Abstracts of papers presented
at the 2010 meeting on

THE CELL CYCLE

May 18–May 22, 2010

Cold Spring Harbor Laboratory
Cold Spring Harbor, New York

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at the 2010 meeting on

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May 18–May 22, 2010

Arranged by

Sue Biggins, *Fred Hutchinson Cancer Research Center*
Nicholas Dyson, *Massachusetts General Hospital Cancer Center*
Johannes Walter, *Harvard Medical School*

Cold Spring Harbor Laboratory
Cold Spring Harbor, New York

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THE CELL CYCLE

Tuesday, May 18 – Saturday, May 22, 2010

Tuesday	7:30 pm	Keynote Speaker **1** Chromosome Cohesin and Condensation
Wednesday	9:00 am	**2** G1 and Cdk Regulation
Wednesday	2:00 pm	**3** Poster Session I
Wednesday	4:30 pm	Wine and Cheese Party *
Wednesday	7:30 pm	**4** S-Phase
Thursday	9:00 am	**5** The DNA Damage Response
Thursday	2:00 pm	**6** Poster Session II
Thursday	7:30 pm	**7** Cell Cycle Regulatory Pathways
Friday	9:00 am	**8** Chromosome Segregation
Friday	2:00 pm	**9** Metaphase / Anaphase
Friday	6:00 pm 7:00 pm	Concert Banquet
Saturday	9:00 am	**10** Proteolysis

Poster sessions are located in *Bush Lecture Hall*

* *Airslie Lawn*, weather permitting

Mealtimes at Blackford Hall are as follows:

Breakfast 7:30 am-9:00 am

Lunch 11:30 am-1:30 pm

Dinner 5:30 pm-7:00 pm

Bar is open from 5:00 pm until late

Printed on 100% recycled paper.

PROGRAM

TUESDAY, May 18—7:30 PM

SESSION 1 CHROMOSOME COHESIN AND CONDENSATION

Chairperson: **D. Morgan,** University of California, San Francisco
J.-M. Peters, Research Institute of Molecular Pathology, Vienna, Austria

KEYNOTE SPEAKER

Douglas Koshland
University of California, Berkeley

"Lessons learned from trying to answer to a higher order"

WEDNESDAY, May 19—9:00 AM

SESSION 2 G1 AND CDK REGULATION

Chairperson: **S. Haase,** Duke University, Durham, North Carolina
J. Lees, Massachusetts Institute of Technology, Cambridge

WEDNESDAY, May 19—2:00 PM

SESSION 3 POSTER SESSION I

WEDNESDAY, May 19—4:30 PM

Wine and Cheese Party

WEDNESDAY, May 19—7:30 PM

SESSION 4 S-PHASE

Chairperson: **J. Diffley,** Cancer Research UK London Research Institute, United Kingdom
B. Duronio, University of North Carolina, Chapel Hill

THURSDAY, May 20—9:00 AM

SESSION 5 THE DNA DAMAGE RESPONSE

Chairperson: **D. Toczyski,** University of California, San Francisco
H. Piwnica-Worms, Howard Hughes Medical Institute, Washington University, St. Louis, Missouri

SESSION 7 CELL CYCLE REGULATORY PATHWAYS

Chairperson: **P. O'Farrell,** University of California, San Francisco
B. Edgar, Universität Heidelberg, Germany

CDK1-cyclin B1-dependent destruction of Mcl-1 during mitotic arrest sensitizes cells to microtubule poisons
Paul R. Clarke, Lindsey A. Allan, Margaret E. Harley, Helen S. Sanderson.
Presenter affiliation: University of Dundee, Dundee, United Kingdom. 208

Cell cycle exit induced by serum withdrawal regulates Rab11 recycling endosome, trafficking Rab8 activation, and the assembly of the primary cilium
Peter K. Jackson.
Presenter affiliation: Genentech, Inc, S. San Francisco, California. 209

Cell cycle exit and re-entry from quiescence in budding yeast
Lihong Li, Shawna Miles, Kate Braun, Amalthiya Prasad, Linda L. Breeden.
Presenter affiliation: Fred Hutchinson Cancer Center, Seattle, Washington. 210

Spontaneous cancer cell quiescence with epigenetic remodeling arising during mitotic telophase
Sridhar Ramaswamy, Anita Wolfer, Ipsita Guha, Ben S. Wittner, Albert Yeh.
Presenter affiliation: Massachusetts General Hospital, Boston, Massachusetts; Harvard Medical School, Boston, Massachusetts. 211

