ORAL AND POSTER PRESENTATIONS

01. THE NEGATIVE REGULATION OF p53 BY HEDGEHOG SIGNALING

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Hedgehog (Hh) signal activation in human cancers is caused by genetic mutations in its signaling molecules that result in the constitutive activation of the Hh signal pathway, such as loss-of-function mutations in the negative regulators of this pathway, e.g. *Patched* and gain-of-function mutations in *Smoothened* (Smo). These genetic alterations are mainly observed in basal-cell carcinomas and medulloblastomas. Recently, it has been shown that aberrant Hh signal activation is widely observed in human cancers. However, the precise role of the Hh signal in cancer development is still unclear.

To analyze the role of Hh signal in oncogenesis, we test the effect of Hh signal molecules against the activity of tumor suppressor p53. We found that mutant Smo efficiently inhibits p53-mediated transcriptional activation and the p53 protein level. Moreover, we found that mutant Smo decreases the endogenous expression level of p53. This inhibition was also observed in the presence of the Hh ligand or with the overexpression of transcription factors Gli1 or Gli2, the downstream effectors of Smo, indicating that this inhibition is specific for the Hh signal pathway. We next analyzed the stability of p53 in response to Hh signal, since amount of p53 in cells is mainly regulated by the ubiquitin-proteasome system. We found that ectopical expression of mutant Smo accelerates Mdm2-mediated ubiquitination of the p53 protein. This result suggests that the Hh signal accelerates the degradation of the p53 protein by the ubiquitin-proteasome system.

Finally, to understand the role of the Hh signal in oncogenesis, we analyzed whether mutant Smo inhibits the DNA-damage-induced accumulation of the p53 protein or not. We found that p53 protein induction in response to adriamycin is suppressed by mutant Smo. Moreover, mutant Smo partially inhibited the p53-dependent apoptosis of oncogene-expressing mouse embryonic fibloblasts. Therefore, the Hh signal may be an accelerator of oncogenesis, that is, it activates cell growth and inhibits the p53-mediated anti-cancer barrier.

02. CHROMOSOME SEGREGATION AND MEIOSIS I PROGRESSION IN *DROSOPHILA* OOCYTE

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Chromosome segregation is achieved as a result of two essential processes: mitosis and meiosis. Defects on the mechanisms on which chromosome segregation relies may result in aneuploidy, causing cancer if they occur in mitotic cells and birth defects if occurring during meiosis.

Meiosis is characterized by two nuclear divisions (meiosis I and II) following a single round of DNA replication. As a result haploid gametes are formed. Chromosome segregation during reductional meiosis I requires three specific events: homologous recombination that results in chiasma formation, monopolar orientation of sister kinetochores and a stepwise degradation of cohesion at anaphase I. Indeed sister kinetochores must remain attached until metaphase II while cohesion is lost on chromosome arms before anaphase I proceeds. Meiosis I specific events must be supported by specific proteins. It was our reasoning for undertaking the screen that resulted in the identification of the *Drosophila* yemanuclein-alpha (yem-alpha), a DNA binding protein specific for the oocyte nucleus (Aït Ahmed et al, 1992). Its localization to the chromosomes is restricted to meiosis I. It is a new component of the synaptonemal complex (SC). After SC disappears yem-alpha remains on chromosome arms and centromeres. Its function is critical for meiosis I progression and homologue segregation; in a recombination defective background a *yem-alpha* mutant oocyte undergoes a single division (1, 2). Such a phenotype has never been reported in *Drosophila* meiosis. Yem-alpha function is genuinely new and will be discussed in the light of these data and other unpublished data.

(1) Oocyte specific *Drosophila* Yemanuclein-alpha is required for reductional division and meiosis I progression (submitted) R. Meyer, M. Delaage, R. Rosset, M. Capri and O. Aït Ahmed.

(2) *Drosophila* Yemanuclein-alpha is a conserved synaptonemal complex associated protein required for meiotic prophase (submitted) R. Meyer, M. Capri, C.Terzian, M. Schoenhals and O. Aït Ahmed.

03. CDC14 DOWN REGULATES RNA POLYMERASE I TRANSCRIPTION IN ANAPHASE TO ALLOW SEGREGATION OF RIBOSOMAL REPEATS

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In addition to cohesin, transcription-dependent linkages hold sister chromatids together in the ribosomal DNA (rDNA). Removal of these linkages depends on the activation of the conserved protein phosphatase Cdc14. However, neither the physical nature of the linkages nor the Cdc14 regulated removal is understood. Here we show that expression of RntA, an A.oryzae ribonuclease, removes transcription linkages in the absence of Cdc14, suggesting that nascent rRNA transcripts block segregation in cdc14 mutants. We find that segregation of rDNA in RntA expressing cells requires condensin, and that rRNA transcription is reduced transiently during yeast anaphase, in a manner dependent on Cdc14. Our findings demonstrate that Cdc14's role in rDNA segregation is to reduce rRNA transcription during anaphase to allow condensin to bind thereby inducing sister segregation through condensation of

ribosomal DNA. These results establish that transcription repression during mitosis is a prerequisite for chromosome segregation and is an active highly regulated process.

04. AURORA KINASE SER349 PHOSPHORYLATION IS REQUIRED DURING XENOPUS LAEVIS OOCYTE MATURATION

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Xenopus laevis Aurora-A is phosphorylated in vivo onto three residues: Ser53, Thr295 and Ser349. The activation of the kinase depends on its autophosphorylation on Thr295. The phosphorylation of Ser53 by still unknown kinase(s) prevents its degradation. The present work focused on the regulation of Aurora-A function via Ser349 phosphorylation. In vitro mutation of Ser349 to alanine (S349A) had few impact on the capability of the kinase to autophosphorylate as well as on its activity. These data in addition to in gel kinase assays and limited proteolytic digestion experiments clearly prove that Ser349 is clearly not an autophosphorylation site. In response to progesterone, *Xenopus laevis* stage VI oocytes microinjected with the Aurora-A S349A mutant proceed normally until germinal vesicle breakdown (GVBD). However the expression of this mutant kinase became toxic soon after meiosis I and lead to oocyte death, demonstrating the importance of this phosphorylation for the regulation of the kinase. Western blot analysis using an anti-phospho-Ser349 antibody reveals a variable level of Ser349 phosphorylation during oocyte maturation and between meiosis I and meiosis II. In this paper our results suggest that the phosphorylation of Ser349 participates to the downregulation of Aurora-A activity observed between MI and MII, an event requisite for oocytes to proceed to metaphase II arrest in response to progesterone.

05. OBSERVATION OF NON-LINEAR CELL GROWTH DURING THE CYCLE NECESSITATES A SIZE-SENSING MECHANISM IN MAMMALIAN CELLS

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Continuously proliferating cells must grow to precisely double their volume during one cell cycle in order to achieve size homeostasis, with protein synthesis predominantly contributing to this growth. To ensure that after mitosis newborn daughter cells can grow at the same rate as their mothers, protein synthesis rates have to double during one cycle. How this doubling takes place remained unclear so far.

Linear growth models conclude that protein synthesis rates stay constant throughout the cell cycle. In consequence, size homeostasis would be achieved without specific control mechanisms. If growth, however, is exponential, the protein synthesis rate steadily increases with volume (larger cells would grow faster), demanding a cell size checkpoint to maintain constant mean volumes among cell populations. This issue represents a long-standing open question in the fundamental biology of eukaryotic cells.

We previously provided evidence for a size-sensing mechanism in various vertebrate cell types. Here, we used an improved cell system of murine myeloid progenitors expressing the IGF-1 receptor, cultivated in the presence or absence of IL-3 and IGF-1. With cytokines, cells were 1.3 times larger and had a shorter doubling time (8.2h) than cells cultivated without cytokines (14.8h). These states could be interchanged within 24 hours. Cells grown under both conditions had to reach the same critical volume in G1 to enter S-phase, implying active size control.

We performed size measurements of single cells in time-lapse studies and of cultures synchronized by centrifugal elutriation, both strongly indicating non-linear growth. In addition, we directly analysed protein synthesis rates at different time points during the cell cycle in synchronized cultures and detected rate increases clearly incompatible with linear growth.

Taken together, we provide strong evidence for non-linear cell growth over the cell cycle and, in consequence, for a size checkpoint acting at a critical volume in G1.

06. MUSCARINIC AGONIST AND ANTAGONISTS CHANGES MUSCARINIC RECEPTOR AND CYCLIN D1 EXPRESSION IN K562 CELLS

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Muscarinic acetylcholine receptors mediate a variety of cellular responses, including inhibition of adenylate cyclase, breakdown of phosphoinositide and modulation of K channels. They are relatively abundant and mediate diverse actions of acetylcholine in the central nervous system, as well as non-nervous tissues innervated by the parasymphatic nervous system. Many cells express a mixture of muscarinic receptor transcripts. G-protein-coupled receptors (GPCRs) are capable of regulating cell growth. Former studies have sugested that acetylcholine and cholinergic agonists alter proliferation of lymphocytes. In the presence of these ligands an increase of lymphocyte mRNA and protein synthesis occurs. We have previously demonstrated that K562 cells express M_2, M_3 and M_4 muscarinic acetylcholine receptors. In this study, we were interested in investigating the effect of muscarinic agonist and antagonist on the expression levels of muscarinic receptors and Cyclin D1 in K562 cells. Our data show that

muscarinic agonist and antagonists treatment leads to changes in muscarinic receptor and Cyclin D1 transcripts.
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07. CONTROL OF CELL CYCLE PROGRESSION BY ACETYLATION OF CELL CYCLE REGULATORY PROTEINS

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Acetylation of lysine residues is a post-translational modification of proteins mediated by a superfamily of enzymes named histone acetyltransferases (HATs). In contrast, removing acetyl groups from proteins is carried out by the so called histone deacetylases (HDACs). Until recently it has been assumed that protein acetylation was mainly related to the regulation of the activity of histones or proteins related with chromatin condensation or transcription. However, nowadays a growing number of non-histones and proteins non-related to chromatin are appearing as substrates for HATs and HDACs, indicating that acetylation-deacetylation of proteins probably is a post-translational mechanism similar to phosphorylation-dephosphorylation that modulates the functional behaviour of most proteins. During the last few years we have been working on the analysis of the possible role of acetylation of cell cycle regulatory proteins (cyclins, cyclin-dependent kinases (cdks) and inhibitors (CKIs)) on the control of cell cycle progression. Our results indicate that at least cyclin A, cdk2 and $p27^{Kip1}$ are substrates of the acetyltransferase PCAF. Acetylation of $p27^{Kip1}$ at specific sites induces an increase in its inhibitory activity on cyclin A-cdk2 complexes. On the other hand, we have also observed that acetylation of cdk2 leads to the inhibition of its kinase associated activity. Thus, cyclin-cdk2 activity might be negatively regulated by two different acetylation-dependent mechanisms: acetylation of p27 that elevates its inhibitory capacity and acetylation of cdk2 that blocks its kinase activity. These data revealed for the first time a new mechanism of cell cycle regulation by direct acetylation of some cell cycle regulatory proteins.

08. MUTATIONS IN THE *TEL1* GENE THAT COMPENSATE FOR Mec1 LACK OF FUNCTIONS IN THE DNA DAMAGE RESPONSE

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In eukaryotes, checkpoint response to DNA lesions is mediated by (PI3K)-related protein kinases, called ATM and ATR in humans, and Tel1 and Mec1 in *S. cerevisiae*. The human Tel1 orthologue, ATM, plays a primary role in sensing and transducing the signals emanating from double-strand breaks and is also required for the generation of IR-induced RPA foci that lead to ATR-dependent checkpoint activation. Conversely, the checkpoint function of *S. cerevisiae* Tel1 appears more furtive, as Tel1-deficient cells do not suffer from obvious sensitivity to DNA damaging agents and there is no major checkpoint defect. This suggests that Tel1 and ATM may differ in their intrinsic kinase activity, in their ability to interact with specific targets and/or with damaged DNA. Because, the ability of Tel1 to sense and transduce the DNA damage signal can be enhanced by increasing Tel1 amount, we asked whether it was possible to isolate mutations in the *TEL1* gene able to compensate for the lack of Mec1 checkpoint functions. We identified seven independent *TEL1hy* mutant alleles, by virtue of their ability to suppress the hypersensitivity to genotoxic agents, as well as the checkpoint defects of Mec1-deficient cells. Suppression of *mec1* cells' hypersensitivity is dominant, suggesting that they encode for gain of function Tel1hy variants. Their suppression effects are not due to increased amounts of Tel1hy proteins compared to wild type Tel1, but it may be due to an increased intrinsic kinase activity. In fact, most of the Tel1hy mutant proteins showed an enhanced kinase activity in vitro compared to wild type Tel1, suggesting that most of the *TEL1hy* mutations result in amino acid changes in domains important to regulate protein kinase activity. Finally, most of the *TEL1hy* alleles cause telomere overelongation, indicating that they promote telomere elongation more efficiently than wild type Tel1.

09. IDENTIFICATION AND ANALYSIS OF HUMAN CELL CYCLE REGULATORS

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Although posttranslational modification, e.g. by CDK-dependent phosphorylation or ubiquitin-dependent proteolysis, is a key regulatory process in mitosis, many human cell cycle regulators are also controlled on the transcriptional level. In order to identify novel human cell cycle regulators we performed two small scale shRNA and siRNA based RNAi screens using 80 genes with expression peaks in late G2-phase of the cell cycle (Whitfield et al. 2002, Walker 2001). Hela cells were transfected with either a pool of 2-3 shRNA expression plasmids targeting one gene or Dharmacon smart siRNA pools and monitored for cellular phenotypes using live cell microscopy for 24-48 hours. CdcA2 and CCDC99 were two of the genes selected for further analysis. Bioinformatic analysis suggest that CdcA2 (also known as Repo-Man) is an intrinsically unstructured protein with poor amino acid sequence conservation and absence of globular domains. Quantitative real-time PCR and immunoblotting revealed that endogenous CdcA2 oscillates during the cell cycle and

peaks in mitosis where it becomes strongly phosphorylated. CdcA2 localises to chromosomes during mitosis and also to chromatin and the nucleolus in other phases of the cell cycle. Overexpression of recombinant protein causes chromosome segregation defects and cell death. Like CdcA2, CCDC99 oscillates during the cell cycle and becomes phosphorylated in mitosis. Immunostaining of this protein suggests that it is localized on the kinetochores and spindle poles of mitotic cells. CCDC99 RNAi causes a strong metaphase arrest followed by cell death.

10. DISSECTING THE TRANSCRIPTIONAL NETWORK OF GROWTH CONTROL

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Cell growth and division is an enigma in science; many genes are associated with it, yet it remains largely unclear how they are regulated and work together in a functional way. The presented interdisciplinary research is directed at uncovering and understanding more of the cell growth signaling pathways. Recent research in our laboratory has identified a set of 570 *Drosophila* genes that result in altered growth phenotypes when knocked down, which makes them good candidates for studying cell proliferation; 44 transcription factors were identified in the screen. Cell cultures of S2 cells of the model organism *Drosophila* will be used in experimental research and computational analyses to determine the role of the individual signaling pathway components, their targets and their interrelations. These results from these studies will be assembled into a model of the cell cycle that is verifiable by well-established experimental procedures.
The specific objectives of the research are the following:
1. Using RNAi knockdown studies to determine the transcriptional regulation of a set of genes known to be involved in cell growth.
2. To experimentally determine the binding specificities of growth regulating transcription factors and to computationally identify these binding sites in the Drosophila genome.
3. To combine the acquired results from these experiments into a systematic model of the transcriptional regulation of the metazoan cell cycle.

11. TEMPORAL ORDERING OF MITOTIC EXIT IN BUDDING YEAST

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Exit from mitosis is an intricate process, implicating numerous controlled events. At the time of anaphase onset the mitotic spindle elongates to segregate sister chromatids, accompanied by condensation and decatenation of the budding yeast rDNA locus. After this, the elongated anaphase spindle is again disassembled, chromosomal origins of DNA replication are reset, and finally cytokinesis finishes cell division by pinching off two new daughter cells.
Ordered progression through mitosis is in part controlled by surveillance mechanisms (checkpoints), but also by intrinsic regulators that coordinate spatial and temporal coupling. Mitotic exit in the budding yeast *Saccharomyces cerevisiae* is controlled by the kinase/phosphatase pair Cdk1/Cdc14. To exit mitosis, the high mitotic Cdk1 activity must be downregulated, and its phospho-substrates dephosphorylated by the Cdc14 phosphatase which is activated at anaphase onset. It is not known whether, and how, Cdk downregulation and Cdc14 activation control the temporal order of mitotic exit.
Here, we aim to understand how the timing of dephosphorylation events is regulated during mitotic exit. Cdk substrates involved in formation of a stable anaphase spindle (e. g. Fin1) are dephosphorylated early during mitotic exit, while the APC activator Cdh1 that re-establishes the G1 state is dephosphorylated later. We address three non-exclusive hypotheses: The phosphorylation status of Cdc14 substrates (i) depends on different Cdk1/Cdc14 ratios, (ii) is time related, or (iii) the ordering of mitotic exit occurs by a dependency of events. Our first results suggest that the ordered dephosphorylation of Cdc14 substrates is mainly controlled by the ratio of Cdk1 to Cdc14. We show that early Cdc14 substrates are dephosphorylated at a relatively high Cdk1/Cdc14 ratio but that late substrates required a lower Cdk1/Cdc14 ratio in order to be dephosphorylated.

