## **Opening Lecture:** "GETTING IN AND OUT OF MITOSIS"

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Cells enter mitosis (more generally, M-phase, and much of our work has been in frog oocytes and eggs and extracts thereof) when CDK1/cyclin complexes are activated. Phosphorylation by these, and other mitotic protein kinases, is responsible for reorganizing the cell and initiating progression to metaphase. We would like to know how many proteins need to be phosphorylated how much to bring about this state of affairs, and have been trying to enumerate the mitotic targets for various cyclin-CDK combinations for some time.

It is well known that the metaphase to anaphase transition occurs when the anaphase-promoting factor (APC/C) is activated and tags a small number of target proteins, including cyclins and securin, with ubiquitin. Chromatids part and move to opposite poles of the cell where they decondense and re-form a functional nucleus. Cytokinesis separates the two daughter cells. Mitotic phosphoproteins revert to their interphase un- or hypo-phosphorylated state.

We recently discovered, more or less by accident, that the activity responsible for this postmitotic dephosphorylation is almost completely inactive in M-phase cell extracts, and is reactivated when cells exit mitosis. This explains how proteins can become almost completely converted to hyperphosphorylated states: not only are kinases activated, but the counteracting phosphatase(s) are concomitantly shut down. I will present the evidence that has led us to this conclusion. It stems from studies of frog egg extracts released from cytostatic factor (CSF) arrest by added $CaCl_2$, and the discovery that calcineurin (protein phosphatase 2B) plays a role in escaping the clutches of CSF.

## S 02. ABNORMAL CELL CYCLE REGULATION IN HUMAN FUSION ONCOGENE CARRYING SARCOMAS.

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Tumor type specific chromosome aberrations that lead to formation of new fusion oncogenes are continuously reported in human sarcomas. Most of these oncogenes encode abnormal transcription factors, and expression analysis of tumors and transfected cells suggests that they cause abnormal expression patterns of many genes. Those fusion oncogenes that have been further analyzed, show transforming activities in cultured cells or in transgenic animals, confirming that they have important roles in tumor development. Several reports suggest that the fusion oncogenes may act directly on cell cycle controlling factors. Expression analysis of cyclins, cyclin dependent kinases and kinase inhibitors, pRB and p53, shows that expression of cyclin D, CDK4/6 are commonly deregulated and considered as early changes, whereas abnormal expression of p16INK4a/p14ARF and mutation/overexpression of P53 seem to correlate with progression to high grade tumors. Several of the fusion oncogenes have been shown to induce expression of cyclin D and E and affect expression of p21(WAF1/CIP1), but the mechanisms are poorly understood. The sarcoma/leukaemia FET group of fusion oncoproteins, share the functionally and structurally similar N-terminal parts of the FUS, EWSR1 and TAF15 proteins and these parts are necessary for their transforming activity. These N-terminal parts have been shown to act as strong transactivator domains of the fusiongene encoded abnormal transcription factors. New data indicate, however, that the N-terminal parts of the FET oncogenes may interact also with components of signal transduction and stress response systems as well as translation regulating proteins. The fusion oncogenes found in sarcomas may therefore directly affect, not only transcriptional regulation but also other functions involved in cell growth and transformation.

## S 03. CONSEQUENCES OF ANEUPLOIDY

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Aneuploidy is a condition frequently found in tumor cells but how aneuploidy affects cellular physiology is not known. We have created a collection of haploid yeast strains that each bear an extra copy of one or more of almost all of the yeast chromosomes. Their characterization revealed that aneuploid strains share a number of phenotypes, including defects in cell cycle progression, increased glucose uptake and sensitivity to conditions interfering with protein synthesis and folding. These phenotypes are observed only in strains carrying additional yeast genes indicating that they reflect the consequences of additional transcription and translation as well as the resulting imbalances in cellular protein composition. Preliminary data further suggest that aneuploidy affects primary mouse cell lines in a similar manner. We conclude that aneuploidy causes not only a proliferative disadvantage but also a set of phenotypes that is independent of the identity of the individual extra chromosomes.

## S 04. DNA DAMAGE CHECKPOINTS: MECHANISMS, LIVE-CELL IMAGING AND RELEVANCE TO HUMAN CANCER

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The lecture will first provide a concise background information on DNA damage response and its biological significance, followed by examples from our recent work on the following issues: First, the emerging role of the DNA damage signalling cascades in coordination of cell cycle checkpoints and DNA repair mechanisms, including unpublished insights into the critical role of protein ubiquitination in DNA damage signalling and repair. Second, novel insights into the spatio-temporal control and dynamics of responses to DNA double strand breaks, focusing on the distinct behavior of various classes of DNA damage response components and repair proteins, as monitored by live-cell imaging approaches in human cells in real time. Third, our data supporting a concept of the DNA damage response machinery as an inducible biological barrier against progression of early stages of human tumours in vivo, and in response to various oncogenes in cell culture models, will be presented. Here, emphasis will be mainly on unpublished results on distinct patterns of DNA damage response activation in diverse types of human malignancies, and discussion of potential cellular and molecular basis of such differences, as well as consideration of the various cancer-associated molecular defects capable of inducing the DNA damage checkpoint machinery. A brief note on emerging and potential future applications of these concepts for biomedicine, particularly oncology, will conclude the lecture.

