice

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Background: Experimental inoculation of Bacillus Calmette-Guérin (BCG) in mice aggravates chronic depressive-like behavior and stimulates the activity of a tryptophan-degrading enzyme, indoleamine-2,3-dioxygenase (IDO) in the brain following the inflammation response. Despite extensive use of acupuncture for treating various psychosomatic disorders in Oriental medicine, there is no experimental study of verifying antidepressant effect of acupuncture on inflammation-associated depression.

Observations: In this study, antidepressant-like activity of acupuncture stimulation at SP6 (Sanyinjiao) or HT7 (Shenmen) acupoint and its neuropharmacological mechanism were investigated in BCG- inoculated mice. Mice were received acupuncture stimulation once a day for 16 days after BCG inoculation. Tail suspension test (TST) was executed on 8th and 16th day and open field test (OFT) on 7th day after BCG-inoculation. After TST, kynurenine (KYN), tryptophan (TRP), monoamine neurotransmitters and metabolites in the mouse brain were analyzed using HPLC. The duration of immobility in TST was significantly increased in the BCG-inoculated control group, and almost restored to normal level in the SP6-treated group on 16th day. There were no significant differences of locomotor activities in OFT between the groups. Turnover ratios of KYN/TRP were significantly increased in the blood and in the brain, and dopamine (DA) level in the hippocampus decreased in the control group. The acupuncture stimulation at SP6 restored KYN/TRP ratio and DA level to the normal.

Conclusions: In summary, acupuncture stimulation at SP6 significantly ameliorated depression-like behavior aggravated by BCG-inoculation without any side-effects due to CNS intervention, implying that acupuncture at SP6 relieved the decrease in DA level in the mouse hippocampus.

C 203 Comparing effects of extremely low frequency electromagnetic fields on cytogenetic characteristics of C3 and C4 plants

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Background: Electromagnetic fields (EMFs) are important environmental factor that can influence the growth and development of plants. We aim to investigate comparing effect of EMFs through growth parameters in higher plants including cytogenetic changes especially the principle endpoints of a genotoxic action: effect on chromosomes and DNA fragmentation. The agricultural plant including Zea mays L (C4) and Brassica napus L(C3) with a major role in the life of people were chosen.

Observations: Exposure to EMFs was performed by a locally designed EMF generator. In the present study, seeds of canola and maize were exposed to EMFs by magnitude of 1 to 7mT in steps of 2mT and the highest intensity was 10mT for 1 to 4 hours in steps of 1 h. Our investigation focused on seedlings grown from pre-treated seeds by 10mT for 2 and 4 h in canola and seedlings grown from pre-treated seeds by 3 and 10 mT, both for 4 h exposure time in maize. These treatments showed the most significant difference of growth characteristics in pre-treated seedlings. The mean value of total, terminal and intercalary chiasmata induced significantly in maize grown from exposed seeds to 3mT intensity, while the same meiotic characteristic of canola reduced significantly, indicating that EMF induces the mean value of the genetic recombination in maize, while EMFs caused reduction in genetic recombination of canola. Other meiotic characteristics including rod bivalent, quadrivalent and univalent formation were reduced significantly in maize, while ring bivalent occurrence induced significantly. On the other hand, ring bivalent and quadrivalent formation were reduced significantly in canola.

Conclusions: All results suggested that maize as a C4 plant and canola as a C3 plant react differently in meiotic characteristics against EMFs. In conclusion, Maize showed a potential for induction of genetic recombination under effects of EMFs, while EMFs caused reduction in genetic recombination of canola.

C 204 Differential Display Analysis of Gene Expression Induced by Polyethylene glycol Treatment in Zea mays

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Background: Differential display technique was used to study the effect of polyethylene glycol (PEG) treatment on two kinds of type II maize callus (regenable and non- regenable) of the HiII genotype. The morphotype of non- regenable callus was changed to regenable callus after PEG treatment.

Observations: Total RNA were extracted from all samples and reverse transcribed using oligo dT primer. Complementry DNA (cDNA) were investigated using seven RAPD primers to study the difference between regenable and non-regenable callus as a result of PEG treatment. Results indicated that highest variations were shown among the treated samples with primers A1, R14, P2 and A13. On the other hand, tow bands, one band and three bands were recorded in control in case of primer A1, R14, and p2, respectively, and disappeared in treated samples. A total of 30 bands showed significant differences in treated samples

comparison to control. Nine of these bands were eluted, purified and sequenced.

Conclusions: Sequence analysis of the DNA fragments and their deduced amino acids revealed that ATFP (Zea mays) protien, putative pol protien, Opie2b gag protien, putative gag-pol protien, aminotransferas classIII and Putative gag-pol precursor (Zea mays) protien were expresed as a result of PEG treatment.

C 205 Phytoremediation of salt contaminated tannery waste water treated lands: Molecular cloning methods for enhanced salt absorption in plants

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Background: The present environment conscientious world is driving researchers to explore phytoremedial methods for treating lands contaminated with high concentration of dissolved solids. Phytoremediation, a low-tech and low-cost method is an emerging cleanup technology for tannery waste water contaminated soils. The leather processing industry mainly associates with the generation of liquid waste with high total dissolved solids (TDS) in the form of salt.

Observations: The present study is based on a newer approach in phytoremediation of high salinity containing tannery waste water lands using the halophytic plant – Salicornia species. The main advantages observed in this plant for phytoremediation are its accumulation capacity and tolerance of high concentration of saline content from tannery waste water treated lands. To develop and use phyto-mining in salted soil, the major apprehension is to increase salt absorption capacity in this plant. The present work involves the growth characteristics of Salicornia species in high salt contaminated lands of tannery waste water treated lands, screening and identification of phytochemical constituents, analysis of chromosomal changes, molecular identification of salt absorption gene, gene transfer methods to isolate and identify the target genes relevant to increased salt absorption.