FRIDAY, May 21—9:00 AM

SESSION 8 CHROMOSOME SEGREGATION

Chairperson: **D. Pellman,** Dana-Farber Cancer Institute, Boston, Massachusetts
T. Kapoor, Rockefeller University, New York, New York

Cooperation of the Dam1 and Ndc80 complexes enhances kinetochore-microtubule coupling and is regulated by Aurora B
Jerry F. Tien, Neil T. Umbreit, Daniel R. Gestaut, Andrew D. Franck, Beth Graczyk, Jeremy Cooper, Linda Wordeman, Tamir Gonen, Charles L. Asbury, Trisha N. Davis.
Presenter affiliation: University of Washington, Seattle, Washington. 212

Loss of pRB causes centromere dysfunction and chromosomal instability
Amity L. Manning, Michelle S. Longworth, Nicholas J. Dyson.
Presenter affiliation: Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts. 220

A mechanism linking whole-chromosome aneuploidy to DNA breaks
David Pellman.
Presenter affiliation: Dana-Farber Cancer Institute, Boston, Massachusetts; Harvard Medical School, Boston, Massachusetts. 221

FRIDAY, May 21—2:00 PM

SESSION 9 METAPHASE / ANAPHASE

Chairperson: **R. Medema,** University Medical Centre Utrecht, The Netherlands
T. Davis, University of Washington, Seattle

Cep192 promotes centrosome-driven spindle assembly by engaging in organelle-specific Aurora A activation
Vladimir Joukov, Arcangela De Nicolo, Alison Rodriguez, Johannes C. Walter, David M. Livingston.
Presenter affiliation: Dana-Farber Cancer Institute, Boston, Massachusetts; Harvard Medical School, Boston, Massachusetts. 222

Cep152 is required for Plk4-induced centriole duplication
Ingrid Hoffmann, Uta Haselmannn-Weiss, Claude Antony, Marc Arnold.
Presenter affiliation: Deutsches Krebsforschungszentrum, Heidelberg, Germany. 223

Aurora A-dependent recruitment of TACC3 to spindles reveals a complex containing clathrin and a novel +TIP
Alexander W. Bird, Nina C. Hubner, Per O. Widlund, Matthias Mann, Anthony A. Hyman.
Presenter affiliation: Max-Planck-Institute of Molecular Cell Biology and Genetics, Dresden, Germany. 224

FRIDAY, May 21—6:00 PM

CONCERT
Grace Auditorium

Einav Yarden, piano
Sergey Ostrovsky, violin

Israeli pianist Einav Yarden has performed as a soloist with the Israel Philharmonic Orchestra, Minnesota Symphony, Calgary Philharmonic, Jerusalem Symphony, Plovdiv Philharmonic, The Israel Symphony, The Spirit of Europe Symphony, under Sir Neville Marriner, Aldo Ceccato, Leon Botstein, Mark Russell Smith, Mendi Rodan and others.

She is a Prize Winner at the prestigious 2006 Minnesota International Piano-e-Competition and was also a finalist at the 2003 Honens International Piano Competition. In addition, she won First Prize at the Aviv Competitions, the most highly acclaimed competition in Israel, taking the Guralnik Prize and the Zilbermann Prize. Other prizes include First Prize at the Katz Piano Competition, the Buchman-Heyman Prize for Excellence and Achievement amongst others. She was a recipient of the AICF scholarships, an important scholarship institution in Israel, between1996-2005.

In 2005 completed four years of extensive study with renowned pianist Leon Fleisher at the Peabody Conservatory, earning her Masters of Music and Graduate Performance Diploma. Before moving to the USA, she graduated with highest honors from the Israel Rubin Music Academy (now the Mehta-Buchman School of Music) under the instruction of Emanuel Krasovsky and prior to that studied with Hadassa Gonen.

She recently came back from a highly acclaimed debut recital at France's "Flâneries des Reims" Festival. Other recent appearances in France include solo recitals at Menton Festival and at Paris's prestigious Salle Cortot. In Switzerland, she was invited by Andras Schiff to take part in a special workshop on Beethoven concerti at the Lucerne Festival last summer.

Aside to her solo performances, she is a highly enthusiastic chamber musician, and devotes herself to regular collaborations with other musicians. She toured in the USA, performing in such series' as the prestigious Ravinia Rising Stars Series, the Dame Myra Hess Recital Series, Jupiter Symphony Chamber Players, Rockefeller University, and many others. She gives solo recitals in various venues in New York City, and in one of her projects initiated and engaged in a unique project with a poet, interweaving piano music and original poetry.