*Our selected references:*

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## S 05. GETTING IN AND OUT OF S PHASE

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The eukaryotic cell cycle coordinates the accurate duplication and apportionment of the genome during proliferation. The large genomes of eukaryotic cells are replicated from multiple replication origins during S phase. These origins are not activated synchronously at the beginning of S phase but, instead, fire throughout S phase according to a pre-determined, cell type specific program. Only after the entire genome is completely replicated do cells proceed into mitosis.

Ensuring that each origin is efficiently activated once and only once during each S phase is crucial for maintaining the integrity of the genome. This is achieved by a two-step mechanism. The first step involves the loading of the Mcm2-7 complex at origins Pre-RCs, which are essential for initiation, can only assemble at origins during G1 phase when cyclin dependent kinase (CDK) activity is low because CDKs inhibit each pre-RC component individually. CDKs then trigger initiation by phosphorylating two additional proteins, Sld2 and Sld3. The regulation of S phase by protein kinases will be described.

Finally, replicated sister chromatids must be decatenated by topoisomerase II before being segregated, Many anti-cancer drugs work by interfering with topo II. A yeast model for how some of these drugs work will be discussed.

## S 06. PHYSIOLOGICAL AND PATHOLOGICAL TOPOLOGICAL TRANSITIONS AT REPLICATION FORKS.

**Rodrigo Bermejo[1], Ylli Doksani[1], Dana Branzei[1], G.Liberi[1], Yuki-Mori Katou[2], Katsuhiko Shirahige[2] and Marco Foiani[1].**

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The checkpoint response and the SUMO-pathway counteract abnormal transitions at replication forks preventing the accumulation of reversed forks and cruciform recombination derivatives resembling hemicatenanes. Although the final targets of these regulatory processes are still unknown, the Sgs1 RecQ helicase and the Top3 topoisomerase have been implicated in the SUMO sub-pathway protecting damaged replication forks. We have investigated whether and how the Top1 and Top2 topoisomerases protect the integrity of replication forks. We found that Top1 and Top2 act within a 600 bp region spanning the moving forks. Top2 exhibits additional S-phase clusters at specific intergenic loci. *TOP1* ablation does not affect fork progression and stability and does not cause activation of the Rad53 checkpoint kinase. *top2* mutants accumulate sister chromatid junctions in S phase without affecting fork progression and activate Rad53 at the M-G1 transition. *top1 top2* double mutants exhibit fork block and processing, and phosphorylation of Rad53 and gH2A in S phase. We have also analyzed the dynamics of replication forks encountering a double strand break and the data will be presented.

Our results indicate that Top1 and Top2 counteract torsional stress and sister chromatid entanglement at the forks, thus preventing the diffusion of topological changes along large chromosomal regions, abnormal chromosome transitions, DNA damage checkpoint activation and chromosome breakage during segregation.

## S 07. CONTROL OF EXIT FROM MITOSIS IN MAMMALIAN CELLS

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Exit from mitosis is controlled by timed APC/C-dependent proteolysis of securin and mitotic cyclins, which is required for the onset of sister chromatid separation and completion of cellular division, respectively. By using RNAi-induced Cdc27 hypomorphic human cell lines, which show an incomplete metaphase arrest phenotype, we found that securin disappears faster from mitotic cells than cyclin B1, causing a fraction of cells to arrest in anaphase. To investigate whether the timing of cyclin B1 degradation is important, we have previously established human cell lines with inducible expression of non-degradable cyclin B1. The observed dose-dependent mitosis arrest phenotypes suggested that phosphorylation thresholds might control late mitotic events, such as hKid-controlled chromosome motility and Ect2-dependent cytokinesis. The decline of CDK activity during exit from mitosis also activates Fzr1/Cdh1, which substitutes Cdc20 as an activator of the APC/C. In order to understand the function of Fzr1 during exit from mitosis, we have generated mice carrying a conditional Fzr1 allele. Fzr1 is most likely essential for murine development since no homozygous Fzr1 deficient mice have been obtained so far. To investigate Fzr1 function at the cellular level, we generated Fzr1 deficient mouse embryonic fibroblasts (MEF), established by Cre-recombinase-mediated excision of exons 2-8. Fzr1 deficient MEFs, however, proliferated as their wild-type controls and did not show any defects in exit from mitosis. The analysis of extracts from synchronised Fzr1 wild-type and Fzr1 deficient MEFs revealed that only substrates that are targeted in early G1-phase but not late mitotic substrates are stabilised in the absence of Fzr1. Although the degradation of mitotic cyclins during mitosis is not affected in the absence of Fzr1, they re-accumulate earlier during G1-phase, suggesting that the main function of Fzr1 is to control G1-phase.

## S 08. LONGEVITY ASSURANCE MOLECULAR PATHWAYS IN HUMAN CELLS

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Ageing and longevity are two multifactorial biological phenomena whose knowledge at molecular level is still limited. We have developed a clonal senescence induced system and we have cloned several senescence associated genes. Analysis of the function of one of the isolated genes, encoding for Clusterin/Apolipoprotein J (CLU), suggests that it is a novel survival factor. CLU is found over-expressed in vitro under a variety of stress conditions and in vivo in samples from patients suffering from various age-related diseases as well as in primary tumours which have acquired chemotherapeutic drug resistance (Int J Cancer 120, 611-622, 2007). In addition, it has been demonstrated that inhibition of endogenous CLU expression by RNA interference induces growth retardation, higher rates of endogenous cellular death and sensitizes human cells to stress (Cancer Res 64, 1834-1842, 2004). Recent findings indicate that effective and sustained CLU depletion by siRNA induces late morphological alterations, growth arrest at the $G_1$/S checkpoint and activation of the mitochondrial axis of apoptosis that engages caspase-9. Moreover, CLU knock-down resulted in down regulation of the BH pro-survival (bcl-2 and bcl-$X_L$) proteins and activation of p53 and its downstream targets, namely $p21^{WAF1/CIP1}$ and bax.