Conclusions: Gene transfer to salicronia species to augment salt absorption efficiency and salt absorption efficiency of plant after transformation through in vitro regeneration and its impact on salt absorption was observed.

C 206 The Regulation of RhoA by Stard13 in Focal Adhesions is Essential for Astrocytoma Cell Motility

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Background: Malignant astrocytomas are associated with high mortality rates. These tumors are highly invasive into adjacent and distant regions of the normal brain rendering them medically unmanageable. Invasion is a multistep process, which ultimately requires the cell to actively migrate through the ECM. Rho-GTPases, mainly RhoA, Rac, and Cdc42, play a major role in the regulation of the processes that ultimately lead to cell migration. StarD13 is a RhoGAP that inhibits the function of RhoA and Cdc42.

Observations: We first aimed at determining the role of RhoA in the progression of astrocytic tumors, a topic that is still controversial in the literature. Looking at the activation of RhoA through the use of a FRET biosensor showed RhoA to localize to the tail as well as to the leading edge of astrocytoma cells undergoing random movement. Our results also showed that knocking down RhoA by siRNA transfection inhibited cell motility. Surprisingly, when we knocked down StarD13 using siRNA we saw an inhibition of cell motility as well. This was mimicked by transfecting the cells with a dominant active RhoA construct. StarD13 mainly localized to adhesion structures, thus RhoA needs to be inhibited at the sites of adhesion. StarD13 knock down cells were unable to move and showed a phenotype of an elongated tail. This showed us that Rho inhibition by StarD13 pays a role in the cell detaching its tail to move forward. Looking at the adhesion structures we also saw complete inhibition of focal complex formation in cells overexpressing active RhoA or where StarD13 was knocked down. This was also mimicked by Rac knock down.

Conclusions: Our study showed that StarD13 regulated the activity of RhoA in astrocytoma cells. StarD13 inhibits RhoA allowing focal adhesions to dissolve for the cells to move forward. StarD13 also inhibits RhoA at the leading edge of cells for Rac to get activated and focal complexes to form.

C 207 Studies on the glycosylation of human guanylyl cyclase C

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Background: Guanylyl cyclase C (GC-C) is a membrane-bound receptor cyclase primarily expressed in the intestinal epithelia. It mediates fluid-ion secretion and exerts a cytostatic effect on the epithelial cells. It is expressed as a differentially glycosylated protein of 130 kDa and 145 kDa. Both forms bind ligand with equal affinity, but only the 145 kDa form is activated by ligands. The critical role of glycosylation in protein folding, trafficking, and mediating protein interactions of GC-C was analyzed.

Observations: Bioinformatic analysis predicted ten glycosylation sites in the extracellular domain of GC-C. The impact of glycosylation on GC-C function was evaluated by inhibiting its glycosylation either by pharmacological intervention or by mutation of all the putative glycosylation sites. The specific sites involved in GC-C function were investigated by systematic mutagenesis of each of the ten putative glycosylation sites. These analyses were carried out in mammalian cells. The importance of glycosylation on GC-C activation was investigated using carbohydrate binding proteins (lectins) which specifically interact with sugars present either only on the 145 kDa form, or on both forms of GC-C. It was found that lectins which interacted with exclusively on the 145 kDa form of GC-C inhibited its activation by ligands, but not ligand binding. The interaction of GC-C with animal lectins such as galectin3 and VIP36 was analyzed in vitro. Galectin3 is found ubiquitously outside and inside cells. VIP36 is a lectin chaperone of the secretory pathway. These lectins are expressed

in the same intestinal cell lines as GC-C, and may be physiologically relevant modulators of GC-C activity.

Conclusions: Glycosylation of de novo synthesized GC-C is required to impart ligand binding ability, 4 crucial sites involved in these processes were identified. GC-C appears to be a client for VIP36, and this interaction may regulate its trafficking. Galectin3 also interacts with GC-C and inhibits its activity.

C 208 Monascin from red mold dioscorea as a novel antidiabetic and antioxidative stress agent in rats and Caenorhabditis elegans

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Background: Monascus-fermented red mold dioscorea (RMD) products have been extensively used in East Asia. We investigated monascin from RMD to test whether this compound acts as an antidiabetic and antioxidative stress agent in Caenorhabditis elegans and rats with streptozotocin (STZ)-induced diabetes. The mechanisms by which monascin exerts its action in vivo were also examined.

Observations: STZ-induced diabetic rats were given monascin at 30 mg/kg/day and sacrificed after 8 weeks. Plasma glucose, triglyceride, cholesterol, and high-density lipoprotein were measured. Antioxidative enzymes in the pancreas were also measured. In addition, monascin was evaluated for stress resistance and potential associated mechanisms in C. elegans. Throughout the 8-week experimental period, significantly lowered plasma glucose, triglyceride, cholesterol, and higher high-density lipoprotein levels were observed in monascin-treated rats. Monascin-treated rats showed lower reactive oxygen species production and higher activities of glutathione peroxidase and catalase in the pancreas compared to diabetic control rats. Monascin-treated C. elegans showed an increased survival rate during oxidative stress and heat stress treatments compared to untreated controls. Finally, monascin extended the lifespan under high-glucose conditions, enhanced small heat shock protein expression, and affected the subcellular distribution of the FOXO transcription factor DAF-16 in C. elegans.

Conclusions: Our study showed antidiabetic and antioxidant effects of monascin in rats, and it conferred an increase in thermotolerance and oxidative stress resistance on C. elegans. The protective effect of monascin is mediated via regulation of the DAF-16-dependent insulin signaling pathway in C. elegans.

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