Other than performing in Israel's most important halls, she performs across the USA and Canada, and in Europe appeared in Austria, Italy, Switzerland, The Netherlands, France, Bulgaria, Scotland, and England.

Sergey Ostrovsky has performed as a quartet member and as a soloist at internationally renowned venues such as Carnegie Hall and Alice Tully Hall (New York), Kennedy Centre and The Library of Congress (Washington), Wigmore Hall and Royal Festival Hall (London), Auditorium du Louvre, Theatre du Chatelet, and Theatre de la Ville (Paris), De Doelen Rotterdam, Sydney Opera House, Tonhalle Zurich, Capetown City Hall, Durban Concert Hall, Cologne Philharmonie, Beethoven House (Bonn, Germany) Wiener Konzerthouse, Tchaikovsky Hall of Moscow Conservatory, Valensia Concert Hall and other venues in Israel, Germany, Austria, England, France, Ireland, Ireland, Holland, China, Canada, Portugal, South Africa, Brazil etc.

As a soloist Sergey performed with orchestras such as Israel Philharmonic, Cape Town Philharmonic Verbier Festival Chamber Orchestra, Jerusalem Symphony Orchestra, Durban KZN Philharmonic, Moscow Philharmonic, Johannesburg Philharmonic, Brooklyn Philharmonic, Israel Chamber Orchestra, “Amadeus” Chamber Orchestra (Poland) Nizhny Novgorod (Gorky) Philharmonic a, Beer Sheva Sinfonet and “Tel Aviv Soloists” ensemble under the baton of Zubin Mehta, Yoel Levi, Philippe Entremont, Maxim Vengerov, Bernard Guller, Zeev Dorman, Uriel Segal, and the others.

Sergey made a few recordings for Naxos label, and EMI Classics with Maxim Vengerov and UBS Verbier Chamber Orchestra.

Sergey Ostrovsky was born in 1975 in Gorky (Nizhny Novgorod) to a family of musicians, began playing violin at the age of 6, and studied with David Lapidus as his first teacher. He made his first concert appearances at the age of 8, and performed for a first time with a symphony orchestra at the age of 13. In the Music College of Gorky Conservatory continued his studies with Lazar Gantman and Yury Gluchovsky. In 1991 immigrated to Israel with his family and continued his studies with Prof. Yair Kless and Irina Svetlova at the Rubin Academy Tel Aviv(from 2005-Buchmann-Mehta High School of Music) In 1996 Sergey founded the Aviv Quartet which moved for studies in Europe with the members of Alban Berg Quartet in Cologne Hochschule (1998-2000), and with members of Daniel Quartet in Rotterdam Conservatory, where he completed his Bachelor Degree Diploma with Natalia Morozova in 2000, and his Master Degree Diploma in Amsterdam Conservatory with Alexander Kerr in 2002.

FRIDAY, May 21

CONCERT

Cocktails 7:00 PM Dinner 7:45 PM

SESSION 10 PROTEOLYSIS

Chairperson: **B. Schulman,** St. Jude Children's Research Hospital, Memphis, Tennessee
J. Pines, University of Cambridge, United Kingdom

Structural studies of ubiquitination machinery
Brenda Schulman.
Presenter affiliation: St. Jude Children's Research Hospital, Memphis, Tennessee.

Structural electron microscopy studies of the COP9 signalosome in complex with neddylated SCF
Radoslav Enchev, Anne Schreiber, Fabienne Beuron, Ed Morris.
Presenter affiliation: The Institute of Cancer Research, London, United Kingdom. 232

Cell cycle control by replication-coupled protein destruction
Robert Duronio.
Presenter affiliation: University of North Carolina, Chapel Hill, North Carolina. 233

CRL4^{Cdt2} regulates G2/M progression by targeting the p53 and histone H4K20 methyltransferase Set8 for degradation
Tarek Abbas, Etsuko Shibata, Jonghoon Park, Sudahkar Jha, Neerja Karnani, Anindya Dutta.
Presenter affiliation: University of Virginia, Charlottesville, Virginia. 234

CRL4^{Cdt2} -dependent destruction of the Set8 histone methyltransferase in S phase and after DNA damage
Richard C. Centore, Courtney G. Havens, Alice Tse, Johannes Walter, Lee Zou.
Presenter affiliation: Massachusetts General Hospital, Charlestown, Massachusetts. 235