We have also attempted an overall molecular and biochemical approach regarding proteasome function in replicative senescence and cell survival. We have observed reduced levels of proteasomal peptidase activities coupled with increased levels of oxidized and ubiquitinated proteins in senescent cells. We have found the catalytic subunits of the 20S complex and subunits of the 19S regulatory complex to be down-regulated in senescent cells. This is accompanied by a decrease in the level of both 20S and 26S complexes (J Biol Chem 278, 28026-28037, 2003). In support, partial inhibition of proteasomes in young cells by specific inhibitors induced a senescence-like phenotype. Stable over-expression of β subunits or POMP in human cell lines resulted in enhanced proteasome assembly and activities and increased cell survival following treatments with various oxidants. Moreover, stable over-expression of $\beta_5$ subunit delayed senescence in human fibroblasts (J Biol Chem 280, 11840-11850, 2005). Finally in search of natural compounds that may activate proteasome, we have identified that the main constituent of olives, oleuropein, exerts stimulatory effects on proteasome. Importantly, continuous treatment of human fibroblasts cultures with oleuropein delays senescence by approximately 15% (Rejuven Res 10, 1570172, 2007).

## S 09. THE ROLE OF THE REPLICATION LICENSING FACTORS CDC6 AND CDT1 IN CARCINOGENESIS

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The accurate execution of DNA replication requires a strict control of the replication licensing factors (RLFs) hCdt1 and hCdc6. The role of these key replication molecules in carcinogenesis has not been, until recently, clarified. Investigation of their status in epithelial lesions covering progressive stages of hyperplasia, dysplasia and full malignancy, mostly from the same patients, revealed how early during cancer development deregulation of these factors occurs. Abnormal accumulation of both proteins occurs early from the stage of dysplasia, while in full blown cancers, elevated levels (more than fivefold) of hCdt1 and hCdc6 characterizes significant proportions of neoplasms. A frequent cause of unregulated hCdc6 and hCdt1 expression is gene amplification, suggesting that these components can play a role *per se* in cancer development. Another source for aberrant hCdc6 and hCdt1 expression is E2F-1, -2-mediated transcriptional overproduction. Forced expression of hCdt1 and hCdc6 promotes re-replication and generates DNA damage, leading to an activated DNA damage response (DDR), which provides the anti-tumor barriers of senescence and apoptosis. Nevertheless, in an inducible hCdt1 cellular system, the continuous deregulated hCdt1 stimulus leads to abrogation of the anti-tumor barriers and results in the selection of clones with more aggressive properties. In addition, stable expression of hCdc6 and hCdt1 in pre-malignant papilloma cells leads to epithelial-mesenchymal transition, producing tumors upon injection into nude mice and thus depicting the oncogenic potential of their deregulation.

## S 10. THE CDK-INHIBITOR P27$^{KIP1}$ LINKS ONCOGENES TO CELL CYCLE CONTROL

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Cell cycle transitions are regulated by the oscillating activities of protein kinases of the family of cyclin-dependent kinases (CDKs). The CDK inhibitor p27$^{Kip1}$ controls cell proliferation by binding to and regulating the activity of CDKs. Usually, binding of the inhibitor results in inactivation of the kinase, however some exceptions have been observed where p27-bound complexes remain catalytically active. p27 is induced by antimitogenic signals and abundant in quiescent cells, where increased protein level can prevent CDK activation and progression through the cell cycle. Levels of the inhibitor protein are elevated in G1 phase of the cell cycle and decline as cells progress towards S-phase. Regulation of p27 expression involves diverse mechanisms including translational control, regulated proteolysis and transcriptional regulation. p27 is haplo-insufficient for tumor suppression and reduced level of the inhibitor frequently correlate with poor prognosis in a large number of human malignancies.

Diverse oncogenic pathways converge at the regulation of p27. We have recently observed that CDK inhibition by p27 and stability of p27 can be directly regulated by tyrosine kinases. Lyn or Bcr-Abl phosphorylate a conserved tyrosine residue of p27, leading to ejection of the inhibitory $3_{10}$-helix of p27 from the catalytic cleft of CDK2. The inhibitor remains bound to the kinase, however its ability to inhibit kinase activity is diminished. In addition, the phosphorylated protein becomes a substrate of CDK2 and this second phosphorylation initiates its degradation by the ubiquitin / proteasome pathway. This novel direct link between transforming tyrosine kinases and p27 can provide an explanation for premature p27 elimination in tumors.