12. CDC25B INVOLVEMENT IN CENTRIOLE DUPLICATION AND MICROTUBULE NUCLEATION DURING INTERPHASE

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There is significant evidence for the involvement of CDK-cyclin complexes in regulating centriole duplication and microtubule nucleation at specific cell cycle stages, although their exact roles in these processes remain unclear. As the activities of CDK-cyclin complexes are themselves regulated by the CDC25 family of phosphatases, we are investigating the role of centrosomally localised CDC25B during interphase. Immunofluorescence analyses revealed that CDC25B together with its negative regulator CHK1, localise asymetrically to the mature centrosome(s) throughout interphase. Inhibition of CDC25B by siRNA results in aberrant centriole duplication, abnormal interphase microtubule arrays, and a marked effect on the centrosomal localisation of □-tubulin. In contrast, forced expression of wild-type CDC25B at the centrosomes in S phase-arrested cells, results in a significant increase in □-tubulin at the centrosomes. This is accompanied by centrosome amplification and aberrant microtubule organisation, such as increased microtubule nucleation from 1 or both centrosomes or microtubule bridging between centrosomes. Centrosome-targeted expression of phosphatase-inactive CDC25B on the other hand, had no effect on the centrosomal localisation of □-tubulin, centrosome duplication or microtubule nucleation. We therefore propose that CDC25B is part of the pathway that

controls the localisation of □-tubulin to the centrosomes, thereby regulating the assembly of daughter centrioles during S phase and the nucleation of microtubules throughout the cell cycle.

13. CLEAVAGE FURROW INITIATION IN CYTOKINETIC IS REGULATED BY MICROTUBULES AND UBIQUITIN-MEDIATED DEGRADATION AND IS CELL CYCLE INDEPENDENT

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Cells segregate the two copies of their replicated genome in metaphase and separate them into two cells during cytokinesis. The timing of cleavage initiation, after the genomes have parted, and the location of the furrow, between the two receding copies of the genome, are of paramount importance for genetic stability. The temporal and spatial regulation of the cytokinetic furrow remains highly controversial. Two, non-exclusive, models were proposed to explain this regulation. The first is that cleavage is induced by signals emanating from the central spindle. The second is that astral spindles inhibit furrowing at the poles, enabling it at a local minimum in the center of the cell. We have used time laps microscopy to follow various cell types and temperature sensitive mutants, upon manipulation of their microtubule in interphase and mitosis. We observed that depolymerization of microtubules in prophase leads to the establishment of ectopic furrows. Furrowing is not a passive consequence of microtubule depolymerization, but depends on the RhoA GTPase signaling pathway and on ubiquitin mediated proteolysis by Cullin-RING ubiquitin ligases. Strikingly in non-adherent cells, as well as adherent cells that have become detached, microtubule depolymerization leads to furrowing anytime throughout the cell cycle. Our results suggest that cytokinetic furrowing is initiated by the controlled relief of the inhibition exerted by astral microtubules, enabling cleavage at the correct time and place. The competence of unattached cells to furrow throughout the cell cycle contrasts with the hypothesis that cleavage is restricted to a mitotic C-phase. We therefore suggest that the initiation of cytokinesis is not an intrinsic cell cycle phase and emphasize its similarity to other cellular processes that involve cell membrane remodeling.

14. INDUCTION OF P53-DEPENDENT APOPTOSIS AND CELL-CYCLE ARREST IN HUMAN CANCER CELL LINES

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Tumour suppressor protein p53 represents one of the most important factors regulating cell proliferation, differentiation and programmed cell death. P53 triggers growth arrest or apoptosis in response to different cellular stress signals and its biochemical function is associated with the ability to operate as a transcription factor. The way of p53 activation seems to be the critical event responsible for the p53 response to DNA damage. Complex interactions of p53 with DNAs are strongly dependent on the dynamics of DNA structure, especially in the context of chromatin. To determine whether genotoxic stress regulates DNA binding by p53 in vivo, we performed chromatin immuno-precipitation (ChIP) assay on tumour and normal cell lines containing wild-type p53. ChIP recovers specifically only p53-dependent promoters DNA, the best target in our set of the promoters are p21, CaN19 and PIG3 DNAs. The p53-DNA binding is dependent not only on the cells condition, but also on the cell type. Our results show that decision between cell arrest and apoptosis may arise, at least in part, from differential affinities for the two groups of promoters, particularly p21, PUMA, CaN19, JAG2 and PIG3 promoters. Biological consequences of p53 activation were tested by flow cytometry. While treatment by UV or doxorubicin induced cell cycle arrest, 5-fluorouracil induced apoptosis in p53 wild type cells. We show by a competition in vitro assay that supercoiling enhances binding of p53 to p21, RGC and PIG3 promoters. This enhancement is more intense in p21 than in the RGC insert.
GAAV 500040502

15. DOWNSTREAM TARGETS OF EZH2 IN MICE

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It is known that the Polycomb group gene Ezh2 is essential for progression of the cell cycle: conditional knock outs usually show arrested phenotype, whereas we have shown that retroviral overexpression in hematopoietic cells provides a proliferative advantage. Ezh2-overexpressing recombinant bone marrow cells could be serially transplanted in mice and a proliferative advantage could be detected with increasing intensity, resulting in a myeloproliferative syndrom. By systematic screening of possible downstream targets we revealed that both a mouse stromal cell line MS5 and unfractionated bone marrow cells transfected with extra copies of Ezh2 showed reproducible downregulation of key cell cycle regulators such as p53, p21, p27, p57. Also levels of Klf1 and Klf4 were downredulated, which is important for erythropoiesis (Klf1) and p53 signaling (Klf4). Downregulation of p21 gene proceeded without DNA methylation of its core promoter, whereas other promoters show indications of the silencing with involvement of the DNA methylation.
These data explain the main proliferative features of cells with up- or down- regulated Ezh2.

16. CDK1 IS SUFFICIENT TO DRIVE THE MAMMALIAN CELL CYCLE.

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Unicellular organisms such as yeasts require a single cyclin-dependent kinase, Cdk1 (also known as Cdc2 in fission yeast and Cdc28 in budding yeast), to drive cell division. This catalytic subunit sequentially binds to activating partners, known as Cyclins, to phosphorylate key targets during the various phases of the cell cycle. In contrast, mammalian cells are thought to require sequential activation of as many as four different Cdks, Cdk2, Cdk3, Cdk4 and Cdk6, to drive cells through interphase (G1 and S phases) as well as Cdk1 to proceed through mitosis. In order to assess the real contribution of these Cdks to the mammalian cell cycle, we have generated mice lacking all interphase Cdks or mice lacking just Cdk1. In the absence of these Cdks, embryos develop until midgestation including organogenesis. In these embryos, Cdk1 binds to all Cyclins resulting in phosphorylation of pRb and expression of E2F-regulated genes. Mouse embryonic fibroblasts derived from these embryos proliferate in vitro with an extended cell cycle due to inefficient inactivation of Rb proteins. Yet, they become immortal upon continuous passage. In contrast, embryos lacking Cdk1 do not develop even at early stages (E1.5-E2.5). These results indicate that Cdk1 is the only essential cell cycle Cdk. Moreover, they demonstrate that in the absence of interphase Cdks, Cdk1 can execute all the events required to drive cell division, at least during early to mid embryogenesis.

17. MEC1 AND TEL1 PHOSPHORYLATION OF HOP1, AN EVOLUTIONARILY CONSERVED MEIOSIS SPECIFIC CHROMOSOMAL PROTEIN

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Budding yeast Mec1, the homolog of mammalian ATR/ATM, is an essential chromosome based signal transduction protein. Mec1 is also required for meiosis and inactivation of the gene leads to a number of meiotic defects including reduction in recombination, loss of inter-homologue bias, loss of crossover control, and failure in meiotic progression. To better understand meiotic roles of Mec1 and its related kinase Tel1, we utilized cytological and biochemical approaches to identify their targets. We found that multiple proteins are phosphorylated by Mec1 and Tel1 during normal meiosis, and identified an evolutionarily conserved meiosis specific protein, Hop1, as a target. The budding yeast Hop1 is a chromosome axis-associated protein, and has been shown to play critical roles in the formation and the subsequent processing of programmed meiotic double strand breaks (DSBs) as well as meiotic chromosome structure development. To address functional significance of Mec1/Tel1 phospohorylation of Hop1, we generated *hop1* alleles where the relevant target sites are eliminated. Our analyses indicate that these mutants, similar to *hop1-null* strains, generate inviable spores, and bypass *dmc1* triggered meiotic prophase checkpoint arrest. Detailed characterization of a selected set of novel *hop1* mutants, and implications regarding role(s) of Mec1 and Tel1 during normal meiosis will be presente

18. CHARACTERIZATION OF PHA-739358, A SMALL MOLECULE INHIBITOR OF AURORA KINASES

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The Aurora kinases are a family of serine-threonine kinases that play a critical and conserved role in the mitotic phase of the cell cycle from yeast to humans. Functions associated with centrosome maturation, spindle organization, chromosome separation and condensation as well cytokinesis have been established. Aurora kinases A & B have been found to be over-expressed in a wide variety of human tumors making them attractive candidates for anticancer therapy. We have identified PHA-739358 as a new potent Aurora kinases inhibitor currently in clinical trials. The compound is a small molecule inhibitor that blocks proliferation, promoting endoreduplication in tumoral cells. In tumor cells the compound induces a morphological phenotype typical of Aurora-B inhibition and molecular markers for Aurora-A and B are affected. In vivo the compound shows good efficacy in different tumor models including xenografts or spontaneous tumors. Profiling of PHA-739358 on a panel of different kinases revealed that the compound inhibits also Ret, Trk-A, FGFRs and Abl. We present here data demonstrating that this compound is capable of inhibiting the wild type Abl as well as the most clinically frequent imatinib-resistant Abl mutants, comprising the T315I mutant. Inhibition is observed both in vitro and in cell line models and it is due to the ability of the inhibitor to bind with high affinity to the Abl kinase domain. We conclude that our novel Aurora kinase inhibitor has promising therapeutic potential in different cancer types and its Abl cross-reactivity holds promise for the treatment of CML patients resistant to the current therapy.

19. A NOVEL CONDITIONAL MOUSE MODEL FOR C-MYC INDUCED PROLIFERATION AND APOPTOSIS IN LIVER

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We have developed a novel mouse model for studying c-Myc function in liver. In this model, the expression of a conditionally active variant of c-Myc, c-MycER, is targeted to the hepatocytes by virtue of the murine albumin promoter. When the transgene product is expressed it is inactive, until it binds the ligand for the modified estrogen receptor (ER) domain: 4-hydroxy tamoxifen. This ligand is administered by intraperitoneal injection of tamoxifen, which is subsequently converted to its 4-hydroxy metabolite systemically. Activation of MycER in this model leads to hepatocyte proliferation within hours, which peaks at around two days of MycER activation and then declines but remains elevated throughout tamoxifen treatment. In line with the described functions of c-Myc, apoptotic cells as measured by the TUNEL assay can be found throughout the livers of tamoxifen treated Alb-MycER mice, but neither in vehicle treated Alb-MycER mice nor in tamoxifen or vehicle treated non-transgenic littermate controls. At the same time, serum ALT levels rise to about 4 times normal levels, indicative for liver damage and similar as to what is observed in patients with cirrhosis. Using this unique mouse model, we show that small molecule inhibitors of anti-apoptotic signalling cascades can be used to modulate the phenotypic outcome of c-Myc activation in vivo (proliferation or apoptosis, net tissue growth or regression). In the mean time, we are seeking to elucidate how c-Myc drives proliferation and apoptosis in liver at the molecular level using microarray technology. Ultimately, understanding how c-Myc function affects liver homeostasis can contribute to the development of better treatments for hepatocellular carcinoma and cirrhosis.

20. DEREGULATED REPLICATION LICENSING CAUSES DNA FRAGMENTATION CONSISTENT WITH HEAD-TO-TAIL FORK COLLISION

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Correct regulation of the replication licensing system ensures that no DNA is rereplicated in a single cell cycle. When the licensing protein Cdt1 is overexpressed in G2 phase of the cell cycle, replication origins are relicensed and the DNA is rereplicated. At the same time, DNA damage response pathways are activated that block further cell cycle progression. We have studied the consequence of deregulating the licensing system by adding recombinant Cdt1 to *Xenopus* egg extracts. We show that Cdt1 induces a DNA damage response and the appearance of small fragments of double-stranded DNA. DNA fragmentation and a strong DNA damage response are dependent on uncontrolled rereplication and do not occur after a single coordinated round of rereplication. The DNA fragments are composed exclusively of double stranded rereplicated DNA. The unusual characteristics of these fragments suggest that they result from head-to-tail collision (rear ending) of replication forks chasing one another along the same DNA template. Preliminary work has been undertaken to examine in more detail the nature of the observed DNA damage response and to establish which proteins are loaded onto DNA during rereplication.

21. A ROLE FOR *Schizosaccharomyces pombe* Cdc14p/Flp1p PHOSPHATASE IN REGULATING FULL ACTIVATION OF Cds1p CHECKPOINT KINASE IN RESPONSE TO REPLICATION STRESS

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Serine/threonine phosphatase Cdc14p/Flp1p is implicated in regulation of mitotic exit by targeting Cdc25p to degradation and thus triggering the reduction of Cdc2p-associated activity. Accordingly, Flp1p is restricted to the nucleolus until mitosis, when it is dispersed throughout the nucleus, mitotic spindle and contractile ring. Upon genotoxic stress, *Schizosaccharomyces pombe* ATM/ATR-related kinase Rad3p activates Chk1p and/or Cds1p which phosphorylate key cell cycle proteins, including Cdc25p, Wee1p and Mik1p, ensuring inactivation of Cdc2p/cyclin complex to prevent entry into mitosis. Given the similarity of regulatory targets of the mitotic exit cascade, and those which delay cell cycle progression upon genotoxic stress, an implication of Flp1p in Cdc25p regulation in response to damaged or unreplicated DNA seemed plausible. Unexpectedly, our results vindicate a role for Flp1p in checkpoint response different from its regulatory function in cell cycle. Replication stress triggers subcellular relocation of Flp1p in a checkpoint dependent manner. In particular Cds1p is required to release Flp1p into the nucleus upon replicative stress. Cds1p and Flp1p proteins interact *in vivo* when cells are exposed to drugs like hydroxyurea and, furthermore, activated Cds1p phosphorylates Flp1p *in vitro,* suggesting that Flp1p is regulated by the S-phase checkpoint kinase. Moreover, Δ*flp1* strains present *in vivo* low levels (of Cds1p) and decreased Cds1p activity upon genotoxic stress. Finally, supporting a role for Flp1p in checkpoint response, *flp1* mutant cells are partially defective in checkpoint response, since Rad22p-recombination foci appear upon HU treatment in this strain.
Our results allow us to suggest that by releasing Flp1 into the nucleus Cds1p checkpoint kinase modulates its own full activation during replication stress.

22. T-LOOP PHOSPHORYLATION OF THE *ARABIDOPSIS* CDC2^{+}/CDC28 HOMOLOG CDKA;1 IS REQUIRED FOR ITS FUNCTION AND CAN BE PARTIALLY SUBSTITUTED BY AN ASPARTATE RESIDUE

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In general, the cell cycle control machinery appears to be highly conserved between the kingdoms. As in other eukaryotes, in plants, progression through the cell cycle is governed by cyclin-dependent kinases – the Cdks. Phosphorylation of a canonical Thr residue within the T-loop by Cdk-activating kinases is required for high enzyme activity in animals and yeast since this very process unblocks the catalytic cleft. By mutating conserved phospho-sites, we showed that the single *Arabidopsis thaliana* Cdc2^{+}/Cdc28 homolog CDKA;1 with the archetypical PSTAIRE hallmark also needs to be phosphorylated in the T-loop at the highly conserved Thr161 to fully activate its function. Interestingly, in *Arabidopsis*, this crucial phosphorylation can be partially mimicked by a Thr>Asp substitution and restore the primary defect of *cdka;1* mutants, that we have isolated in our lab. Although this CDKA;1 variant displayed a dramatically reduced kinase activity, homozygous mutant plants could be recovered. These partially rescued plants, however, showed pronounced abnormalities such as a severe failure of the meiotic program, shedding light on different requirements for CDKA;1 function during the course of early gametogenesis. Furthermore, we explored the regulatory context of CDKA;1 *in planta*. We found that different phospho-site mutants of CDKA;1 demonstrate a differential ability to bind substrates and in yeast two-hybrid screens we have identified a number of potential novel interactors.