COHESIN AND SORORIN ARE RECRUITED TO CHROMATIN BY DISTINCTLY DIFFERENT MECHANISMS

Jane Song, Andrea L Lafont, Susannah Rankin

Oklahoma Medical Research Foundation, Cell Cycle and Cancer Biology, 835 NE 13th St, Oklahoma City, OK, 73104

Sister chromatid cohesion ensures that replicated chromosomes are held together from the time they are made until cell division. Sister chromatid cohesion is established and maintained by the actions of several proteins on cohesin, a large multi-subunit protein complex that is thought to entrap the sister chromatids. The mechanistic and topological details of how cohesin holds chromosomes together, and how cohesion is enhanced or affected by components of the replication fork are not known.

In an effort to better understand how cohesion is regulated, we have investigated the dependencies among various cohesion factors, with particular emphasis on factors implicated in cohesion establishment. Using extracts from *Xenopus* eggs, we have tested the effects of DNA replication on chromatin loading of the two vertebrate cohesion establishment factors, Eco1 and Eco2. We have also tested the dependencies among cohesin, the Eco proteins, and sororin, a protein required for cohesion in vertebrates.

Our data show that cohesin and sororin are recruited to the chromatin by distinctly different mechanisms. We show that sororin binds to a site that is depends on DNA replication, the cohesin complex, and the Eco family of acetyltransferases. Together our data suggest that sororin is a cohesion maintenance factor, recognizing and perhaps stabilizing cohesin that is modified during DNA replication.

IDENTIFICATION AND CHARACTERIZATION OF A NOVEL COHESIN PROTEINASE MEDIATING SISTER CHROMATID SEGREGATION

Anil K Panigrahi, Debananda Pati

Texas Children's Cancer Center, Baylor College of Medicine, Department of Pediatric Hematology, Oncology, Molecular and Cellular Biology, 1102 Bates Ave., Houston, TX, 77030

From S phase until the onset of anaphase, the sister chromatids are held together by a multi-protein ring-like structure called Cohesin. The cohesin ring comprises of four proteins: Rad21, Smc1, Smc3 and one of SA1 or SA2. In higher eukaryotes, the resolution of sisters during mitosis is accomplished by a two-step removal of cohesin from the chromosomes. The bulk of cohesin is removed from chromosome arms in prophase in a process triggered by the kinase PLK1. The residual centromeric cohesin is removed at Metaphase-to-anaphase transition when cleaved by Separase, a CD-clan proteinase. Such cleavage opens the cohesin ring and releases the sisters, allowing them to segregate. Thus, maintenance and regulation of sister chromatid cohesion and separation is thought to be the primary function of Rad21. In recent years, however, Rad21 has been implicated in regulation of boundary elements, transcription and DNA damage repair. Our laboratory had earlier shown that Rad21 is cleaved during apoptosis at sites distinct from Separase cleavage, and functions both as a responder to, and stimulator of, apoptotic signals.

In an effort to identify and purify the proteinase responsible for apoptotic cleavage of Rad21, we prepared nuclear extract from etoposide-treated Molt4 cells and fractionated over several chromatographic steps. The enriched active fractions were then bound to a Rad21-affinity matrix. The bound proteins were analyzed by LC/MS-MS, which revealed a single peptidase among 96 proteins identified. The lone proteinase is identified as the catalytic subunit of Calpain. We demonstrate that Calpain cleaves Rad21 in vitro; and also in vivo when intracellular calcium content is increased. Calpain has been implicated in apoptosis, necrosis, development and differentiation. However, Calpain-mediated cleavage of Rad21 is not associated with apoptosis or necrosis. Instead, Rad21 cleavage by calpain results in precocious segregation of the sister chromatids. The Calpain cleavage site on Rad21 is distinct from the ones cleaved by Separase. This work identifies Calpain as a novel cohesin-proteinase promoting sister chromatid separation, and suggests existence of a novel chromosome-segregation pathway that integrates chromosome biology with cellular calcium signaling.