## S 11. REGULATION OF CENTRIOLE DUPLICATION IN MAMMALIAN CELLS

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The centrosome organizes the microtubule cytoskeleton and consists of a pair of centrioles surrounded by pericentriolar material. During the cell-division cycle the centrosome is duplicated near the G1/S transition leading to formation of the spindle apparatus that segregates the duplicated chromosomes into the daughter cells. Polo like kinases (Plks) perform crucial functions in cell-cycle progression and during mitosis. We have previously shown that polo-like kinase-2, Plk2, is activated near the G1/S phase transition, and plays a role in the reproduction of centrosomes during the cell cycle. We find that the polo-box of Plk2 is required both for association to the centrosome and centriole duplication. Plk2 is localized to centrioles during early G1 phase where it only associates to the mother centriole and then distributes equally to both mother and daughter centrioles at the onset of S phase. In addition, we found that Plk2 cooperates with both Cdk2 and Plk4 in regulating centriole duplication. Moreover, our studies indicate that Plk2 is involved in the regulation of cyclinE protein stability since RNAi-mediated down-regulation of Plk2 results in a decrease of cyclinE protein levels. This results in a diminution of phosphorylation levels of centrosomal cyclinE/Cdk2 substrates, namely NPM/B23 and CP110. Ablation of Plk2 function by RNAi also leads to the formation of abnormal mitotic spindles again supporting a function of the kinase in the reproduction of centrioles. Thus, Plk2, among a few known centrosomal proteins, is an important player in orchestrating centriole duplication. Determination of the substrates of Plk2 relevant to centriole duplication will ultimately be necessary to unravel the mechanism by which it controls centriole duplication and shed more light on the regulatory pathways that govern this process.

## S 12. p14/MP1-MEK1 SIGNALING REGULATES ENDOSOMAL TRAFFIC AND CELLULAR PROLIFERATION DURING TISSUE HOMEOSTASIS

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The extra-cellular signal-regulated kinase (ERK) cascade regulates proliferation, differentiation and survival in multi-cellular organisms. Scaffold proteins regulate intracellular signaling by providing critical spatial and temporal specificity. The scaffold protein MEK1 partner (MP1) is localized to late endosomes by the adaptor protein p14. Using conditional gene disruption of *p14* in mice we now demonstrate that the p14/MP1-MEK1 signaling complex regulates late endosomal traffic and cellular proliferation. This function is essential for early embryogenesis and during tissue homeostasis as revealed by epidermis specific deletion of *p14*. These findings show that endosomal p14/MP1-MEK1 signaling has a specific and essential function *in vivo* and therefore implicate that regulation of late endosomal traffic by extra-cellular signals is required to maintain tissue homeostasis.

Work in the Huber laboratory is supported by the Special Research Program “Cell Proliferation and Cell Death in Tumors” (SFB021, Austrian Science Fund).

## S 13. CENTROMERES AND THE CONTROL OF CHROMOSOME SEGREGATION IN MITOSIS

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Chromosome segregation and hence genetic stability depends on assembly of functional kinetochores within centromeric regions. Centromeric DNA and kinetochore protein sequences have diverged very rapidly during evolution. Particularly in Drosophila it has been difficult to identify homologs of centromere and kinetochore proteins known in other eukaryotes, raising speculations that Drosophila centromere biology might be unusual (as clearly established in the case of telomeres). However, apart from the centromere-specific histone H3 (Cenp-A/Cid), we have identified Drosophila Cenp-C and KMN network components. A few diverged Drosophila components identified by co-immunoprecipitation and mass-spectrometry cannot be recognized as orthologs. Nevertheless, our findings indicate that Drosophila kinetochores are organized according to a shared eukaryotic design.

To define the spatial organization of these shared eukaryotic kinetochore components, we have exploited some unique advantages of Drosophila melanogaster. Cenp-A/Cid, Cenp-C, and KMN components were mapped along the inter sister kinetochore axis with an apparent precision below 10 nm. This revealed a polar orientation of not only the Ndc80 complex but also of Cenp-C. The C-terminus of Cenp-C was observed near but well separated from the innermost component Cenp-A/Cid. The N-terminus of Cenp-C is further out, clustered with Mis12 and the Spc25 end of the rod-like Ndc80 complex which is known to bind to microtubules at its other more distal Ndc80/Nuf2 end. Analyses of strechted chromatin fibers suggest that Cenp-C is involved in stabilization of higher order centromeric chromatin structure.

Mitotic spindle checkpoint proteins are only transient kinetochore components. Using centromere-targeting domains of constitutive kinetochore components, we have generated Mps1- and BubR1 variants which persist at the kinetochore throughout mitosis in order to address the role of spindle checkpoint protein shedding via kinetochore MTs in checkpoint silencing before anaphase onset. These variants have failed to provide evidence supporting the importance of shedding.

## S 14. MODULATING $p27^{kip1}$ EXPRESSION LEVELS BY A SMALL MOLECULE AS A THERAPEUTIC STRATEGY IN THE TREATMENT OF COLON CANCER

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The cyclin kinase inhibitor $p27^{kip1}$ acts as an important tumor suppressor protein in a variety of human cancers. Its expression levels closely correlate with the prognosis of the affected patient and also predict the outcome of various treatment modalities. While many tumors express low levels of p27 its genomic locus is rarely mutated in cancer cells. We previously showed that ubiquitin dependent turnover of p27 is part of the progression sequence of intestinal cancers. Using a mouse system in which p27 turnover is partly inhibited we were able to demonstrate that interference with p27 degradation results in a block to tumor cell de-differentiation in the intestine. Based on these results we decided to conduct a small molecule screen to identify substances which block p27 degradation. We identified HZI150006, a natural compound derived from myxobacteria as a potent inducer of p27 expression in a variety of cancer cells. Treatment with HZI150006 induces apoptosis in human colon carcinoma cells *in vitro* and in xenotransplant tumors derived from these cell lines *in vivo*. Moreover HZI150006 also leads to a dramatic reduction of tumor vasculature in xenotransplants and an inhibition of HUVEC tube formation *in vitro*. All of these effects are dependent on p27 expression as cells derived from p27 knockout mice or cells in which p27 expression was lowered by siRNA treatment did not respond to HZI150006. The high efficiency with which HZI150006 targets cancer cells and tumor vasculature combined with its very low toxicity *in vivo* makes a p27 directed therapy using HZI150006 worthy of further clinical development.