Our data suggests that parts of the molecular mechanistics of Cdk regulation is conserved between yeast, animals, and plants. However, the regulatory circuits controlling overall Cdk activity appear to be strikingly different.

23. SCREENING FOR FISSION YEAST MUTANTS DEFECTIVE IN DNA REPLICATION INITIATION.

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We identified a novel phenotype associated with mutants defective in DNA replication initiation. Unlike mutants defective in DNA replication elongation, initiation mutants show no sensitivity to loss of the Cds1 kinase when incubated at semi-permissive temperatures. However, under identical conditions, deletion of Chk1 leads to loss of viability as a result of cells entering mitosis in the absence of a complete round of DNA synthesis. Even though Cds1 is not required to maintain the viability of these mutants at the elevated temperature, both Swi1 and Mrc1 are both essential. This implies that Swi1 and Mrc1 have additional functions in DNA replication that are not related to their ability to activate Cds1 kinase. Consistently, Chk1 phosphorylation is observed in the replication initiation mutants in the absence of Mrc1. We have now shown by indirect immuno-fluorescence of Rad22 foci, that DNA damage does occur in these mutants and is therefore likely to be the cause of Chk1 activation. It is not yet known whether the damage occurs at initiation sites or is caused by replication stress associated with a decrease in the number of active replication forks.

Based on our findings, we have now developed a screen to identify mutants defective in DNA replication initiation. Over 80,000 temperature-sensitive mutants defective in cell cycle progression were screened for synthetic lethality when combined with either the *cds1* or *chk1* deletion strains. Two mutants were identified that were sensitive to the loss of Chk1, but not Cds1. Both mutants were found to correspond to genes required for DNA replication initiation, thus underscoring the selectivity of our screen.

24. TIPIN IS REQUIRED FOR THE STABILITY OF REPLICATION FORKS AND THE DNA REPLICATION CHECKPOINT IN XENOPUS LAEVIS EGGS EXTRACT

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We identified Xenopus laevis tipin as a substrate for Cyclin E/CDK 2 that is phosphorylated in interphase and undergoes further phosphorylation upon entry into mitosis. We mapped the interphase phosphorylation site at Ser326 and we are currently trying to identify the mitotic kinase(s) and the site(s) targeted. Tipin was originally identified as tim-1 interacting partner in a yeast two-hybrid screening. We showed that tipin and tim1 interact also in Xenopus egg extract. They are part of a large protein-protein complex that localizes at the replication fork and plays an important role in the S-phase checkpoint. In fact, depletion of tipin from Xenopus extracts results in a defect in the activation of chk1 in response to aphidicolin treatment, probably due to a failure in loading the checkpoint mediator protein, claspin, onto chromatin in the tipin-depleted extract.

During unperturbed DNA replication, the tipin/tim1 complex is bound to chromatin, and we were able to detect interactions between tipin and the MCM helicase. Depletion of tipin from Xenopus egg extracts did not significantly impair normal replication, but substantially blocked the ability of stalled replication forks to recover after removal of the block imposed by aphidicolin. Our data strongly suggest that, as in yeast, tipin and tim are part of a pausing complex that prevents the uncoupling of the DNA polymerase and helicase activities, stabilizing the replication fork.

In 'cycling' extracts, the tipin/tim1 complex is loaded onto the chromatin when DNA replication starts and is released from the chromatin when the extract enters mitosis. An interesting hypothesis is that mitotic phosphorylation regulates the release of tipin from the chromatin at the entry in mitosis, inactivating the S-phase checkpoint. Further experiments will be directed to understand the role and the biological relevance of this phosphorylation.

25. FUNCTIONAL STUDIES OF NOVEL CELL CYCLE REGULATORY GENES

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Unlike in the yeast, analysis of cell cycle regulation in multicellular organisms had usually not been based on loss-of-function genetics. To identify genes involved in cell cycle regulation, Prof. Jussi Taipale's laboratory has carried out genome wide RNA interference screens in *Drosophila* cells[1]. Novel regulatory genes that control cell size, cytokinesis, cell death/apoptosis, and G1 and G2/M phases of the cell cycle were identified in this study. The RNAi screens were carried out with very high confidence level (99.99994%) providing us reliable list of genes involved in all the steps of cellular growth control, and a solid platform to continue with analysis of function of the identified genes in regard with cell cycle regulation.

To understand how the identified genes regulate metazoan cell cycle progression and more specifically, to map them into different regulatory pathways, we are going to study the localization of the interesting regulatory proteins (and possible changes during the cell cycle), interactions with other proteins, and functions as kinase substrates. For these purpose we are producing our own monoclonal antibodies against the proteins of interest and against the related negative regulators (such as p27 and Rbf) of the cell cycle. We are expressing genes of interest as fusion with human IgG Fc-domain[2,3] in S2 cell-line followed by purification with Protein A Sepharose column. We will firstly immunize mice with ten antigens as pilot experiment (up to five antigens per mice). If the process works well, we plan to extend the work to cover all of the genes of interest (around 300-500). Experimental results of this protocol will be presented.

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26. HUMAN MOB1 IS REQUIRED FOR CYTOKINESIS BY DESTABILIZING MICROTUBULES AT THE MIDBODY

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Mob1 is an essential gene in S. cerevisiae, mutants of which arrest late in mitosis with an incomplete cytokinesis. In Drosophila melanogaster Dmob1 is also an essential gene and, furthermore, mosaic animals develop large tissue masses with increased cell proliferation and defective apoptosis, indicating that Dmob1 is a tumor suppressor gene. We have recently identified and characterized the human Mob1-like proteins. By immunofluorescence and live cell imaging we have observed that HsMob1 localizes to the centrosomes throughout the cell cycle and in the midbody at cytokinesis. Reducing, by RNAi, the levels of human HsMob1 from human tissue cultured cells results in a failure to complete cytokinesis in a way such that the cells stay connected by long intercellular bridges. It also induces prolonged centriole separation and multinucleation. In contrast, centrioles fail to split when HsMob1 is overexpressed. Surprisingly, Mob1-depleted cells gain mobility, moving rapidly in random directions. These cells also possess microtubules with increased resistance to depolymerization by cold and drug treatments. Furthermore we detect a significant increase in acetylated microtubules in cells after Hmob1 depletion, in particular in the midbody region at the end of cell division, suggesting that the cytokinesis failure is caused by an overstabilization of the microtubules in the intrecellular bridge.
Finally, we have isolated centriolin as a molecular partner of Mob1, which when depleted from human cells cause a phenotype extremely similar to the one observed after Mob1 depletion. We conclude that Hmob1 is required for proper cell abcission to occur but, unlike the yeast gene, it is not required for cells to exit mitosis and re-enter interphase.

27. ROLE OF HUMAN CLASPIN IN CHECKPOINT RECOVERY AND APOPTOSIS

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In response to genotoxic stress, cells can undergo a checkpoint-mediated cell cycle arrest or undergo apoptosis. The DNA damage checkpoint pathway is mediated by phosphorylation and activation of effector kinase Chk1 by ATR, a process that critically depends on adaptor protein Claspin. In this study we investigated the role of Claspin in the recovery from checkpoint arrest as well as in apoptosis. In non-damaged cells the abundance of Claspin is regulated through proteasomal degradation. After DNA damage, Claspin is transiently stabilized, and its expression depends on Chk1 kinase activity. In addition, Claspin is degraded upon mitotic entry, a process that is dependent on the β-TrCP-SCF ubiquitin ligase and Polo-like kinase-1 (Plk1). We demonstrate that Claspin interacts with both β-TrCP and Plk1 and that inactivation of these components or the β-TrCP recognition motif in Claspin prevents its mitotic degradation. These results show that Claspin levels are tightly regulated, both during unperturbed cell cycle and following DNA damage. Interestingly, expression of a non-degradable Claspin-mutant inhibits recovery from the G2 arrest induced by triggering the DNA damage checkpoint, demonstrating that degradation of Claspin at the onset of mitosis is an essential step for checkpoint recovery.
During DNA damage-induced apoptosis, Claspin is degraded both by cleavage of Asp25 and Asp1072 by Caspases 3 and 7. Importantly, in addition to cleavage by Caspases, we observed a proteasome-dependent degradation of Claspin under apoptotic conditions. To mimic these conditions, we downregulated Claspin by siRNA, which resulted in an increase in apoptotic induction both in the presence and absence of DNA damage. Therefore, we conclude that Claspin has anti-apoptotic activity and is

degraded by two different pathways during apoptosis. Together, these data demonstrate that Claspin is an important mediator in multiple pathways involved in maintaining the integrity of the genome.

28. CDK1 CONTRIBUTES TO DNA REPLICATION CONTROL DURING S PHASE IN RAT HEPATOCYTES.

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Liver has the unique capacity to regenerate after resection or injury. This process mainly involves hepatocytes which are highly quiescent differentiated cells. The understanding of cell cycle control of these differentiated cells is crucial since chirurgical resection and transplantation are based on the liver regeneration potential.

To study cell cycle control in hepatocytes, we used two/third hepatectomy in rat and primary cultures. Taking advantage of the synchronous progression of hepatocytes throughout the cell cycle *in vivo* and in primary culture, we have demonstrated that CDK1, cyclin A and cyclin B1 exhibit a biphasic pattern of expression correlating S phase and G2/M transition, respectively. Unexpectedly, immunoprecipitations of CDK1 and CDK2 followed by kinase assays activity revealed that CDK1 and CDK2 formed active complexes with cyclins A or B during the S-phase. Expression and activation of CDK1/cyclins A/B complexes seem to be a hepatocyte specific feature since CDK1 is not detected during S phase in synchronized human foreskin fibroblasts. In order to investigate the role of CDK1 and CDK2 during S phase in hepatocytes, their expression was knocked down using siRNA in primary culture of hepatocytes. CDK1/2 siRNA transfected cells showed a significant decrease in BrdU incorporation compared to control siRNA-treated cells. Moreover, using long-term hepatocyte coculture model in which the hepatocyte DNA synthesis is governed by pro-inflammatory cytokines (TNF□) and growth factors (EGF), as *in vivo* during liver regeneration, we showed that CDK1 induction in G1/S transition is dependant on extracellular matrix degradation following TNF□stimulation and MMP9 induction. Together these data indicated that CDK1 is a marker of the G1/S transition in normal rat hepatocytes and contributes along with CDK2 to S phase initiation and/or progression, under the control of cell-cell communications.

30. A DELICATE BALANCE BETWEEN GLUCOCORTICOID RECEPTOR EXPRESSION AND ITS LIGAND DETERMINES SENSITIVITY AND KINETICS OF GLUCOCORTICOID-INDUCED APOPTOSIS

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Glucocorticoid (GC)–induced apoptosis is an essential component in the treatment of acute lymphoblastic leukemia (ALL) and related malignancies. Although the pathway leading to cell death is not well understood, it is clear that it is initiated by the GC receptor (GR), a ligand activated transcription factor of the nuclear receptor family, and that the response somehow depends on expression of the GR and its ligand. Since GR expression differs between patients and the amount of GC administered varies between treatment protocols, the details of this receptor-ligand interplay are clinically relevant. Therefore we decided to address the effects of GR expression and GC concentration on kinetics and extend of GC-induced cell death. To this end, we generated a childhood acute lymphoblastic leukemia cell line (CCRF-CEM) expressing a tetracycline-responsive transactivator but lacking functional endogenous GR and stably transfected it with a GR^{wt}-ires-GFP construct. In the resulting cell line, GR levels can be modulated at will by varying the exposure to doxycycline and be readily monitored by fluorimetric analysis of GFP expression. Moreover, this cell lines allowed us to mimick, and study the effects of, GR autoinduction that is observed in GC-sensitive (but not GC-resistant) ALL cell lines and some T-ALL patients. Our results show a dramatic effect of the investigated parameters on "therapy outcome" in this model, i.e., GC resistance in cells with low GR levels could be overcome by exposure to high GC concentrations and cells expressing high levels required very low amounts of GC to undergo high degree of apoptosis. GR induction during GC exposure (mimicking GR autoinduction) resulted in a more delayed cell death. Very high GR levels were associated with a paradox response, i.e. high GC induced less apoptosis than low GC. Our data suggest that monitoring GR expression and auto-regulation in ALL patients might have relevance for selecting appropriate GC doses in future "individualized medicine" protocols.

31. HCF-1 INTER-SUBUNIT COMMUNICATION INVOLVED IN THE REGULATION OF THE CELL CYCLE.

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The goal of this project is to understand the control of human-cell proliferation, by elucidating the functions of the protein Host-Cell Factor-1 (HCF-1). HCF-1 is unusual in that it undergoes a process of proteolytic maturation that results from cleavages at six centrally located 26 amino acid repeats called HCF-1PRO repeats to generate a heterodimeric complex of stably associated amino- (HCF-1N) and carboxy- (HCF-1 C) terminal subunits. The absence of the HCF-1N or HCF-1 C subunit leads predominantly to either G1 or M phase defects, respectively.

We have hypothesized that HCF-1 forms a heterodimeric complex to permit communication between molecules involved in regulating different phases of the cell cycle. Indeed, there is evidence for such inter-subunit communication because a point mutation in the HCF-1N subunit in the temperature-sensitive hamster cell line tsBN67 causes, in addition to G1- phase defects associated with the HCF-1N subunit, M-phase defects similar to the defects seen upon loss of HCF-1C function. Furthermore inhibition of HCF-1 proteolytic maturation by deletion of the six HCF-1PRO repeats (HCF-1 ΔPRO) also leads to M-phase defects, specifically cytokinesis defects leading to binucleation, indicating loss of HCF-1C function in the absence of subunit maturation.
With a recombinant HCF-1 ΔPRO molecule containing a single re-inserted HCF-1PRO repeat and thus active for proteolytic maturation, we tested if the loss of HCF-1C function in HCF-1 ΔPRO is the result of lack of processing or of the deletion of the proteolytic processing region. We found that the rescue of the proteolytic processing rescues the cytokinesis defects showing that the proteolytic region is not important except for the processing of the two subunits for HCF-1C to work. We also show that unprocessed HCF-1 decreases histone H3 S10 phosphorylation (H3S10P) levels and increases histone H4K20 dimethylation (Me2) levels, properties also observed in the absence of HCF-1C function.

32. MOLECULAR PROFILING OF THE NOVEL EPOTHILONE ZK-EPO: ACTION ON CELL CYCLE PROGRESSION AND APOPTOSIS IN TUMOUR CELLS

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ZK-EPO is a novel epothilone that binds to the tubulin subunits of the cellular microtubule network, stabilizing microtubules. Therefore, it inhibits the dynamic instability of the microtubule spindle, crucial for chromosome segregation during mitosis. ZK-EPO has demonstrated highly significant activity in both sensitive and in multidrug-resistant (MDR) tumour models *in vitro* and *in vivo*. To reveal the molecular mechanism of action of ZK-EPO, several *in vitro* experiments were performed in cancer cells.
ZK-EPO inhibited proliferation of a broad panel of tumour cell lines at sub-nanomolar concentrations. It caused microtubule bundling and disturbance of the mitotic spindle as investigated by laser scanning microscopy and tubulin polymerization assays. Treatment of tumour cells with ZK-EPO lead to aberrant mitosis and micronucleation as well as cell cycle arrest in G2/M in a concentration dependent manner, presumably due to activation of the spindle assembly checkpoint. Moreover, induction of apoptosis and mitotic catastrophe by ZK-EPO was detected using FACS analysis and measurement of caspase activity.
Thus, the comprehensive *in vitro* characterization of ZK-EPO presented here, supports its extensive evaluation in clinical trials and reported techniques represent surrogate markers for drug activity.