## S 15. POSTTRANSLATIONAL MODIFICATION WITH SUMO - THE E3 LIGASE RANBP2

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Small ubiquitin related modifier SUMO-1 and its homologs can be reversibly conjugated to a large number of cellular proteins. Depending on the specific target, SUMOylation may regulate protein interactions, alter intracellular localization, influence stability, or alter activity. The E1 activating enzyme Aos1/Uba2 and the E2 conjugating enzyme Ubc9 are sufficient for modification of some targets, but most require E3 ligases for efficient modification. We identified the nucleoporin RanBP2/Nup358 as a unique E3 ligase that conjugates SUMO to unknown proteins at the NPC, and catalyses sumoylation of Sp100 and HDAC4 in vitro (1). In vivo targets are still unknown. RanBP2's 30 kD E3 ligase domain is unrelated to other SUMO- or ubiquitin E3 ligases, as it is a natively unfolded protein fragment that assumes partial structure only in association with the SUMO E2 conjugating enzyme Ubc9 (2). In addition to its role as a SUMO E3 ligase, RanBP2 has long been known to serve as a docking site for nuclear transport complexes in and out of the nucleus. More recently, it has also been implicated in kinetochore functions during mitosis, this time in association with the transport receptor Crm1 (reviewed in 3). Interestingly, RanBP2's SUMO E3 ligase domain and binding sites for transport receptors, the GTPase Ran and sumoylated RanGAP1 all are contained within an 80 kD RanBP2 fragment. This suggests that transport receptors and Ran may have an influence on RanBP2's E3 activity and/or target selection, a hypothesis that we are currently following.

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3. Dasso M. Biochem Soc Trans. 2006, 34, 711-715 (review).

## S 16. A ROLE FOR MAMMALIAN APC-Cdh1 IN CONTROLLING DNA REPLICATION AND GENOMIC STABILITY

**Irene García-Higuera[1], Eusebio Manchado[2], Marcos Malumbres[2] and Sergio Moreno[1]**
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Progression throughout the cell cycle is modulated by different E3 ubiquitin ligases that target cell cycle regulators for degradation in the proteasome. The Anaphase-Promoting Complex (APC) is an E3 ubiquitin ligase that associates with two co-activators, Cdc20 and Cdh1. APC-Cdh1 is active from anaphase until the end of G1 and promotes the degradation of cyclins A and B, Cdc20, Aurora A, Aurora B and some DNA replication regulators, such as Cdc6 and geminin. To understand the specific roles of Cdh1 and the possible overlap with Cdc20 function, we have recently generated Cdh1 conditional knock out mice and analyzed cell cycle progression in the absence of Cdh1. Cdh1-deficient mouse embryonic fibroblasts grew in culture with slower kinetics than wild-type cells. These mutant cells displayed deficient exit from mitosis and abnormal S-phase entry after stimulation with serum. The relative levels of APC-Cdh1 substrates will be presented and the consequences of deregulated APC-Cdh1 activity on DNA replication and genomic instability will be discussed.

## S 17. DOES COHESIN CONCATENATE SISTER DNAS?

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Sister chromatids are held together by multi-subunit complex called cohesin whose Smc1 and Smc3 subunits are rod shaped proteins with ABC-like ATPases at one end of 50nm long intra-molecular anti-parallel coiled coils. At the other ends are pseudo-symmetrical hinge domains that interact to create V shaped Smc1/Smc3 heterodimers. N- and C-terminal domains within cohesin's third subunit, kleisin, bind to Smc3 and Smc1 ATPase heads respectively, thereby creating a huge tripartite ring whose integrity is essential for holding sister DNAs together. Proteolytic cleavage of kleisin by separase opens the cohesin ring and triggers sister chromatid disjunction. We have proposed that cohesin associates with chromosomes by trapping DNA inside its ring and that it holds sister DNAs together by trapping them within the same ring. This hypothesis makes three key predictions: 1) linearization of a small circular chromosome should permit chromatin fibres to slide through the cohesin ring and should therefore sever the connection between sister DNAs, 2) introduction of site-specific chemical cross links at the three interfaces between cohesin's Smc1, Smc3, and Scc1 subunits should trap circular sister DNAs inside a single, albeit huge, circular cohesin molecule even after protein denaturation in the presence of a strong ionic detergent at high temperature, and 3) entry of DNA inside a cohesin ring must involve transient dissociation of one it's three interfaces. My talk will discuss experiments that test all three predictions.

## S 18. CELL CYCLE CONTROL: HOW TO PRESERVE CHROMOSOME NUMBERS DURING CELL DIVISION ?

**E.A. Nigg**

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The error-free segregation of duplicated chromosomes during cell division is vital to the development and health of all organisms. Chromosomal instability and imbalances (aneuploidy) are typical of many solid human tumors and often correlated with malignancy. Many chromosome aberrations are likely to result from the deregulation of mitotic progression, a defective spindle checkpoint and/or centrosome abnormalities. Thus, our research aims at elucidating the role of protein kinases (and phosphatases) in the control of cell division, with a special emphasis on the function of the spindle assembly checkpoint and the regulation of the centrosome cycle. In this lecture, I will describe our phospho-proteomic studies on the human spindle apparatus and our recent discovery of PICH, a novel key component of the spindle assembly checkpoint.

(Selected recent references: Habedanck et al., (2005), Nat Cell Biol., 7:1140-1146; Nousiainen et al. (2006), Proc. Natl. Acad. Sci. USA, 103:5391-6; Silljé et al., (2006), Curr. Biol., 16: 731-42; Hanisch et al. (2006), EMBO J. 25:5504-15; Nigg (2006), Int J Cancer. 119:2717-23; Nigg (2007), Trends Cell Biol. 17:215-21; Baumann et al. (2007), Cell 128:101-14).

## S 19. REGULATION OF SISTER CHROMATID COHESION IN MAMMALIAN CELLS

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Sister chromatids are connected by cohesin complexes. This cohesion is essential to allow the bipolar attachment of chromosomes to the spindle, whereas the subsequent removal of cohesin from chromosomes is required for anaphase. In vertebrates, the bulk of cohesin is removed from chromosome arms already in prophase by a mechanism that depends on the mitotic kinases Plk1 and Aurora B, and on the protein complex condensin I. Cohesin at centromeres is protected from this "prophase pathway" by a protein called Sgo1. In metaphase, the protease separase is activated, cleaves the Scc1 subunit of centromeric cohesin and thereby initiates anaphase.

To identify additional proteins that might control the association of cohesin with chromatin we have purified human cohesin and analyzed associated proteins by mass spectrometry. One protein we identified is an ortholog of the Drosophila protein Wapl (wings-apart like) that has been implicated in heterochromatin formation and chromosome segregation. Human Wapl is specifically associated with cohesin throughout the cell cycle and, like cohesin, is associated with chromatin from telophase until prophase of the next mitosis. Depletion of Wapl blocks cohesin dissociation from chromosomes during the early stages of mitosis and prevents the resolution of sister chromatids until anaphase, which occurs after a delay. FRAP experiments revealed that Wapl depletion also increases the residence time of cohesin on chromatin in interphase. Based on our data we propose that Wapl is part of a molecular device that is required to unlock cohesin from a particular state in which it is stably bound to chromatin.

Another protein that we detected in cohesin samples purified from chromatin is sororin, a protein that was known to be required for cohesion. To obtain further insight into sororin's function we have addressed when during the cell cycle sororin is required for cohesion. Our data indicate that sororin is dispensable for the association of cohesin with chromatin but that sororin is essential for proper cohesion during G2-phase. Like cohesin, sororin is also needed for efficient repair of DNA double strand breaks in G2. Finally, sororin is required for the generation of normal amounts of the stably chromatin-bound population of cohesin in G2. Our data indicate that sororin is a chromatin protein that functions during the establishment or maintenance of cohesion in S and G2-phase, respectively.

## S 20. GETTING INTO AND OUT OF MITOSIS

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We are trying to understand how cells divide to ensure that their two daughter cells receive an identical copy the genome. This is clearly essential to genomic stability and defects in the checkpoints controlling chromosome segregation may contribute to tumorigenesis. We have developed a FRET sensor that is specific for cyclin B1-Cdk1 activity and are using this to determine when and where cyclin B1-Cdk1 is activated and inactivated in living, dividing cells. Once cells have entered mitosis, the key to the control of chromosome segregation is the regulation of the Anaphase Promoting Complex/Cyclosome (APC/C) by the spindle assembly checkpoint. We have developed a live cell assay to monitor APC/C activity and its inhibition by the spindle checkpoint. This has revealed that the checkpoint is intrinsic to the timing of mitosis, and partially explains the ability of the APC/C to select the right substrate at the right time. We have found that the APC/C is recruited to unattached kinetochores by the checkpoint proteins that may explain the tight temporal control on the APC/C by the checkpoint. We are currently testing the idea that this temporal control requires that the checkpoint and ubiquitination machineries interact at the spindle. Moreover, the interplay between the APC/C and its regulators are essential to coordinate mitosis with DNA replication.

## S 21. DYSREGULATION OF CELL CYCLE CONTROL GENES AND GENOMIC INSTABILITY

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Proteins that normally contribute to cell viability by controlling cell cycle progression must be properly regulated in order to not become deleterious to the genomic integrity of the cell. Cyclin E1, as well as, the Cks protein paralogs, Cks1 and Cks2 are found overexpressed in a broad spectrum of human cancers, suggesting that these proteins when deregulated contribute to malignancy. In order to understand the role(s) of these proteins in oncogenesis, we have investigated the cellular consequences of their deregulation and overexpression. Cyclin E deregulation appears to promote genomic instability by several independent mechanisms: by impairing pre-replication complex assembly and by blocking the metaphase-anaphase transition. The former leads to replicative stress and possibly chromosome instability, whereas the latter leads to polyploidy. Cks1 and Cks2 share a redundant function required for transcription of mitotic regulatory genes *CCNB1*, *CCNA2*, and *CDK1*. Depletion of both Cks proteins in somatic cells leads to G2 arrest followed by polyploidy or cell death, depending on the cell type. Surprisingly, overexpression of Cks proteins leads similarly to G2 arrest and polyploidy, although the mechanism is completely different. These results suggest that dysregulation of cell cycle control genes often promotes oncogenesis by causing genomic instability and ultimately aneuploidy.