33. THE RB2-P53 CONNECTION IN CELLULAR SENESCENCE

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Cellular senescence, a pathway activated in response to a variety of stress signals, stalls cells irreversibly in G1/S transition. The appearance of senescent cells *in vivo* in premalignant lesions but not in malignant tumors emphasizes the role of cellular senescence as a tumor suppressor mechanism. Cooperative functions of the p53-p21 and p16INK4a-pRb pathways are thought to be of fundamental importance in the induction of cellular senescence. As we showed recently, wild-type p53 can activate cellular senescence in rat C6 cells lacking p16INK4a, arresting the cells in late G1 phase between cyclin E to cyclin A- mediated steps. This suggests that the p16INK4a-pRb pathway may be dispensible for the induction of cellular senescence. Instead of an accumulation of pRb we observed a strong increase of Rb2 in senescent C6 cells. We assumed that this retinoblastoma protein functions as a critical mediator in cellular senescence. We now show that this sequence of events is also characteristic of wild-type p53-expressing human tumor cell lines and human primary cells treated with DNA damaging agents. This shows that the cooperation between Rb2 and p53 is of fundamental importance in the induction of cellular senescence and might have an impact on the outcome of cancer therapy.
This work is supported by Deutsche Krebshilfe.

34. EXPRESSION OF *ATCYCD2;1* IN ARABIDOPSIS INCREASES CELL OUTPUT FROM MERISTEM REGIONS OF THE PLANT AND TESTS THE ROLE OF CELL DIVISION IN PLANT GROWTH.

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Plant growth is indeterminate and it has seemed likely that growth may be driven by cell formation and autonomous expansion. The Arabidopsis thaliana *CYCD2;1* gene introduced in genomic form increased cell formation in the Arabidopsis root apex and leaf by reducing G1 phase duration and reduced size of cells at S phase and division. Plants with a homozygous single insert of genomic *CYCD2;1* grew with normal morphology and without accelerated growth of root or shoot, not providing evidence that cell formation or CYCLIN D2 controls growth of post embryonic vegetative tissues. At the root apex, cells progressed normally from meristem to elongation but their smaller size enclosed less growth and a 40% reduction in final size of epidermal and cortical cells was seen. Smaller elongated cell size inhibited endoreduplication, indicating a

cell size requirement. Leaf cells were also smaller and more numerous during proliferation and epidermal pavement and palisade cells attained 59 % and 69 % of controls while leaves reached normal size. Autonomous control of expansion was therefore not evident in abundant cell types that formed tissues of root or leaf. Cell size was reduced by greater number formed in a tissue prior to cell and tissue expansion. Neither the boundary of the root apical meristem nor termination of elongation correlated with constant number or size of cells contained, therefore signals that act across cell boundaries appear most likely to define the areas of division and the limits of cell expansion, with no significant contribution to growth from cell autonomous development.

35. MOLECULAR ANATOMY OF SCC2, A PROTEIN REQUIRED FOR COHESIN LOADING ONTO CHROMOSOMES

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Scc2/Scc4, a complex conserved from yeast to human, has an essential role in loading the cohesin complex to chromosomes and establishing sister chromatid cohesion. Scc2, the lager subunit, is predicted to consist of HEAT repeats and have potential to interact with multiple targets. To understand the functions of Scc2 at a molecular level, we carried out domain analysis of *S. cerevisiae* Scc2. First, we divided Scc2 into three pieces, expressed each of them in yeast, and examined which of the fragments has the ability to bind to the Scc4 subunit or to the cohesin complex. The results indicated that Scc2 interacts with Scc4 and cohesin through the N- and C-terminal domains, respectively. Next, we sequenced *scc2-4* to identify the temperature-sensitive mutation site. This proved to be a missense mutation within a well-conserved segment in the middle domain. The Scc2-4 mutant protein still retained the ability to interact with Scc4 and cohesin. The mutant protein, however, failed to bind to chromosomes, as shown by ChIP-on chip analysis. Combined with the fact that Scc4 is required for Scc2 binding to chromosomes, our data suggest that the N-terminal and middle regions of Scc2, complexed with Scc4, interact with chromosomes, whereas the C-terminal domain binds to the cohesin complex. We have successfully isolated several new scc2 ts mutants whose mutations locate within the N- or C-terminal domain. By characterizing their phenotypes, we plan to verify the model mentioned above.

36. NDR KINASES AND REGULATION OF CYTOKINESIS IN DICTYOSTELIUM DISCOIDEUM

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During later stages of mitosis cytokinesis is initiated to accomplish cell division. Many cytoskeletal proteins of Dictyostelium discoideum have been described to play a role in cytokinesis. Much less is known about signaling mechanisms triggering cell division. For activating contraction of the actomyosin-ring at the cleavage furrow, it is predicted that signals also emerge from centrosomes. In Schizosaccharomyces pombe and Saccharomyces cerevisiae, the regulating pathways involved in cytokinesis are the septation initiation network (SIN) and the mitotic exit network (MEN), respectively. The social amoeba D. discoideum has an almost complete set of homologous components of these signaling pathways. Septase, a homologue to the SIN-kinase Cdc7p, was identified during a screen for cytokinesis-defective mutants and is crucial for proper cleavage furrow formation. Potential regulators upstream and downstream of septase are a polo-like kinase (Plk1), a GTPase activating protein (Bub2), a GTPase (Spg1), four NDR kinases (NdrA-D) and three Mob1 proteins (MobA-C), which were identified by database searches. The D. discoideum polo-like kinase Plk1 localizes to centrosomes and spindles during mitosis. Bub2, Spg1 as well as MobB and MobC permanently localize to centrosomes. By tandem affinity purification NdrB and MobB were found to interact, but the subcellular distribution of NdrB and the other NDR kinases has not been investigated so far.
To further characterize this network of signaling proteins, immunoprecipitation and tandem affinity assays are used. For identifying specific interactions and functions, full length and truncated constructs of septase and NDR kinases are purified and tested in in-vitro assays. Mutants of the NdrA kinase were generated and are currently characterized. Furthermore, GFP-tagged constructs are tested for their localization in live cells. A putative upstream effector of NdrA and NdrB is the Ste20-like severin kinase SvkA, which is tested whether it binds to the conserved phosphorylation motifs present in the NDR kinases.

37. CELL CYCLE START AFTER FERTILIZATION

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Molecular machineries for DNA replication and cell cycle control have been remarkably clarified in recent years. Nonetheless, it remains a major unsolved subject in biology how fertilization initiates S-phase and the following embryonic cell cycle in animal eggs. Model organisms to analyze this issue are mouse, frog and echinoderm starfish. While unfertilized mature eggs of most vertebrates arrest at metaphase of meiosis II (meta-II), those of starfish arrest at G1-phase after completion of meiosis II. Meta-II arrest in frog eggs is performed by an APC/C inhibitor, Erp1/Emi2 that is stabilized and activated through phosphorylation by Mos-MAPK-p90Rsk. Here we show that the same Mos-MAPK-p90Rsk pathway is necessary and sufficient for G1-phase arrest in unfertilized starfish eggs. Our data indicate that to prevent initiation of the embryonic cell cycle, two branches are likely to split away downstream of the Mos-MAPK-p90Rsk pathway: one branch negatively regulates initiation of S-phase through prevention of Cdc45 loading onto chromatins, and the other branch negatively regulates

initiation of M-phase through prevention of accumulation of cyclins A and B. Shutdown of the Mos-MAPK-p90Rsk pathway by fertilization cancels these negative regulations, resulting not only in the Cdc45 loading to initiate S-phase but also in the activation of Cdc2 associated with M-cyclins for entry into M-phase. Such a starfish system makes a great contrast to frog system in which shutdown of the Mos-MAPK-p90Rsk pathway is not sufficient for initiation of the embryonic cell cycle.

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38. DETECTING THE ROLE OF THE MITOTIC SPINDLE CHECKPOINT PROTEIN BUB1 DURING MITOSIS AND CARCINOGENESIS

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Bub1, a conserved protein kinase acting at kinetochores, performs two crucial functions during mitosis. It is essential for the spindle checkpoint and is required for correct kinetochore-microtubule attachment. Bub1 depletion inactivates the spindle checkpoint and causes chromosome alignment defects leading to chromosome missegregation, aneuploidy and genetic instability, a hallmark of cancer cells. A correlation between Bub1 mutations and carcinogenesis has already been described underlining the importance of a functioning Bub1 in normal cells. An important question is how Bub1 contributes at the molecular level to its different functions and whether an impairment of the functions is sufficient to cause a malignant phenotype. To address these questions, we screened Bub1 mutants for separation of function and carcinogenicity in human cells by RNAi complementation. These different Bub1 mutants lack conserved domains or carry point-mutations found in human cancers. We are now testing whether the different mutations lead to an impairment in the spindle checkpoint control and/or kinetochore-microtubule attachment defects in HeLa cells. Our preliminary data indicate that the kinase activity of Bub1 is essential for a fully functioning checkpoint. Currently, we are applying the same procedure in untransformed RPE-hTERT cells to investigate the effect of several mutants regarding the development of a malignant phenotype. In a second step, we aim to use those tools to better understand at a molecular level how the spindle checkpoint failure might lead to cancer development.

39. A MECHANISM FOR THE ACTIVATION OF DNA REPLICATION CHECKPOINT THROUGH *S.CEREVISIAE* MRC1.

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DNA replication checkpoint cascade plays a crucial role for the maintenance of genome integrity. However, the molecular entity of this cascade is not well understood. We have previously shown that budding yeast Δmrc1 or Δtof1 fails to arrest DNA replication fork stably when cells are treated with HU (hydroxyurea). This arrest is independent of Mec1 (ATM in human) and Rad53 (Chk2 in human), sensor and effector kinases for DNA replication checkpoint, respectively. To understand functional domains required for fork arrest and checkpoint activation in Mrc1 protein (1096 a.a.), I started to search for the protein(s) which interact with Mrc1 directly. For this purpose, we overexpressed in yeast various proteins in the replication machinery, and found that two subunits in MCM helicase (Mcm6, Mcm7) and DNA Pol alpha-interacting factor Mcm10 were able to bind to Mrc1. Among these interactions, interaction between Mcm6 (1017 a.a.) and Mrc1 was the strongest and specific. For the interaction with Mcm6, the middle region of Mrc1, containing the coiled-coils was essential. For Mcm6, C-terminal (168 a.a.) was essential for Mrc1 binding. Introduction of 2 amino acids alanine substitution mutations at Mcm6 C-terminal region abolished interaction with Mrc1 in vivo. In this mutant MCM complex was stably formed and cells grew normally under normal condition. Replicative stress induced by HU could trigger DNA replication checkpoint normally in this cells, however when cells were treated with MMS, replication checkpoint was not activated at all. Function of Mcm6 protein will be discussed as a candidate of sensor for replicative stress.

40. ARRESTING BREAST CANCER CELLS IN G1/S BY TREATMENT WITH THE PROGESTERONE RECEPTOR ANTAGONIST ZK230211

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Discovery of nuclear steroid receptors, and their function in normal and malignant breast has been successfully translated into the first targeted drugs in oncology. Estrogen receptor antagonists besides aromatase inhibitors (inhibitors of estrogen synthesis) currently are the best available options for the treatment of breast cancer. But luminal-epithelial breast cancer cells are not necessarily exclusively responsive to estrogen receptor antagonists, as the progesterone receptor is highly expressed in this compartment as well and may be an additional target for therapeutic intervention.

We have developed a highly potent progesterone receptor antagonist (ZK230211) that has a considerable potential not only for therapeutic intervention but also for prevention of breast cancer. New results from translational research

efforts elucidating the progesterone receptor antagonist mode of action will be reported.
The outcome of ZK230211 treatment of breast cancer cells has been investigated *in vitro* and *in vivo*. In brief, the growth of T47D breast cancer cells is dependent on estradiol, which activates estrogen receptor related pathways. The growth stimulatory effect is almost completely abolished after treatment with subnanomolar concentrations of ZK230211, which act on the endogenous PR. FACS based cell cycle analyses under various conditions disclose a G1 arrest of treated T47D cells. To gain a better insight into the mechanisms leading to G1 arrest, we also investigated the expression and phosphorylation status of diverse cell cycle regulatory proteins (Cyclins, Retinoblastoma protein, etc.) and proteins relevant for PR signalling (PR, MAPK Erk, etc.). Based on our data we conclude with a putative mechanism of cell cycle inhibition *in vitro*, which may also confer to *in vivo* data.

41. AN RNAI SYNTHETIC INTERACTION SCREEN TO DISSECT THE TP53 PATHWAY

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RNAi screens have recently contributed major advances to the field of system biology and genomics. We have used a genome-wide enzymatically prepared siRNA (esiRNA) library in search for genetic interactions with components of the TP53 pathway. We used 2 isogenic HCT116 cell lines that differ only in their TP53 status and compared their viability in the context of depleted gene function by RNAi.
Using an automated microscopy system we monitored and compared the proliferative advantage or disadvantage of knockdowns in the specific cell types. This comparison allowed us to eliminate the 'non-specific' and 'general' phenotypes, affecting both cell types. Interestingly, we have discovered genes, which depletion gave rise to a viability phenotype only in one of the TP53 positive, or TP53 negative cells.
The majority of the found hits lead to general cellular stress and thus to TP53 pathway activation, which specifically eliminates TP53 wild type cells. However there are a handful of hits, which knockdown specifically ceases TP53 knockout mutant's proliferation. These potentially therapeutically interesting hits are currently under further investigation.

42. ROLE OF EB1 IN SPINDLE DYNAMICS AND DISASSEMBLY IN XENOPUS EGG EXTRACT

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EB1 is a microtubule stabilizer in Xenopus egg extract. In this study, we have examined its role in assembly and disassembly of the meiotic spindle. As expected for spindles assembled in absence of microtubule stabilizer, ΔEB1 spindles are 40% shorter then the control spindles. In addition, we also observed 30-50% more multipolar spindles in ΔEB1 extract and strong reduction of microtubule density in the spindle mid-zone. Spindle mid-zone is also where Eg5, a motor that slides apart antiparallel microtubules and thereby acts as a driver of poleward microtubule flux, is localized. Poleward microtubule flux as well as dynamic instability of microtubules are two major contributors to spindle dynamics. By fluorescence speckle microscopy we measured 60-75% slower poleward flux speed in ΔEB1 depleted spindles compared to control spindles, indicating that density of spindle mid-zone microtubules is important for proper poleward flux speed. Furthermore, this suggests for the first time that microtubule polymerization could provide a pushing force for poleward microtubule flux. Since poleward microtubule flux is considered to be the major driving force of chromosome segregation in Xenopus egg extract, we followed behavior of ΔEB1 spindles during metaphase to interphase transition that was triggered by Ca2+ addition. Interestingly, ΔEB1 spindles that exhibit slower poleward microtubule flux neither segregate their chromosomes nor reorganize their microtubules into interphasic network although the extract biochemically enters interphase (APC/C was activated and cdk1 activity dropped). Additionally, in ΔEB1 extract spindles persisted long after Ca2+ addition. These spindles have shrunk to 50% of their metaphase length and have only few bundled microtubules. We are currently trying to understand the nature of these bundled microtubules and molecular mechanism involved in their stabilization.
Taken together, our results demonstrate that EB1 as a microtubule stabilizer plays an essential role in the assembly of functional spindle in Xenopus egg extract. In particular, it is required for maintenance of proper poleward microtubule flux that is necessary for spindle disassembly and chromosome segregation.

43. MECHANISM OF LATE G1 PHOSPHOINOSITIDE 3-KINASE ACTIVITY MEDIATED G1-> S-PHASE TRANSITION

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Phosphoinositide 3-kinase (PI3K) is one of the early signalling molecules induced by growth factor receptor (GFR) stimulation and is necessary for cell growth and cell cycle entry. PI3K activation occurs at two distinct time points during G1 phase. The first peak is observed immediately following GFR ligation, and the second in late-G1, 3 to 4 h before S phase entry. This second activity peak is essential for transition from G1 to S phase; nonetheless, the mechanism by which this peak is induced and regulates S phase entry is poorly understood. Here, we show that activation of Ras and Tyr kinases are required for late-G1 PI3K activation. To look for the mechanism by which PI3K

regulates G1-S transition, we inhibited late-G1 PI3K activity by using LY294002 (PI3K specific inhibitor) and checked for levels of different protein which regulate S-phase entry and initiation of DNA replication. We found that inhibiting PI3K activity in late G1 results in low c-Myc and cyclin A protein levels, impaired Cdk2 activity, and reduced MCM2 (minichromosome maintenance) loading onto chromatin. Considering our observations and earlier reports mentioning similar defects on inhibiting c-Myc, we postulated that the primary consequence of inhibiting late-G1 PI3K can be to regulate c-Myc protein levels. We used a conditional c-Myc construct and activated it in advanced G1 to test this hypothesis. In an alternate approach to examine this hypothesis we used a more stable form of c-myc i.e. c-MYC^{T58A} and studied the effect of PI3K inhibition in late G1 on cell cycle progression in c-Myc ^{T58A} expressing cells . We will present the results of these studies.

44. DUAL ROLES FOR WIP1 IN CHECKPOINT RECOVERY

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A cell containing damaged DNA arrests its cell cycle to prevent transmission of mutations and to allow time for repair. In G2 phase, this arrest is achieved by checkpoint signaling cascades which inactivate components of the signaling network that initiates mitotic entry. However, little is known on how the inactivation of the checkpoint signaling and the entry into mitosis are performed after repair of the DNA-damage. In an RNAi-based screen for proteins necessary for DNA-damage checkpoint recovery we identified the p53-induced phosphatase Wip1. Wip1 has previously been reported to inactivate multiple targets in the checkpoint signaling cascades. We found that when Wip1 levels are reduced, p53 mediated repression of transcription is enhanced, leading to a failure to enter mitosis because of low levels of mitotic inducers. When both Wip1 and p53 levels are reduced, cells are delayed in recovering from DNA-damage because of reduced inactivation of other checkpoint components.
We propose that Wip1 has dual roles in checkpoint recovery: a long term effect enabling the accumulation of mitotic inducers during the checkpoint, and a short term effect, allowing the activation of mitotic inducers by inactivating parts of the checkpoint signaling.

45. PHYSIOLOGICAL FUNCTIONS OF MAMMALIAN CDC20 AND CDH1/FZR1 IN VIVO

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Mitotic progression crucially depends on the inactivation of specific cell cycle regulators by protein degradation. Some of these events are mediated by the Anaphase-Promoting Complex (APC) in association with two adaptor proteins, Cdc20 and Cdh1. These two subunits target specific proteins for ubiquitin-mediated degradation although their specificity for particular substrates is not clear. In addition, these two proteins have distinct patterns of expression in specific mammalian tissues and their expression is differentially altered in tumor cells. Thus, it is not clear how APC modulates cell cycle progression in specialized cell divisions. We have recently generated gene-targeted mice with conditional knock out alleles for either *Cdc20* or *Cdh1* (also known as *Fzr1* in mammals). Both proteins are required for mouse embryonic development although their genetic ablation causes specific phenotypes at different developmental stages. In particular, Cdh1 ablation results in placental defects accompanied by defective endoreduplication of trophoblast cells. In addition, the absence of Cdh1 provokes specific alterations in the nervous system of mid-stage embryos, in agreement with the high levels of expression of this protein in post-mitotic neurons. Cdc20, on the other hand, is required for cell divisions during early embryonic development. The phenotype of mice heterozygous for these *Cdc20* or *Cdh1-null* alleles and their susceptibility to tumor development will be presented. In addition, we will discuss on the effect of eliminating Cdc20 or Cdh1 in vivo using acute expression of Cre recombinase under inducible regulation or in specific genetic backgrounds.

46. CELL CYCLE-DEPENDENT REGULATION OF THE FORKHEAD TRANSCRIPTION FACTOR FOXK2

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Processes such as proliferation, differentiation and survival have progressively been linked to the highly conserved forkhead box (FOX) transcription factor family.
To date little is known about the forkhead transcription factors of the K class. FOXK1 has been implicated in the regulation of myogenic progenitor cells and it can interact with the Serum Response Factor (SRF) to regulate SRF-dependent transcription (our unpublished data). We are studying the regulation and function of FOXK2 as a potential relative to the yeast protein Fkh2p. Fkh2p controls cell cycle-dependent gene expression of a whole gene cluster in G2/M-phase. Strikingly FOXK proteins are the only mammalian forkhead factors within a family of over 40 members that share close homology to Fkh2p, yet their functional implication in cell cycle regulation remains to be analyzed.
Our data show that FOXK2 protein levels vary in a cell cycle-dependent manner. More importantly FOXK2 is phosphorylated in specific phases of the cell cycle prior to its degradation. We demonstrate that FOXK2 is a substrate for the cyclinA/cyclin-dependent kinase 2 complex (ClnA/CDK2). Furthermore FOXK2 seems to control the transcription of the cell cycle regulator $p21^{CIP}$ in reporter assays, suggesting that FOXK2 indeed plays a role in mammalian cell cycle control. We are currently investigating the mechanism of FOXK2 phosphorylation and its impact on FOXK2 activity. Additional studies are probing the possible regulation of FOXK2 by extracellular signals.

47. SHUGOSHIN PREVENTS SISTER CHROMATID SEGREGATION

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During DNA replication, sister chromatids become physically linked along their length by protein complexes called cohesins. In meiosis, the conserved kinetochore protein Shugoshin (Sgo1) is required to maintain this sister chromatid cohesion at centromeres beyond meiosis I, thereby preventing sister chromatid segregation until meiosis II. Sgo1 is associated with kinetochores until the onset of anaphase II, consist with a model whereby Sgo1 protects the centromeric cohesin complex from cleavage at meiosis I. We have tested this idea by asking if *SGO1* overexpression can inhibit cohesin loss during mitosis. Our findings indicate that Sgo1 can inhibit sister chromatid segregation by preventing the removal of cohesins from chromosomes. These results have also provided us with a platform to screen for proteins involved in the Sgo1 pathway.

48. CDK1 PHOSPHORYLATION OF THE CDC42 GTPASE MODULE IS REQUIRED FOR NORMAL CELL GROWTH

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The cell cycle must be integrated with ongoing cell growth to ensure that growth and division are orchestrated correctly. The mechanisms that ensure this integrity are largely unknown. Recent work from our lab showed that cyclin dependent kinase 1 (Cdk1) coordinates cell surface growth with the cell cycle. When Cdk1 activity was inhibited, polarised secretion was rapidly interrupted and cell surface growth stalled. G1 cyclin-Cdk1 complexes specifically phosphorylated multiple components of the Cdc42 GTPase module, including the guanine nucleotide exchange factor (GEF), GTPase activating protein (GAP) and two associated adaptors. Cdk1 target sites for the entire complex have now been mapped, with the aim of understanding the mechanisms by which Cdk1 activity controls membrane delivery. Phosphorylation of the Cdc42 module occurs as cells initiate polar growth and is required for normal growth. We have analysed membrane trafficking events including the movement of endocytic and exocytic vesicles in Cdk1 phosphorylation-site mutants. We propose that signals relayed from Cdk1 via the Cdc42 module ensure a balance of endocytosis and exocytosis that maintains polarised growth during the cell cycle.

49. INSIGHTS INTO THE MOLECULAR ACTIONS OF THE HOST CELL FACTOR 1 (HCF-1) PROTEIN DURING M PHASE

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The human HCF-1 protein is involved in transcriptional regulation through interaction with site-specific transcription factors and histone modifiers. It is also implicated in cell-cycle progression, especially of the G1 and M phases. It is synthesized as a precursor protein of 300 kD, which is proteolytically processed into amino (HCF-1_N) and carboxy (HCF-1_C) terminal subunits that remain non-covalently associated. This heterodimeric complex is associated to the chromatin throughout the cell cycle except mitosis. The role of HCF-1 during M phase has been previously associated with the HCF-1_C subunit as its presence rescues the formation of binucleated cells and chromosome segregation defects observed in the absence of the endogenous HCF-1. Using live-cell microscopy, we observed a delay in anaphase onset upon depletion of HCF-1, due to a slower chromosomal alignment observed with H2B-GFP fusion protein. Whereas the presence of HCF-1_N partially rescues this delay and leads to many M-phase defects, the presence of HCF-1_C enhances the anaphase onset delay and rescues M-phase defects. These results suggest that the absence of HCF-1 impairs chromosome alignment and HCF-1_C promotes the activation of the mitotic checkpoint. This activation would give more time for the chromosomes to align and therefore reduce the number of abnormal cells. To understand the molecular mechanisms behind these phenotypes, we analysed HCF-1_C-interacting proteins and identified a number of mitotic proteins and other proteins involved in cell-cycle progression. Using microarrays, we identified genes whose expression is disregulated following depletion of the HCF-1. We observed that the presence of HCF-1_N or HCF-1_C rescued the expression of 79% and 57% of the disregulated genes, respectively. We believe that the disregulated genes and HCF-1_C-associated proteins might reveal the role of HCF-1_C in M phase.

50. SELF-ASSEMBLY OF CENTRALSPINDLIN IS CRITICAL FOR CYTOKINESIS

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Microtubule-bundle structures, such as the central spindle and midbody, play critical roles in cytokinesis, from positioning of the cleavage furrow to separation of daughter cells. Centralspindlin is a protein complex of a mitotic kinesin (ZEN-4 in *C. elegans*, Pavarotti in *Drosophila* and MKLP1 in mammals) and a Rho-family GAP (CYK-4 in *C. elegans*, RacGAP50C/tumbleweed in *Drosophila* and MgcRacGAP in mammals) that is essential for the formation of these microtubule-based structures. We have previously reported that centralspindlin oligomerizes under

physiological conditions. However, the biological significance of this was unclear. Here, we show that the kinesin subunit ZEN-4 contains a small region at the C-terminal end of its predicted coiled coil that is essential for

self-assembly of centralspindlin. Mutant versions of ZEN-4, deficient specifically in self-assembly by deletion of this region, were defective in microtubule-organization in vitro. In *C. elegans*, self-assembly-deficient *zen-4* transgene rescued *zen-4* null animals much less efficiently than wild type *zen-4* transgene. Moreover, self-assembly defective ZEN-4 failed to accumulate to the midbody, leading to abortive cytokinesis. These data indicate that self-assembly of centralspindlin is critical for its functions in both formation of the midbody and completion of cytokinesis.

51. NON-CDK-BOUND P27 IS A MARKER FOR TGF-BETA INDUCED GROWTH ARREST AND CELLULAR STRESS

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Transforming growth factor-beta reversibly arrests epithelial and endothelial cells in the G1 phase of the cell cycle. The arrest involves TGF-beta-induced expression of p15, which by binding to Cdk4/6-cyclin D-complexes facilitates the subsequent binding of p27 to Cdk2-cyclin E thus blocking the activities of both G1 and G1/S-phase complexes.

We show that TGF-beta growth arrest also involves a rapid accumulation of p27 in a form unbound to Cdk-cyclin complexes (p27-NCDK for non-cdk-bound), whereas TGF-beta has no effect on the total levels of p27 in the TGF-beta-responsive mink lung epithelial (Mv1Lu) -cells. p27-NCDK is detected by a specific monoclonal antibody recognising a native form of p27 in the nucleus. We have mapped the epitope of the antibody to the cdk-binding region of p27.

In contrast, hepatocyte growth factor can release TGF-beta arrested cells back into the cycle and revert the effect of TGF-beta on p27-NCDK. Using chemical inhibitors we identified the PI3K-pathway as a major regulator of p27-NCDK. Furthermore, we find that several types of cellular stress (metabolic, osmotic, temperature) also induce p27-NCDK. We are currently investigating signalling pathways involved in p27-NCDK regulation. These findings indicate that p27-NCDK is a marker for both cell cycle arrest and cellular stress.

52. INCOMPLETE CYTOKINESIS IN THE LIVER: A NEW DEVELOPMENTAL PROGRAM CONTROLLED BY INSULIN

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Generally correlated with tumorigenesis, tetraploidy status is nevertheless a physiological feature of liver development. Whereas hepatocytes of newborn are exclusively diploid, during post-natal liver growth, the liver parenchyma undergoes progressive polyploïdization, with notably the successive appearance of binuclear 2x2n and mononuclear 4n hepatocytes. In a previous study using time-lapse videomicroscopy, our group has clearly demonstrated that appearance of tetraploid progenies (binucleated 2x2n) was due to a modified cell-cycle, characterized by a process of incomplete cytokinesis. We have now deciphered the molecular mechanism regulating this event. Indeed, we show *in vivo* and *in vitro* that this physiological process is characterized by an absence of cell elongation from anaphase to telophase, leading to a lack of cytoskeleton rearrangement to the cleavage plane, normally controlled by the ROCK/myosinII pathway. Moreover, microtubules networks and particularly astral microtubules are totally disorganized and are not able to contact the equatorial cortex. Consequently, molecular signals normally delivered to the future site of cleavage furrow are impaired, preventing activation of RhoA signalling, and thus formation of a contractile cytokinesis ring. Furthermore, we also demonstrated that weaning triggers this process. Indeed, if animals are weaned at 15 days, we clearly observed that the incomplete cytokinesis process is largely initiated at 19 days in contrast with animals that stay with the mother with no access to her nutriment. Weaning period is clearly associated with physiological changes, such as modification of nutritional diet, hormonal variations and circadian clock appearance. We are now trying to define which cell signalling pathways trigger incomplete cytokinesis. We are currently assessing the effect of modifying nutrient supply (high/low fat diet) or the insulin level in the induction of binucleation process. Our preliminary results indicate that insulin is a major player since when insulin signalling is impaired, binucleation process is not initiated.

53. EXPLORING DEVELOPMENTAL AND CELL CYCLE RELATED ROLES OF THE *C. ELEGANS CLK-2* DNA DAMAGE CHECKPOINT GENE

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clk-2 is conserved from yeasts to mammals and is essential for viability. We previously, in studies recently confirmed in yeast and humans, implicated *clk-2* in multiple DNA damage checkpoint response pathways. Using *C. elegans* we are dissecting the multiple essential functions of this conserved gene during embryogenesis and germ line progression. *clk-2* deletion worms can progress through embryogenesis and larval development due to maternal rescue but become sterile as germ cell become arrested in the cell cycle. While replication still occurs and centrosomes duplicate and separate normally, mitotic spindles cannot be detected and germ cell nuclei exhibit high levels of phosphorylated histone H3. Two *clk-2*

temperature sensitive mutants when grown at restrictive temperature exhibit prolonged early embryonic cell cycles. This phenotype is largely suppressed by atr-1/chk-1 checkpoint mutants, suggesting a role of *clk-2* in embryonic S-phase progression. Interestingly, the prolonged embryonic cell cycles are also suppressed by depleting the primordial *C. elegans* p53 like gene *cep-1*. However, *cep-1* does not suppress delayed cell cycle timing occurring in other S-phase mutants. In addition to its potential role in S-phase progression *clk-2* appears to be required for mitotic spindle formation as *clk-2* mutants at 25.5°C exhibit microtubule nucleation and spindle pole body defects. Furthermore, *clk-2* mutants genetically interact with genes involved in chromosome segregation and spindle assembly. We are currently investigating if the spindle defects seen in *clk-2* mutants are due to centrosome maturation or spindle assembly defects.

54. CELL CYCLE INHIBITOR P16INK4 CONTROLS NF-κB BY INHIBITION OF NF-κBP65

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The nuclear factor κB (NF-κB) regulates the expression of multiple genes including genes essential for cell cycle control. NF-κB is found in most cells in a dormant state in the cytoplasm bound to the inhibitory family IκB (e.g. IκBα) via an ankyrin repeat domain. In response to various exogenous stimuli IκBα is phosphorylated on Ser32/Ser36 residues by IκB kinase β (IKKβ), which is part of an IKK complex containing two catalytic subunits (IKKα and IKKβ) and the regulatory subunit IKKγ. IKKβ-phosphorylated IκBα is rapidly degraded *via* the ubiquitin proteolysis pathway (UPP). Consequently, functional NF-κB molecules are free to enter the nucleus. Ubiquitinylation of IκBα is promoted by the cullin-RING ubiquitin ligase (CRL) Skp1/Cul1/F-box protein $SCF^{\beta TrCP}$ containing the F-box protein β-TrCP as substrate adapter, which specifically recognises and binds to Ser32/Ser36-phosphorylated IκBα. The active dimeric NF-κB complex translocates into the nucleus and transactivates target promoters. Here we report that a family of proteins containing ankyrin repeats, the inhibitors of Cdk4 (INK4) are able to bind NF-κBp65 and inhibit its nuclear transport and transactivational ability. Association of p16INK4 with NF-κBp65 takes place, if the NF-κB inhibitor IκBα becomes degraded in response to TNFα stimulation. Overexpression of INK4 molecules suppresses the transactivational ability of NF-κB significantly. In contrast to INK4 proteins, overexpression of the cell cycle inhibitor p27 enhances NF-κB transactivation activity. Thus, the effect of INK4 proteins on NF-κB function possibly modifies NF-κB mediated transcriptional activation of cell cycle associated factors.