## S 22. HOW TO KEEP THE ORDER RIGHT WHEN THE TIMING IS WRONG? INSIGHTS INTO MECHANISMS COUPLING S PHASE AND MITOSIS IN BUDDING YEAST

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In eukaryotes the duplication and segregation of chromosomes occur during discrete periods of the cell cycle, S phase and mitosis, which are separated by an intervening G2 phase. In fact two types of controls, **timing** and **checkpoints**, ensure that chromosomes are completely duplicated and faithfully segregated during cell division, thus preserving genome stability. Surveillance mechanisms (checkpoints) delay mitosis when DNA is damaged or under-replicated, or when sister chromatids are not properly attached to the mitotic spindle. In yeast neither the DNA damage (Mec1, Chk1, Rad53) nor the spindle assembly (Mad1-3, Bub1, 3) checkpoint proteins are essential for viability, unless cells are stressed by conditions that damage DNA, stall replication forks or impede kinetochore-microtubule attachment.

To identify mechanisms coupling M phase to the completion of S phase when **replication forks progress normally**, we changed the **timing** of S phase to make it coincide with mitosis. We will show that cells in which the onset of S phase is delayed, but that nevertheless replicate chromosomes with normal pace and order, crucially rely on both *MEC1* and *MAD2* pathways for their viability. When chromosomes replicate late, anaphase is delayed through inhibition of Cdc28 on Tyr19 as well as by Mec1- and Mad2-dependent stabilization of Pds1/securin. Removing both Mec1 and Mad2 causes mitotic catastrophe with Rad52 foci suggestive of chromosomal breakage. Our results indicate that phosphorylation of $Cdc28_{Tyr19}$ does play a role, after all, in coupling S and M phases in budding yeast. They also underscore the importance of temporal control of DNA replication for correct chromosome segregation and suggest that DNA damage and spindle checkpoints might have evolved at a time when both S and M were triggered by a unique ancestral cyclin B-Cdk1 complex.

## S 23. F-BOX DEPENDENT AND INDEPENDENT FUNCTIONS OF RCA1

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Rca1/Emi proteins are essential inhibitors of the anaphase-promoting-complex/cyclosome (APC/C). In *Drosophila,* Rca1 is required during G2 to prevent premature cyclin degradation by the APC/C-Fzr complex. All members of the Emi1/Rca1 family share a conserved zinc binding region (ZBR) which is required for its inhibitory activity towards the APC/C. The Emi1/Rca1 family proteins also belong to the class of F-Box proteins that are known to act as substrate recognition subunits in SCF-E3-Ligase complexes. The F-Box in Emi1 and Rca1 is functional as both can interact with members of the SKP family. However, no F-box dependent function has been ascribed to these proteins.
We have performed a structure/function analysis of Rca1 demonstrating that a C-terminal fragment is sufficient for APC/C inhibition. We find that the F-box of Rca1 is dispensable for APC/C-Fzr inhibition during G2. Nevertheless, we demonstrate that Rca1 has an additional function at the G1-S transition, which requires the F-box. Overexpression of Rca1 accelerates the G1-S transition in an F-box dependent manner. Conversely, S-phase entry is delayed in cells in which endogenous Rca1 is replaced by a transgene lacking the F-box. We show that Rca1 interacts with SCF-components like Skp and Cullin and present our efforts to identify possible targets of the SCF-complex in which Rca1 acts.
To define the function S-phase function of Rca1, we have tested the effect of Rca1 overexpression in salivary glands, a tissue that undergoes endoreplication and lacks a mitotic program. Furthermore, it is thought that APC/C-Fzr is not required once the endoreplication has been initiated. By using this tissue, we could bypass the G2-function of Rca1 of mitotic cycles. Surprisingly, we observed that Rca1 blocks the endoreduplication cycles by promoting expression of several mitotic genes that are usually downregulated. We thus propose that Rca1 acts as an F-box protein in an SCF-complex whose function is to promote expression of mitotic genes. Rca1 might therefore be crucial for the maintenance of the mitotic program and the balance between proliferative and endoreduplication cycles.

## S 24. MITOTIC CHROMOSOME CONDENSATION AND SEGREGATION

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Mitotic chromosome structure depends on the chromosomal condensin complex. Without condensin, metaphase chromosomes remain undercondensed and lack structural stability. We have asked where along budding yeast chromosomes the condensin complex associates, and what we can learn from its binding pattern about the mechanism of chromosome condensation. Our results suggest that condensin, like its relative the cohesin complex, is loaded onto chromosomes by a loading factor, the Scc2/4 complex. Unlike cohesin, that moves away from its loading sites after the loading reaction, condensin remains at the loading sites. We discuss the implications of these patterns for mitotic chromosome structure.

Furthermore, condensin is required during anaphase to promote sister chromatid resolution. In the absence of condensin, strong anaphase bridges and segregation defects are observed. How condensin promotes sister chromatid resolution is unknown. We have used the budding yeast rDNA as a model locus, whose segregation depends on condensin activity during anaphase. We show that anaphase bridges in a condensin mutant are resolved by ectopic expression of a foreign (Chlorella virus) but not endogenous yeast topoisomerase II (topo II). This suggests that catenation prevents sister rDNA segregation, and that yeast topo II is ineffective in decatenating the rDNA, and maybe other chromosomal regions, in the absence of condensin.