55. REGULATION AND FUNCTION OF RINGO/SPEEDY PROTEINS

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Cell cycle progression is driven by the periodical activation of cyclin-dependent kinases (CDKs), which is brought about by temporal expression of activating subunits named cyclins. Recent reports indicate that some CDKs can be also activated by non-cyclin proteins named RINGO or Speedy. These proteins were originally identified as regulators of the meiotic cell cycle in *Xenopus* oocytes. Subsequently, several RINGO/Speedy proteins have also been found in mammals. RINGO/Speedy proteins lack sequence similarity to cyclins but can directly activate both CDK1 and CDK2. All RINGO/Speedy family members share a conversed core sequence that is critical for CDK binding and activation. We have recently shown that the expression of *Xenopus* RINGO/Speedy is tightly regulated during the meiotic cell cycle by two proteasome-mediated proteolytic mechanisms, which involve the ubiquitin ligases SCF-βTrCP and Siah-2, respectively. We are now characterizing the regulation and function of the mammalian RINGO/Speedy proteins. We have found that RINGO/Speedy A is periodically expressed during the cell cycle and accumulates in G1 phase. Rapid changes in the expression level of this protein are facilitated by its very short half-life and are mediated by the ubiquitin-proteasome system. Our results indicate that the stability of the RINGO/Speedy A protein is probably controlled by different mechanisms. Expression of a RINGO/Speedy A form with increased stability induces abnormal chromatin condensation and interferes with cytokinesis, suggesting that proper control of RINGO/Speedy A protein levels is important for normal cell cycle progression of mammalian cells. We will also discuss results addressing the substrate specificity of RINGO/Speedy-activated CDK1 and CDK2 versus the equivalent CDK-cyclin complexes.

56. CELL SIZE REGULATION AND ER RELEASE BY THE J-CHAPERONE YDJ1 IN LATE G1

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The G1 cyclin Cln3 coordinates cell growth and proliferation to set cell size in budding yeast. It is generally assumed that Cln3, which is present throughout G1, accumulates passively in the nucleus until a threshold is reached to trigger cell cycle entry. We found that Cln3 is retained bound to the ER in early G1 cells, and it is partly released in late G1 allowing its nuclear accumulation. ER retention and stable binding to cyclin-dependent kinase Cdc28, both require a chaperone-regulatory J_i domain present in Cln3, whereas the J-chaperone Ydj1 is essential for release and nuclear accumulation of Cln3 in late G1. Finally, Ydj1 is limiting both *in vivo* and *in vitro* for ER release of Cln3. As protein synthesis and ribosome

assembly rates compromise chaperone availability, we propose that Ydj1 may transmit growth capacity information to the cell cycle for setting efficient mass/ploidy ratios.

57. CONTROL OF ANAPHASE PROMOTING COMPLEX ACTIVITY BY THE SPINDLE ASSEMBLY CHECKPOINT

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The anaphase promoting complex (APC/C) is a large multi-subunit ubiquitin ligase responsible for targeting specific substrates for degradation. Two key substrates during mitosis are Securin and Cyclin B1. Degradation of Securin allows sister chromatid separation by activating the protease Separase while Cyclin B1 destruction allows mitotic exit. APC/C activity is tightly controlled during mitosis by the spindle assembly checkpoint (SAC). The SAC ensures that the APC/C only becomes active once all kinetochores have attached to the mitotic spindle thus ensuring equal segregation of sister chromatids. The target of the SAC is Cdc20, the mitotic co-activator of APC/C. The SAC components Mad2, Bub3 and BubR1 have been reported to inhibit Cdc20 activity by directly binding to the co-activator.

We are focusing on investigating how APC/C activity is controlled during mitosis by employing biochemical approaches. We have developed an affinity approach for purifying the APC/C from mitotic HeLa extracts, based on a monoclonal antibody directed against the APC4 subunit. Purification of the APC/C from cells that have an active SAC and cells with an inactive SAC reveals that the latter APC/C is much more active in *in vitro* ubiquitination assays of Securin. This indicates that the purified APC/C reflects the *in vivo* situation and that potential regulatory factors are present in the preparations. We are currently performing mass spectrometry of these samples to identify factors associated with the APC/C in the two mitotic states. In addition to this we performed a candidate approach, which revealed that Cdc20 was more abundantly associated with the APC/C when the SAC was inactive.

58. SHWACHMAN-DIAMOND SYNDROME AND LEUKEMIA; A ROLE FOR SBDS IN THE CELL DIVISION PROCESS?

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Shwachman-Diamond Syndrome (SDS) is characterized by several hematological defects, including neutropenia and defective neutrophil migration. Additionally, SDS patient have a highly increased risk of developing myelodysplasia and/or acute myeloid leukaemia (MDS/AML) of 20-30% at the age of 20-30 years old. Recent identification of SDS-associated mutations in the *SBDS* gene at chromosome 7 has provided the molecular basis for further investigations to the underlying molecular mechanisms defects. To date, little is known about the cellular function that SBDS plays. To gain more insight into the cellular and molecular function of SBDS, we have characterized the SBDS expression profile and subcellular localization in several differentiating (hematopoietic) cell types. In neutrophil differentiation studies with PLB-985 and CD34+ cord blood cells we observed that SBDS expression is downregulated during the differentiation process. Immunofluorescence studies revealed that SBDS is predominantly localized in the nucleus and to a lower extent in the cytoplasm. Interestingly, we observed in many cell types that SBDS co-localizes with the microtubule organizing centre (MTOC) and in dividing cells SBDS is localized at the mitotic spindle. To further investigate the role that SBDS might play in cell division-related processes, we compared the proliferative potential of SBDS-deficient SDS patient T-cells to control cells. Preliminary data show that SDS patient T-cells proliferate less than control cells.
Altogether, our data show that SBDS expression is downregulated upon neutrophil differentiation. The observed co-localization of SBDS with the MTOC and the mitotic spindle together with the decreased T-cell proliferation of SDS patient cells strongly suggests that SBDS plays a role in the cell division and/or chromosome segregation process. Defects in either of these processes could potentially contribute to neutropenia and/or leukemia. Our future studies will be directed to gain more insight into the role that SBDS plays in cell cycle-related processes in normal and leukemic cells.

59. ALIEN INHIBITS E2F1 GENE EXPRESSION AND CELL PROLIFERATION

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Alien was previously characterized as a corepressor for specific members of the nuclear hormone receptor superfamily including the thyroid hormone receptor (TR), vitamin D3 receptor (VDR), the androgen receptor (AR) and DAX1. Using a proteomic approach we have identified Alien as a novel interacting factor of the cell cycle regulator E2F1. The transcription factor E2F1 plays a crucial role in the regulation of cell-cycle progression at the G1-S transition. Furthermore, E2F1 is associated with DNA

repair and apoptosis. In line with that, deregulation of the transcription factor E2F1 is a common event in most human cancers. The interaction between Alien and E2F1 was confirmed by *in vitro* binding assays suggesting a direct binding of E2F1 and Alien. However it was unclear whether this interaction influences cell proliferation and endogenous E2F1 target gene expression. Here, we show by chromatin immuno-precipitation (ChIP) that Alien is recruited *in vivo* to the E2F binding sites present in the E2F1 gene promoter, inhibits the transactivation of E2F1 and represses endogenous E2F1 gene expression. Interestingly, using synchronized cells to assess the expression of Alien profile during cell cycle the levels of endogenous Alien is increased during G1, G1/S and G2 phase. Furthermore, stable transfection of Alien leads to reduction of cell proliferation. Thus, the data suggest that Alien acts as a co-repressor for E2F1 and is involved in cell cycle regulation.

60. BUB1 IS ESSENTIAL AND MAINTAINS CENTROMERIC COHESION BY ACTIVATION OF THE SPINDLE CHECKPOINT

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Bub1 is a component of the spindle assembly checkpoint (SAC), a surveillance mechanism which ensures genome stability by delaying anaphase until all the chromosomes are stably attached to spindle microtubules via their kinetochores. To define Bub1's role in chromosome segregation, embryogenesis and tissue homeostasis, we generated a mouse strain in which BUB1 can be inactivated by administration of tamoxifen, thereby bypassing the pre-implantation lethality associated with the Bub1-null phenotype. Tamoxifen-induced ablation of Bub1 shows that it is also essential following gastrulation and the initiation of organogenesis. Bub1 is also required for proliferation of mouse embryo fibroblasts (MEFs). In the absence of Bub1, MEFs undergo one highly aberrant mitosis but then do not divide again. In adults, tamoxifen-induced inactivation of Bub1 occurs very efficiently in testes, resulting in infertility. Consistent with an anti-proliferative effect, mitotic cells and mature spermatids were less apparent in Bub1-deficient seminiferous tubules. Thus, in the four different scenarios we have examined, Bub1 is essential for cellular proliferation. By analysing cultured MEFs, we also examined the effect of Bub1 inactivation on chromosome segregation and SAC function. These issues have previously been addressed by several RNAi-based studies in HeLa cells, yielding conflicting reports. We confirm our earlier observation that Bub1 is required to target BubR1 to kinetochores. In addition, we show that Bub1-deficient fibroblasts fail to align their chromosomes or sustain SAC function. We also observed premature centromere separation in nocodazole-arrested Bub1-null cells. Because these MEFs require Bub1 to prevent anaphase in the presence of nocodazole, we reasoned that the loss of cohesion could be due to SAC-override, rather than a direct effect on Sgo1-dependent protection of centromeric cohesion. Indeed, inhibition of the proteasome rescues the apparent cohesion defect, indicating that Bub1 maintains sister chromatid cohesion by activation of the spindle checkpoint.

61. THE E3 UBIQUITIN LIGASE SCF^{SKP2} REGULATES DIFFERENTIATION INDEPENDENT FROM THE CELL CYCLE.

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The SCF ^{Skp2} complex is an E3 ubiquitin ligase that is known to target a number of cell cycle regulators, including cyclin-dependent kinase inhibitors, for proteolysis. Skp2, the F box recognition subunit of the complex, is elevated in a variety of human tumours, where it is thought to dysregulate cell proliferation.

Using Xenopus as a model system, here we demonstrate that Skp2 has an additional role in regulation of differentiation of primary neurons, the first neurons to differentiate out of the neural plate. Xenopus Skp2 (XSkp2) shows a dynamic expression pattern in neural tissue of the early embryo and depletion of XSkp2 results in generation of extra primary neurons. In contrast, overexpression of XSkp2 inhibits neurogenesis in a manner dependent on its ability to act as part of the SCF ^{Skp2} complex. Moreover, inhibition of neurogenesis by XSkp2 occurs upstream of the proneural gene NeuroD and prior to cell cycle exit. We have previously demonstrated that the Xenopus cyclin dependent kinase inhibitor Xic1 is essential for primary neurogenesis at an early stage, and before these cells exit the cell cycle. We show that SCF ^{Skp2} degrades Xic1 in embryos and this at least partially explains the ability of Skp2 to regulate neurogenesis. Thus, we postulate that the SCF ^{Skp2} complex may have functions in the control of cellular differentiation additional to its role in cell cycle regulation that may contribute to the oncogenic potential of Skp2.

62. CONTROL OF DNA REPLICATION BY MITOCHONDRIAL METABOLISM

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We followed cell cycle progression of *Saccharomyces cerevisiae* cells in continuous culture systems, where the growth rate can be altered separately from nutrient-dependent variables. Although it is commonly assumed that metabolism plays a "permissive", not an "instructive", role in the timing of DNA replication, we found that mitochondrial functions are not simply required for, but they can also promote DNA replication. Increasing the mitochondrial DNA content of the cell through genetic means, by over-expression of the Abf2p mitochondrial

DNA maintenance protein, markedly accelerates DNA replication in the nucleus. We also found that DNA replication is significantly accelerated in cells lacking the NAD^+-dependent de-acetylase Sir2p. Our results are consistent with the following model: Over-expression of Abf2p increases NADH levels, which in turn inactivates Sir2p, and leads to increased acetylation and activation of origins of DNA replication. These findings identify pathways that link mitochondrial biology with DNA replication. They also suggest that chromatin modifications may serve as a read-out of the metabolic requirements for DNA replication.

63. TRIIODOTHYRONINE LEADS TO INCREASED PROLIFERATION OF CLEAR CELL RENAL CELL CARCINOMA CELLS BY CHANGING EXPRESSION OF RETINOBLASTOMA FAMILY PROTEINS

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One of the most frequent reasons of cancerogenesis is improper G1/S phase transition. Triiodothyronine (T3) is an important regulator of proliferation, apoptosis and differentiation. Clear cell renal cell carcinoma (ccRCC) the most common type of renal cancers. It was shown that the expression of some G1/S key proteins and is the signal pathway of T3 is disturbed in ccRCC. The aim of the study was to investigate the role of T3 in regulation of G1/S transition in ccRCC and the effect of T3 on retinoblastoma protein (pRb) and p107.
This was examined in cell lines (ccRCC cell lines – Caki-1, Caki-2 and line from human kidney proximal tubules – HK-2). T3 increased proliferation of cancer cells and decreased proliferation of healthy cells. Differences in proliferation were reflected by changed number of cells entering S phase. Expression of thyroid hormone receptors (TRs), pRb and p107 expression was measured by western blotting at early G1, late G1 and at early S phase. The overall TRs expression was several times higher in HK-2 than in cancer cells, but TRβ in nucleus was unchanged. This might be a result of lower TRα expression in ccRCC or more efficient nuclear import of TRβ. In Caki-1 and HK-2 the expression of TRs was rising during cell cycle progression, while in Caki-2 was decreasing. Several times higher level of pRb in cancer cells and increased number of cells entering S - phase suggests improper action of this protein. T3 treatment of cancer cells resulted in dramatic decrease of p107 expression at late G1 phase. This could explain changed T3 action in ccRCC cells.
In conclusion we show that T3 acts differently on healthy and cancerous cells, leading to the increased proliferation of the latter. T3 exerts its effect by changing the expression profile of key G1/S transition proteins.

64. INITIATING SPINDLE DISASSEMBLY IN ANAPHASE REQUIRES PP2A AND CALCIUM BUT NOT THE APC/C

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Stable inheritance of genetic information requires faithful chromosome segregation, which is implemented by the mitotic spindle in anaphase. In the *Xenopu*s model system, the majority of all spindle microtubules shorten by depolymerisation to accompany chromosome movement towards the spindle poles [1, 2]. The trigger for anaphase in fertilised *Xenopus* eggs as well as in egg extracts is a transient increase in free Calcium [3, 4 5]. Calcium activates Calmodulin-dependent kinase II (CaMKII), which induces the activation of the Anaphase Promoting Complex/Cyclosome (APC/C) [6, 7]. The APC/C destines securin and CyclinB for degradation, the first allowing sister chromatids to separate and the latter permitting exit from M-phase [8, 9]. However, it is unclear what triggers the concomitant depolymerisation of microtubules. Using RanGTP-mediated microtubule structures as well as complete spindles in the *Xenopus* cell free system, we show that Calcium induces microtubule depolymerisation at anaphase onset through a pathway, which is independent of the APC/C. We demonstrate that neither CyclinB degradation nor a drop in Cdk1 activity, but the activity of Protein Phosphatase 2A (PP2A) is required for microtubule depolymerisation. Importantly, this conclusion is supported by experiments in human somatic cells, in which the knock-down of PP2A subunits leads to defects in chromosome segregation caused by hyper-stable spindles. We postulate that in vertebrates microtubule depolymerisation in anaphase does not depend on a reduction in Cdk1 activity, but is dominated by the activity of PP2A. Calcium coordinates anaphase onset through the activation of the APC/C to promote securin degradation and at the same time by activating a novel signalling pathway, which changes PP2A activity, induces microtubules to depolymerise and disassembles the mitotic spindle. The identification of this novel pathway that ensures faithful chromosome segregation contributes to the understanding of cell cycle mechanisms, which, if disturbed lead of a variety of human diseases, such as cancer and other disorders characterized by chromosome instability and aneuploidy.

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65. CHANGING THE ACETYLATION STATUS AT SPECIFIC CIRCADIAN TIMES HAS DIFFERENTIAL EFFECTS ON THE EXPRESSION LEVELS OF CELL CYCLE GENES IN N2A CELLS

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Changing the acetylation levels of histones and other cellular non histone proteins with histone deacetylase (HDAC) and acetylatranferase (HAT) inhibitors has dramatic effects on the expression levels of many cell cycle genes. In malignant cells, this can lead to changes in cell cycle progression and/or to the induction of apoptosis. Recent results have shown that the expression levels of certain cell cycle genes are regulated by circadian clock component proteins. Amongst clock-regulated cell cycle genes are the G1-specific *cyclin D1*, the G2/M-specific *wee-1* and *c-myc*. In this report, we modify the acetylation levels in cells of the neuroblastoma N2A mouse cell line at specific circadian times in order to study changes in the expression levels of the above-mentioned cell cycle genes that may be dependent on circadian phasing. For this purpose, we used trichostatin A (TSA), an HDAC inhibitor and curcumin, a inhibitor of the HAT, p300. TSA is known to increase acetylation levels, while curcumin is expected to lower the acetylation status of proteins. Our results indicate that circadian time is critical in modulating the effect of TSA or curcumin on the expression levels and the phase of circadian clock component genes and of circadian clock-regulated cell cycle genes.

66. KINETOCHORE-ASSOCIATED CYTOPLASMIC DYNEIN IS REQUIRED FOR STABILIZING KINETOCHORE FIBER MICROTUBULES DURING MITOSIS IN MAMMALS

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During mitosis, cytoplasmic dynein plays key direct and indirect roles in organizing microtubules (MTs) into a functional bipolar spindle. During this process dynein is also recruited to the sister kinetochores on each chromosome, but its role(s) in the function of these organelles remain vague in part because globally inhibiting dynein disrupts spindle assembly. However, dynein can be selectively depleted from kinetochores by disrupting ZW-10, and recent studies using this approach conclude that kinetochore-associated cytoplasmic dynein (KD) functions primary to silence the spindle assembly checkpoint (SAC). Here we use antibody microinjection and siRNA of ZW-10, as well as video-LM of GFP-tagged cells, to explore the role of KD in chromosome behaviour during spindle assembly. We find that depleting KD inhibits the rapid poleward motion of attaching kinetochores, but not kinetochore fiber (K-fiber) formation. However, once chromosomes attach to the spindle KD is required for timely and proper chromosome congression, as evidenced by the fact that congression fails in ZW-10 depleted cells even when mitosis is prolonged by MG132. In these cells the distance between sister kinetochores on congressed chromosomes, which reflects tension, is significantly reduced relative to controls. Moreover, compared to controls, the K-fibers in ZW-10 depleted cells are more labile to cold shock. Thus, KD is required during spindle assembly to stabilize kinetochore MT attachments, likely by generating tension on the kinetochore, and in its absence chromosome congression is delayed and often defective. This, combined with premature anaphase onset in cells lacking ZW10, generates the characteristic "lagging" chromosome phenotype seen during anaphase. Finally, as shown previously, depleting dynein from kinetochores reduces the velocity of anaphase poleward chromosome motion by ~ 40%. Together our results reveal that, in addition to silencing the SAC, KD is also required for stabilizing K-fiber MTs and for powering poleward chromosome motion during spindle assembly.

67. T-LOOP PHOSPHORYLATION OF CDK4 IS A DIRECT TARGET FOR CELL CYCLE REGULATION

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CDK4 is a master integrator of mitogenic and antimitogenic extracellular signals. It is also crucial for many oncogenic transformation processes. Various molecular features of CDK4 activation remain poorly known or debated, including the regulation of its association with D-type cyclins, its activating Thr172-phosphorylation and the roles of Cip/Kip CDK "inhibitors" in these processes. T-Loop (Thr172) phosphorylation of CDK4, like that of the other cell cycle CDKs, is considered to be performed solely by CAK (cyclin H-CDK7), which is nuclear and constitutively active. Binding to p21 or p27 prevents CDK4 activation by CAK.
Thr172-phosphorylation of CDK4 was re-investigated using two-dimensional gel electrophoresis in various experimental systems, including human fibroblasts and

tumor cell lines stimulated by serum, thyroid cells stimulated by thyrotropin via cAMP or growth factors, and transfected cells. Thr172-phosphorylation of cyclin D3-bound CDK4 was directly regulated by various mitogenic and antimitogenic factors. This identifies it as a determining target for cell cycle control by extracellular factors. CAK was unlikely to be responsible for regulated CDK4 phosphorylation: (i) Thr172-phosphorylation of CDK4 was enriched in CDK4 complexes containing p21 or p27, even at inhibitory levels of p27 that precluded CDK4 activity; (ii) deletion of p27 NLS sequence relocalized cyclin D3-CDK4 in the cytoplasm, but it did not impair CDK4 phosphorylation; (iii) in thyrocytes, growth factors stimulated the phosphorylation and activity of CDK4 bound to cyclin D1, but not the activity of cyclin D3-CDK4 complexes, which were specifically activated by TSH and cAMP; (iv) within the same cyclin D3 immunoprecipitations of T98G cells, T-loop phosphorylation of CDK4, but not of CDK6, depended on serum, while CDK6 appeared as a better substrate than CDK4 for recombinant CAK; (v) CAK expression and activity were not regulated in these systems. Collectively, these unexpected observations suggest that regulated CDK4-activating kinase(s) remain(s) to be uncovered.

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68. THE HUMAN SPINDLE-ASSOCIATED PROTEIN ASAP IS PHOSPHORYLATED BY AND INTERACTS WITH AURORA-A : FUNCTIONAL SIGNIFICANCE *IN VIVO*

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We have recently characterized **ASAP** (**AS**ter **A**ssociated **P**rotein), a novel human spindle-associated protein. One of the main characteristics of its overexpression is the presence of numerous monopolar spindles. Depletion of ASAP by RNA interference results in severe mitotic defects: it provokes aberrant mitotic spindles, delays mitotic progression with chromosome congression and segregation defects and leads to defective cytokinesis or cell death. We show here that ASAP co-localizes with Aurora-A at the centrosomes in interphase, at the centrosomes and spindle poles during mitosis and at the spindle midzone in cytokinesis. ASAP is phosphorylated *in vitro* and *in vivo* by Aurora-A. Both proteins interact *in vivo* and the depletion of Aurora-A leads to a proteasome-dependant degradation of ASAP, indicating that the interaction and/or the phosphorylation of ASAP by Aurora-A might be necessary to stabilize ASAP. We have identified one phosphorylated site of ASAP by Aurora-A *in vivo*. This site is phosphorylated at early mitosis at centrosomes and the phosphorylated form is observed throughout mitosis. The functional significance of this phosphorylation is currently being investigated and the data will be presented at the meeting. ASAP plays a crucial role in spindle formation and cell cycle and deciphering its function will help to understand mechanisms that are often deregulated in tumor cells.

69. DISTINCT POOLS OF CDK1/CYCB INDEPENDENTLY DRIVE CYTOPLASMIC AND NUCLEAR MITOTIC CYCLES IN DROSOPHILA EMBRYOS

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Entry into mitosis is characterized by dramatic remodelling of nuclear and cytoplasmic compartments. The mechanisms that co-ordinate mitotic entry of nuclear and cytoplasmic components are poorly understood. To address this issue, we injected purified Cyclin B (CycB) and Cyclin A (CycA) into drosophila embryos at precise times during syncytial cycles and monitored their effects on cytoplasmic and nuclear CDK1 activation. Injection of CycB during interphase induced premature nuclear envelope breakdown (NEB), spindle assembly and entry of cytoplasmic markers into mitosis. These data demonstrate that CycB is rate limiting for cytoplasmic and nuclear CDK1 activation. Closer inspection revealed that CycB induced cytoplasmic mitotic entry prior to NEB, indicating that cytoplasmic CDK1/CycB activation precedes its nuclear activation. In contrast, injection of CycB following inhibition of DNA replication induced premature cytoplasmic but not nuclear mitotic entry. This suggests that distinct pools of CDK1 drive these events. The discrepancy in nuclear and cytoplasmic CDK1 activation is abolished in the absence of a functional DNA replication checkpoint. Preliminary data suggest a role for this checkpoint in coordinating nuclear and cytoplasmic pools of CDK1 by regulating CycB nuclear import. Finally, we present evidence that additional control of nuclear and cytoplasmic remodelling may occur via the substrate specificity of CDK1/CycB and CDK1/CycA.

70. AURORA-A IS INVOLVED IN RESUMPTION OF MEIOSIS AND SPINDLE FORMATION IN MOUSE OOCYTES

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Aurora-A is a centrosome-localized serine/threonine kinase crucial for cell cycle control. This kinase is mainly involved in centrosome maturation and spindle assembly in somatic cells. The active T288 phosphorylated Aurora-A appears on centrosome in the late G2. Additionally, it spreads to the minus ends of the mitotic spindle microtubules. Aurora-A activates centrosomal CDC25B and is also involved in recruitment of the cyclin B1 to centrosomes. Centrosomal CDC25B and cyclin B are key initial activators of CDK1 during mitosis entry.

We study the role of Aurora-A during meiotic maturation of mouse oocytes. Total Aurora-A is present in the nucleus in GV-stage oocytes (G2 equivalent). Additionally, active Aurora-A is localized entirely to the centrosome (MTOC)

shorly before germinal vesicle breakdown (GVBD). Compared to somatic cells, where active Aurora-A is at the centrosomes and the spindle poles, active Aurora-A is strictly localized on MTOCs at metaphase I in oocytes. We show that activation of centrosomal Aurora-A is independent on PI3K-PKB and CDK1 signaling pathways. This was proved by cultivation of oocytes in presence of roscovitine (CDK1 inhibitor), LY-294002 (PI3K inhibitor) and SH-6 (PKB inhibitor). Treated oocytes show high phosphorylation of Aurora-A on T288 and centrosome amplification despite the presence of intact nuclear envelope. Silencing of Aurora-A by RNA interference induces incorrect spindle assembly. Oocytes are arrested in prometaphase I and unable to reach metaphase II. After microinjection of eGFP-Aurora-A mRNA into GV-stage oocytes, overexpression of Aurora-A leads to distortion of MI spindle organization as well. Our results indicate that Aurora-A is the key centrosomal player in meiotic maturation, essential for proper spindle formation and metaphase I-metaphase II transition.

71. ROLE OF REACTIVE OXYGEN SPECIES (ROS) IN SWITCHING MITOGENIC TO DEATH SIGNALING

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Reactive oxygen species (ROS) are critical intermediates in the cell death caused by many stress agents and pro-apoptotic signaling pathways. However, ROS are also recognized as essential second messengers in mitogenic signaling and cellular transformation, which control these events through distinct modifications of intracellular signaling proteins. To get a better understanding of a possible role for ROS in switching mitogenic to death signaling, we used IL-3 dependent cell lines as model systems. While the presence of antioxidants dramatically delayed growth in IL-3, it also protected cells against apoptotic death following growth factor removal. Further experiments in 32D cells demonstrated that IL-3 withdrawal resulted in growth arrest and dramatically increased ROS production, which was causally linked to cell death induction. In the absence of IL-3 these changes were actively prevented by oncogenic C-RAF and inhibiting RAF signaling in the presence of IL-3 was sufficient for arresting proliferation and inducing apoptosis. RAF also delayed the onset of apoptosis, following treatment with staurosporine (STS) or the oxidative stress-inducing agent *tert*-butyl hydroperoxide (*t*-BHP). Apoptotic cell death is preceded by a ROS-dependent increase in mitochondrial Ca^{2+}, which was absent in cells expressing transforming C-RAF. Prevention of mitochondrial Ca^{2+} overload after IL-3 deprivation delayed apoptotic cell death. In summary, our data show that the transition from the proliferative stage to growth arrest and cell death is critically controlled by ROS. Mitogenic and survival proteins like RAF kinases actively prevent this process by controlling ROS production and mitochondrial Ca^{2+} homeostasis.

72. THE RETINOBLASTOMA RELATED PRBL2/P130 IS FUNCTIONALLY REGULATED BY ACETYLATION.

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p130, an Rb-related protein and member of the pocket protein family, exerts regulatory functions during the cell cycle. Its functional activity in the nucleus is regulated by phosphorylation. Recombinant p130 and truncated versions of the protein were analyzed for the presence of modified subspecies of p130, especially acetylated p130. Overexpression using an insect-cell system, in-vitro acetylation using CBP/p300 as the acetyltransferase, and analysis by autoradiography revealed acetylation of the C-terminus of p130; mass spectrometry pinpointed the primary acetylation site to K1079, which is located within a nuclear localization sequence.
In exponentially growing NIH-3T3 cells, p130 is present in the cytoplasm as well as in the nucleus, but acetylation is restricted to the nuclear p130. Thus nuclear extracts of synchronized NIH-3T3 cells were analyzed for acetylation and phosphorylation of p130 during the cell cycle. During the S/G2 transition, the main proportion of p130 appears in two hyperphosphorylated states of which one is acetylated as well.
Focusing on cells synchronized at the S/G2 transition, acetylated and hyperphosphorylated p130 forms of nuclear extracts co-elute after anion exchange and size exclusion chromatography. Acetylated and hyperphosphorylated p130 could also be immunoprecipitated from synchronized NIH-3T3 cells. Pull-down experiments combined with mass spectrometry were used to determine acetylation specific interaction partners. NIH-3T3 cell lines stably expressing FLAG-tagged p130 as well as K1079R and K1079Q mutated forms are being generated.
We present evidence that the regulation of p130 is not only established by phosphorylation and cellular localization, but also by cell cycle dependent acetylation.

73. LINKS BETWEEN FORK STABILIZATION MECHANISM AND COHESION ESTABLISHMENT

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In mitotic S-phase, cells not only duplicate their genetic information but also have to prepare for the faithful partition of chromosome (they have to establish sister

chromatids cohesion). So far, it was shown that cohesin was loaded onto chromosomes at the beginning of S-phase, and Eco1 is essential for the cohesion establishment during S-phase. Eco1 is also essential for viability. In addition to Eco1, many factors have been identified as "cohesion establishment" factors which are non-essential for viability.

To investigate the mechanism of cohesion establishment, we analyzed binding profiles of four factors, Ctf18, Chl1, Ctf4, and Eco1 onto the *S. cerevisiae* genome during normal and HU perturbed S-phase using the ChIP-chip. Our data demonstrated that Ctf4 was at replication forks during unperturbed and perturbed S-phase, but Ctf18 and Chl1 were present at the forks only in HU. There was no specific localization of Eco1. Furthermore, our analyses using *ctf4* and *ctf18* deletion mutants suggested that those factors were involved in DNA replication checkpoint directly. In the deletion mutant of *ctf18*, DNA replication forks became unstable and activation of late firing origins took place under the HU arrest, which suggested that the deletion of *ctf18* resulted in the loss of DNA replication checkpoint. Interestingly, a mutant deleted for *ctf4* did not show any defects in the activation of DNA replication checkpoint, but interaction between MCM helicase and DNA Polα complex became specifically unstable, and DNA Polα no longer associated stably with replication forks under HU. Ctf4 was also phosphorylated in a Rad53 dependent manner.

Eco1 belongs to an another class of protein as its mutant does not show any defects in DNA replication checkpoint or fork structure. We identified several loci of extra genetic suppressors of *eco1-1* mutant by microarray based technique. Characterization of two suppressor loci will be presented.

74. CDC25A IS ABLE TO INDUCE RESUMPTION OF MEIOSIS BUT COMPROMISES METAPHASE I – METAPHASE II TRANSITION IN MOUSE OOCYTES

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Resumption of meiosis and meiotic maturation are characterized by nuclear envelope disintegration and dynamic changes in microtubules followed by first polar body extrusion. These events are CDK1 dependent. CDC25A/B/C dual phosphatases are activators of cyclin-dependent kinases (CDKs). Generation of CDC25B -/- mice revealed essentiality of CDC25B in CDK1 activation during resumption of meiosis. CDC25B -/- oocyte are unable to resume meiosis and form metaphase spindle.

CDC25C -/- mice are viable and fertile suggesting unnecessity of CDC25C both for mitotic and meiotic cell cycle regulation. CDC25A -/- mice are embryonic lethal and therefore potential role of CDC25A in meiosis is unknown.

We have shown that CDC25A protein is expressed in GV-stage (G2 equivalent) oocytes but decreases in CDK1 dependent manner during meiotic maturation. In metaphase I and metaphase II stages very low level of CDC25A protein is present with comparison to GV-stage.

Resumption of meiosis is blocked by high level of cAMP. CDC25A, as well as CDC25B, are able to overcome this cAMP block (induced by phosphodiesterase inhibitor IBMX). This suggests that CDC25A can be involved together with CDC25B in resumption of meiosis.

Using microinjection of eGFP-CDC25A and eGFP-CDC25B mRNAs constructs we have revealed that CDC25A is exclusively nuclear protein until nuclear envelope disintegration (GVBD – germinal vesicle break-down). Diversely, CDC25B localizes to cytoplasm at GV-stage oocytes and translocates to nucleus shortly before GVBD.

Overexpression of eGFP-CDC25A, to interfere with CDC25A degradation during meiotic maturation, results in metaphase I block. Oocytes with high level of CDC25A are unable to reach metaphase II compared with control eGFP-expressing oocytes. This metaphase I block is not accompanying with considerable changes in CDK1 or MAPK activity.

These data suggest that CDC25A can be involved together with CDC25B in CDK1 activation and resumption of meiosis in mouse oocytes but CDC25A degradation after GVBD is essential for proper metaphase spindle formation and metaphase I - metaphase II transition.