## S 25. DEVELOPMENTAL CONTROL OF CELL DIVISION IN *C. elegans*

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Animal development requires close coordination between cell division and differentiation. We focus our research on the integrated execution of these processes. On the one hand, we study how asymmetric cell divisions generate daughter cells that differ in developmental potential. In addition, we search for control mechanisms that prevent cell-cycle entry of terminally differentiated cells.

Asymmetric division of stem-cell like progenitors usually generates a new uncommitted progenitor (self-renewal) and a more differentiated daughter. Critical in this process is the plane of cell cleavage, which is determined by the position of the mitotic spindle. Previously, we have shown that the coiled-coil protein LIN-5 is required for chromosome segregation and spindle positioning in *C. elegans*. LIN-5 localizes to the spindle apparatus as well as the cell cortex. LIN-5 acts together with G protein regulators (GPR-1/2) of the Pins/LGN family as well as Gai/o subunits and a non-receptor exchange factor (RIC-8). Through evolutionarily conserved interactions, these proteins control microtubule-associated pulling forces and thereby the position of the spindle. We have identified novel candidate partners of LIN-5, one of which is related to *Drosophila* ASP and human ASPM (Abnormal SPindle-like, Microcephaly-associated). Functional interaction between LIN-5 and ASPM-1 controls the cleavage plane in meiosis.

A second project starts from the observation that deregulated cell-cycle control in *C. elegans* causes extra divisions only in certain lineages. Inactivation of critical negative regulators and/or ectopic expression of Cyclin/CDKs does not induce S phase in differentiated cells. Apparently, additional levels of control prevent S phase entry of such cells. To identify such controls, we combine deregulation of candidate genes with random gene inactivation in strains carrying cell-cycle reporters.

Our goal is to better understand the developmental decision to proliferate or differentiate, ultimately to acquire improved control over this decision in cancer treatment and regenerative medicine.

## S 26. INTERACTION OF A TPR MOTIF IN BUBR1 AND BUB1 WITH A KINETOCHORE PROTEIN ENSURES SPINDLE CHEKPOINT AND CHROMOSOME ALIGNMENT IN HUMAN CELLS

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The spindle checkpoint control and chromosome alignment are the fundamental features during mitotic progression, and require, respectively, BubR1 and Bub1 among others. While BubR1 and Bub1 are temporally recruited to kinetochores in mitosis, the kinetochore-docking site for these proteins remains unknown. We found that a human kinetochore oncoprotein blinkin/AF15q14 links BubR1 and Bub1 to kinetochores. Our rescue experiments indicate that direct association of Bub1 and BubR1 with blinkin/AF15q14 assures spindle checkpoint and chromosome alignment. The phenotypes of blinkin RNAi, accelerated mitosis by the failure of checkpoint control and chromosome misalignment due to the lack of kinetochore and microtubule attachment, resemble the double RNAi phenotypes of Bub1 and BubR1. While the carboxy domain of blinkin/AF15q14 associates with the hMis13 and hMis14 subunits of the hMis12 complex in the inner kinetochore, the association of the amino and middle domain of blinkin/AF15q14 with the TPR (tricotetrapeptide repeat) domains in the amino termini of BubR1 and Bub1 is essential for BubR1 and Bub1 to execute their distinct mitotic functions. Blinkin/AF1q14 may be the center in the network of generating kinetochore-based checkpoint signaling.

## S 27. CHROMOSOMAL INSTABILITY AND TUMOUR PROGRESSION

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Chromosomal instability in terms of numerical aberrations (aneuploidy) and structural rearrangements (deletions, gene amplifications and translocations) is likely to be essential and rate limiting for tumour progression. Defects in cell cycle control, particularly defects in the cell cycle checkpoints, seem to play a central role in chromosomal destabilization. We study the role of chromosomal instability in tumour progression, particularly in the development of distant metastases.

Most of the human tumours can be divided into two major groups on the basis of gross ploidy level, highly aneuploid tumours (A-tumours) and pseudo-diploid tumours (D-tumours). The A-tumours develop distant metastases rapidly, and usually kill a large proportion of the patients within a few years. The D-tumours usually progress slowly. This difference in rate of tumour progression between A- and D-tumours most likely reflects differences in rate of mutation due to chromosomal instability.

Combining high-resolution microarray-CGH analysis (ROMA) with quantitative multigene fluorescence in situ hybridization (QM-FISH) provides the opportunity to detect and validate a wide range of chromosomal rearrangements, such as gene amplifications, deletions and duplications. We have used these combined techniques to examine 243 aneuploid and pseudo-diploid breast tumours (A-tumours and D-tumours), for which long term follow-up and detailed clinical information were available.

D-tumors are particularly useful subjects for this approach, revealing complex rearrangements and repeated sequential amplification events on certain chromosomes that provide unique insights into the genomic progression of the disease. First, the fine structure of these amplification clusters, as detected by ROMA and quantitatively validated by QM-FISH, provides extremely high resolution “pointers” to potential novel oncogenes, since many of the detected amplicons contain only one or two known or prospective genes. Second, QM-FISH patterns provide a means for interpretation of the mechanism of these events. Third, the reproducibility and frequency of these events, especially in very early stage of tumour development, provides insight into the earliest chromosomal events in breast cancer. Finally, we have identified very strong correlations between certain sets of rearrangement events and clinically relevant parameters such as development of distant metastasis and long-term survival. These correlations may enable novel and powerful prognostic indicators for breast and other cancers when more samples can be examined.