75. A NOVEL CUL3-BASED E3-LIGASE REMOVES AURORA B FROM MITOTIC CHROMOSOMES, THEREBY REGULATING MITOTIC PROGRESSION AND COMPLETION OF CYTOKINESIS IN HUMAN CELLS

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Faithful cell cycle progression is tightly controlled by the ubiquitin-proteasome system. Here we identify a novel human Cullin3-based E3-ligase (Cul3), which is essential for mitotic division. In a complex with the substrate-specific adaptors KLHL9 and KLHL13, Cul3 is required for correct chromosome alignment in metaphase, proper midzone and midbody formation and completion of cytokinesis. This Cul3-based E3-ligase removes components of the chromosomal passenger complex (CPC) from mitotic chromosomes and allows their accumulation on the central spindle during anaphase. Aurora B directly binds to the substrate recognition domain of KLHL9 and KLHL13 *in vitro*, and co-immunoprecipitates with the Cul3-complex during mitosis. Moreover, Aurora B is ubiquitylated in a Cul3-dependent manner *in vivo*, and by reconstituted Cul3/KLHL9/KLHL13-ligase *in vitro*. We thus propose that the Cul3/KLHL9/KLHL13 E3-ligase controls the dynamic behavior of Aurora B on mitotic chromosomes, and thereby coordinates faithful mitotic progression and completion of cytokinesis.

76. THE DROSOPHILA MITOTIC INHIBITOR FRÜHSTART SPECIFICALLY BINDS TO THE HYDROPHOBIC PATCH OF CYCLINS

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The hydrophobic patch of cyclins interacts with cyclin-dependent kinase (Cdk) substrates and p27-type Cdk inhibitors. Although this interaction is assumed to contribute to the specificity of different Cdk-Cyclin complexes, its role in specific steps of the cell cycle has not been demonstrated. We show that in Drosophila the mitotic inhibitor Frühstart (Frs) binds specifically and with high affinity to the hydrophobic patch of cyclins. In contrast to p27-type inhibitors, Frs does not form a stable interaction with the catalytic centre of Cdk and allows phosphorylation of generic model substrates, such as histone H1. Consistent with a 2.5 times stronger binding to CycA than to CycE in vitro, ectopic expression of frs induces endocycles, in a manner similar to that reported previously for downregulation of CycA or Cdk1. We propose that binding of Frs to cyclins blocks the hydrophobic patch to interfere with Cdk1 substrate recognition.

77. LOSS OF THE SUV39H1 HISTONE METHYLTRANSFERASE PROMOTES MYC-DRIVEN B-CELL LYMPHOMAGENESIS AND CONFERS POOR THERAPY OUTCOM

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The Myc oncogene is frequently found activated in human lymphomas, causing an ARF/p53 dependent apoptotic response. To further advance to a full-blown malignancy, cells with constitutive Myc expression select for apoptotic defects. We have previously reported that Myc-driven lymphomas bearing a targeted apoptotic defect may retain the capability of executing drug-inducible senescence. Moreover, our lab recently identified the histone-H3 lysine 9 methyltransferase Suv39h1 as a critical part of the oncogene-induced senescence machinery, acting as an early barrier against Ras-initiated lymphomagenesis. This finding raised the question whether treatment sensitivity of Myc-driven lymphomas would be compromised by loss of Suv39h1 function.

Eμ-myc transgenic mice were intercrossed to mice harboring targeted deletions at the Suv39h1 locus. Myc-transgenic mice with no additional defined genetic lesions (hereafter referred to as controls) produced B-cell lymphomas with a median onset of 117 days. Surprisingly, mice lacking one or both *Suv39h1* alleles developed lymphomas at a median age of 58 days. Lymphomas arising in female Suv39h1+/- mice invariably lost expression of the X-linked Suv39h1 gene, explaining indistinguishable tumor latencies in Suv39h1+/- and Suv39h1-/- mice. TUNEL staining *in situ,* and short-term cytotoxicity assays in response to the DNA damaging drug adriamycin *in vitro* demonstrated no difference in the apoptotic capability between both groups. However, when cells in cycle where assessed by Ki67 staining *in situ*, fraction of control lymphoma cells appeared to be negative, while virtually all Suv39h1-deficient cells stained positive. Assessment of oncogene-induced senescence by senescence-associated-□-galactosidase activity unveiled positive staining of a significant fraction of cells in control lymphomas, while no positive cells were found in Suv39h1-deficient lymphomas. This finding was recapitulated by immunofluorescent analysis of H3K9 tri-methylated cells. Data addressing cell-autonomous and non-autonomous contributions to this phenotype will be reported.

Anticancer treatment *in vitro*, using adriamycin, produced a strong senescent phenotype in control cells in the presence of an apoptotic block, while no senescence response was detectable in Suv39h1-deficient lymphoma cells. Accordingly, mice harboring Suv39h1-deficient lymphomas achieved a significantly inferior outcome to treatment compared to mice bearing control lymphomas, irrespective of presence or absence of an additional apoptotic block mediated by Bcl2.

Our data show a significant role of the oncogene-induced senescence program during tumor formation, and genetically dissect the role of drug-inducible senescence as a critical component of the long-term-outcome to cancer therapy *in vivo*.

78. IDENTIFICATION AND CHARACTERIZATION OF NEW KINETOCHORE PROTEINS

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Kinetochores are large complex protein structures that assemble at the centromeric regions of each sister chromatid and attach chromosomes to the spindle, co-ordinate microtubule dynamics to chromosome movement along the spindle, and generate the 'wait' signal that prevents anaphase onset until all the chromosomes are correctly aligned on the spindle. Surprisingly the subunits of this multiprotein complex reamin to be identified and characterized, with the exception of the yeast S. cerevisiae kinetochore. In order to identify and characterize kinetochore proteins in *Drosophila* we have developed a method to obtain enriched mitotic kinetochore protein fractions. The protein profile pattern of such fractions was characterized by separation by two-dimensional electrophoresis followed by peptide mass fingerprinting. We have identified 92 proteins in the protein fraction. Some (like CID and Cenp-E) had previously been described as kinetochore components. Others, like Sgt1, Ndc80, Nuf2 and Mis12 had been described in other organisms but not in

Drosophila. Finally, 16% of the proteins show no obvious homologues in other organisms. We have characterized in detail the function of DmSgt1, Ndc80, Nuf2, Mis12 and four of the new genes (that we named Cms1, 2, 3 and 4). We have determined the dynamics of these proteins at the kinetochore by expression in S2 cells GFP-tagged vertions of the proteins and RNAi experiments show that they are required for proper chromosome congression and segregation. Furthermore, mutant analysis show that mutations in the Cms3 and DmSgt1 genes cause early embryonic or 1st instar larval lethality. Finally, pull-down assays using the newly identified proteins as baits have allowed us to start assembling the Drosophila kinetochore structure.

79. ANAPHASE ONSET BEFORE COMPLETE DNA REPLICATION WITH INTACT CHECKPOINT RESPONSES

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Completion of DNA replication before mitosis is essential for genome integrity. Cellular checkpoints prevent mitosis in the presence of ongoing or stalled replication forks. Here we show that in *smc5-smc6* mutants, related to cohesin and condensin, replication is delayed, most significantly at natural replication-impeding loci like the ribosomal DNA (rDNA) gene cluster. In the absence of Smc5-Smc6, chromosome non-disjunction occurs as a consequence of mitotic entry with unfinished replication despite intact checkpoint responses. Eliminating processes that obstruct replication, such as removal of replication barriers or inactivation of ribosomal RNA transcription prevents rDNA non-disjunction in *smc5-smc6* mutant cells. Similarly, replication fork blocks induced by a DNA alkylating agent (MMS) prevent *smc5-smc6* mutants to complete whole genome replication. We also present data showing that a G1 pulse of low-dose MMS, which does not induce the DNA damage checkpoint, is sufficient to prevent completion of chromosome replication in *smc5-smc6* mutants. In spite of not having finished replication of most chromosomes, *smc5-smc6* mutants do enter anaphase. Our results demonstrate the lack of regulatory mechanisms to monitor the completion of S phase before mitosis and reveal a function for the Smc5-Smc6 complex in mediating stable replication through challenging DNA templates.

80. THE KINASE MPS1 IS REQUIRED FOR CORRECT CHROMOSOME SEGREGATION IN FEMALE MOUSE MEIOSIS I

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The mitotic spindle assembly checkpoint (SAC) verifies during each cell division in metaphase the correct attachment of sister kinetochores to the bipolar spindle. The presence of one unattached kinetochore leads to a SAC-dependent cell cycle arrest in metaphase until proper attachment has been achieved. Furthermore, components of the SAC are implicated in the correct timing of prometaphase. The dual-specificity kinase Mps1 is an essential component of the SAC. Mps1 is required for the kinetochore localization of certain SAC components such as Mad2 upon checkpoint activation in metaphase.
Meiosis I is a specialized cell division, as paired chromosomes (each consisting of two sister chromatids), and not paired sister chromatids (as in mitosis and meiosis II) are separated towards opposite poles. Because of this fundamental difference we are interested in the role of the SAC in meiosis I in mammalian oocytes to elucidate the molecular mechanisms controlling the first meiotic division. We and others have shown previously that the SAC is functional in meiosis I in mouse oocytes, and depends on the SAC protein Mad2. To analyse the importance of SAC control during the first meiotic division we generated an oocyte-specific conditional hypomorphic mouse model of Mps1. In the present study we demonstrate that the SAC kinase Mps1 is essential for several aspects of meiosis I. Timing of early meiosis I events is strongly perturbed, and chromosome missegregations occur at high rates. Aneuploid metaphase II ocytes are generated. Surprisingly, oocytes harboring only the mutant allele of Mps1 still arrest upon nocodazole treatment in meiosis I and recruit Mad2 to kinetochores. Furthermore, a nearly complete loss of fertility was observed in female mice harboring oocytes expressing only the Mps1 mutant. Our results demonstrate the importance of one component of the SAC for several key events in meiosis I.

81. HISTONE ACETYLATION AND CIRCADIAN CLOCK GENE REGULATION: EFFECTS ON THE EXPRESSION OF CELL CYCLE GENES

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The molecular mechanism that drives the mammalian circadian clock has been studied in depth during recent years. Most cells in the brain and the peripheral tissues contain a molecular clock consisting of at least 12 component genes. This central pacemaker rhythmically controls the transcriptional activity of ~10% of the genome, ~10% of which are proliferation-related genes. Increasing experimental evidence has accumulated indicating that certain cell cycle-related genes are controlled by components of the mammalian circadian clock. Findings, revealing that the rhythmic gene expression of the key clock components, *per1, per2* and *cry1,* is synchronized to the rhythmic acetylation of histone H3 in their promoters,

support the idea that circadian clock gene expression is regulated by histone acetylation and the action of histone acetyltransferases (HATs) and histone deacetylases (HDACs). The aim of the present work is to study the effect of two acetylation-related inhibitors on the expression levels of clock genes and the concomitant changes that may occur in the expression levels of cell cycle related genes. The inhibitors used in this investigation are trichostatin A, an HDAC inhibitor and curcumin, an inhibitor of the HAT, p300. The cells, NIH3T3 mouse fibroblasts, were rhythmically synchronized by the glucocorticoid, dexamethasone. The results show that these two inhibitors differentially affect the expression levels of both clock and cell cycle genes as a function of the specific circadian time of their administration.

82. MOLECULAR MECHANISMS OF SUBSTRATE RECOGNITION AND UBIQUITYLATION BY THE APC/C UBIQUITIN LIGASE

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The ubiquitin pathway is an ATP-dependent tagging system for protein degradation. The anaphase-promoting complex/cyclosome (APC/C) is an essential E3 ubiquitin ligase that ubiquitylates numerous proteins at specific times in the cell cycle. We believe that substrate recognition is a key issue in understanding how the APC/C ubiquitylates its substrates. To understand the mechanisms how the APC/C recognises the substrates, we have been using several model substrates including Nek2A (a centrosome kinase), Cdc13 (B-type cyclin) and Mes1 (a meiotic APC/C substrate). Although we haven't found the universal mechanism yet, there are some interesting relationships between the mode of recognition and timing/role of destruction. Nek2A that is destroyed in prometaphase, like cyclin A, directly binds to the APC/C through the C-terminal methionine-arginine (MR) dipeptide tail. This interaction is not dependent on Cdc20/Fizzy, a target of the spindle assembly checkpoint (SAC). This partly explains why Nek2A can be ubiquitylated and destroyed by the APC/C even when SAC is active. In contrast, the D-box/KEN-box of Mes1 directly interacts with the Fizzy family of proteins in the absence of the APC/C. Since Mes1 is a potent APC/C inhibitor, it makes sense to interact with an essential activator to block the function. Cdc13 apparently interacts with both APC/C and Fizzy family of proteins, which presumably allows formation of a stable ternary complex for ubiquitylation. Moreover, we have recently identified Rhp54 (S. pombe Rad54 homolog) as a new APC/C substrate. Rhp54 is specifically destroyed in G1-phase in a KEN-box-dependent manner. Our preliminary data suggest that Rhp54 destruction plays a pivotal role in DNA damage response pathway. We will discuss the physiological relevance with the latest data.

83. ROLE OF CDC20 IN CYCLIN A AND B1 DEGRADATION

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The activity of mitotic Cyclins (A- and B-type Cyclins in mammalian cells) is tightly regulated. The amount of mitotic Cyclins in the cells is regulated at the level of transcription, translation and degradation. The Anaphase-Promoting Complex (APC/C), a ubiquitin ligase, is responsible for ubiquitinating mitotic Cyclins and therefore targeting them to degradation. Temporal regulation of Cyclin degradation during mitosis is not completely understood. In particular, it is still unclear how Cyclin A is degraded in the presence of Spindle Assembly Checkpoint (SAC) activity while Cyclin B1 degradation is inhibited. The SAC inhibits the Cdc20-dependent APC-mediated degradation of Cyclin B1 until all chromosomes have bipolar attachment and thus prevents premature exit from mitosis. Cdc20 has been shown to be targeted by the SAC. It has been shown that Cdc20 interacts with the SAC components and the expression of Cdc20 mutants that are defective in this interaction results in aberrant cell cycle progression and insensitivity to factors that would normally inhibit progression in mitosis through the SAC activation.
We have investigated how Cdc20 depletion affects Cyclin A and B degradation. Cdc20 was depleted in Hela cells using siRNA. The majority of Cdc20 RNAi cells were able to exit mitosis, but took longer than normal. The degradation of microinjected fluorescently-tagged Cyclin A and B in individual cells was followed using time-lapse DIC-fluorescence microscopy. Surprisingly, it was observed that Cyclin A degradation is impaired while Cyclin B degradation is unaffected. These results were confirmed for endogenous Cyclin A and B by analyzing fixed Hela cells. We concluded that there could be two possible explanations for this observation: 1. Cyclin B1 degradation does not depend on Cdc20 or other factors can fulfill Cdc20's role in Cdc20 RNAi cells. 2. The small amount of Cdc20 left in the cells is sufficient to promote degradation of Cyclin B1, but not Cyclin A. From other publications, we expected the latter to be the case. Thus, we tried to optimize the knock-down of Cdc20. With optimized knock-down, cells were arrested at metaphase. Moreover, none of the microinjected fluorescently-tagged Cyclin A and B was degraded.
We conclude that Cyclin A degradation is greatly dependent on Cdc20. In contrast, only a small amount of Cdc20 suffices for Cyclin B degradation. It is interesting to investigate how Cyclin B1 is recruited to APC in the presence of a small amount of Cdc20. It is possible that Cyclin B1 can interact directly with APC and does not require Cdc20 for recruitment, and transient interaction of Cdc20 with APC can activate the complex to ubiquitinate Cyclin B1. Supporting this possibility, a study done in Xenopus egg extracts showed that Cyclin B1 destruction-box binds the APC/C, but not Cdc20.

84. GENOME-WIDE RNAI SCREENING OF NOVEL COMPONENTS INVOLVED IN BMP SIGNALING

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Bone morphogenetic proteins (BMPs) constitute the largest subfamily within the transforming growth factor-β (TGF-β) superfamily and exert profound effects on cell division, differentiation, and cell death. Dpp, BMP type ligand in Drosophila, has been well studied being involved in both cell growth and differentiation. The basic structure of the pathway has been well established. However, there are still some unknown components in the pathway which are necessary for understanding cell growth control. In order to identify novel components that regulate BMP signalling, we are performing a genome-wide RNAi screening in *Drosophila* S2 cells. We have established the assay system in the high throughput screening based on the imaging analysis. Using dsRNA library which covers >90% of *Drosophila* genome, we have performed the primary and secondary screenings, and found about 200 candidate genes, which were selected for further analysis. BMP receptors (tkv and punt) and Smad1 (mad) were found as candidate genes, indicating that this approach could be very useful to investigate the loss-of-function of BMP signalling. Furthermore, several candidate genes were found in the previous screening on pathways regulating cell size and cell-cycle progression (1). With genetic and biochemical approaches, we characterize the novel genes found at the screening how they are involved in cell growth and differentiation.

(1) Bjorklund *et al., Nature 439***:** 1009-1013, 2006