(Supported by Award Number 1RO1 CA109478 from National Cancer Institute)

COHESIN SUBUNIT SCC1 PROMOTES CASPASE ACTIVATION AND CELL DEATH IN MITOSIS

Laura A Diaz-Martinez[1,2], Zemfira Karamysheva[1], Soonjoung Kim[1], Hongtao Yu[1,2]

[1]UT Southwestern Medical Center, Pharmacology, Forest Park Rd, Dallas, TX, 75390, [2]Howard Hughes Medical Institute, HHMI, Forest Park Rd, Dallas, TX, 75390

Faithful chromosome segregation in mitosis relies on the precise regulation of sister chromatid cohesion. Sister chromatids ought to be cohered from the time of DNA replication to the onset of anaphase when cohesion is dissolved to allow chromosome segregation. Key to the regulation of sister chromatid cohesion is the spindle checkpoint. This surveillance mechanism delays anaphase onset in response to chromosome attachment defects, thus preventing aberrant chromosome segregation and genomic instability. Continued checkpoint activation, such as that caused by microtubule poisons (e.g., taxol), induces a prolonged mitotic arrest followed by apoptosis.

Through a genome-wide siRNA screen we identified the cohesin subunit Scc1/Rad21 and the pro-apoptotic factor NOXA as factors that promote apoptosis during mitotic arrest. Depletion of Scc1 or NOXA delays apoptosis during taxol-induced arrest. Scc1 or NOXA depletion also increases the number of cells that are able to escape the taxol-induced arrest and become polyploid. Interestingly, the apoptotic role of Scc1 seems to be independent of its role in sister chromatid cohesion since depletion of other subunits of the cohesin complex does not delay apoptosis.

Scc1 is a known caspase substrate that is cleaved when interphase cells undergo apoptosis in response to DNA damage. We provide evidence that Scc1 is also cleaved in a caspase-dependent manner during mitotic cell death. Surprisingly, we also found that when Scc1 is depleted caspase-3 activation is abolished. These results suggest the existence of a feedback loop in which Scc1 is both a promoter and a target of caspase activity. Because of the known role of cohesin at chromosomes during mitosis and in light of this novel role for Scc1 as a pro-apoptosis factor during mitotic arrest, we hypothesize that cohesin might serve as a link between the mitotic machinery and the apoptosis network.

CONDENSIN AND MICROTUBULE TENSION DRIVE DE-CATENATION BY TOPOISOMERASE II THROUGH STABLE MODIFICATION OF DNA TOPOLOGY

Jonathan Baxter[1], Jorge B Schvartzman[2], John F Diffley[3], Luis Aragon[1]

[1]MRC Clincial Sciences Centre, Cell Cycle Group, Hammersmith Hospital, Du Cane Rd, London, W12 0NN, United Kingdom, [2]Centro de Investigaciones Biologicas , Departamento de Bioliogia Celular y del Desarrelo, Ramiro de Maeztu 9, Madrid, 28040, Spain, [3]Cancer Research UK , Clare Hall Laboratories, Blanche Lane, South Mimms , EN6 3LD, United Kingdom

The de-catenation of sister DNA molecules by topoisomerase II during mitosis is an absolute requirement to segregate replicated chromosomes. Topo II (Top2 in yeast) mediates topological change by generating a transient double stranded break in one segment of DNA (the G segment) and then transporting an unbroken segment of duplex DNA (the T segment) through the break before re-ligation. Thus the operation can either be intra-molecular, which relaxes supercoils or inter-molecular to resolve catenations. A longstanding question of mitosis is what provides the directionality of Top2 action to remove all catenations on the replicated sister chromatids before cell division is completed

Here we show that catenated yeast minichromosomes undergo a topological transition during mitosis that provides a stable substrate for topo II to resolve inter-plasmid catenanes in preference to intra-plasmid supercoils. This transition requires a centromere, microtubule tension and Condensin function. Analysis of the supercoiling of plasmids purified from mitotic cells indicates that the transition is characterised by overwinding of the replicated DNA.

We therefore describe the first experimental evidence that Top2 de-catenation during mitosis is driven by a topological modification of DNA mediated by condensin and spindle forces.

REGULATION OF SISTER CHROMATID COHESION AND CHROMATIN STRUCTURE BY COHESIN

Jan-Michael Peters

Research Institute of Molecular Pathology, Peters Lab, Vienna, , 1030

Throughout interphase, numerous sites in mammalian genomes are bound by cohesin complexes. During DNA replication these complexes establish physical connections between the newly synthesized sister chromatids. The resulting cohesion is essential for chromosome segregation and for DNA damage repair. Cohesion establishment depends on cohesin acetylation and coincides with particularly stable binding of cohesin to DNA. This binding mode depends on a cohesin associated protein, called sororin. Although cohesin is best known for its role in mediating cohesion, we and others discovered that cohesin has also important roles in gene regulation. We suspect that these functions are the reason why cohesin binds to chromatin already before cohesion is established and why cohesin associates with DNA even in postmitotic cells, which will never establish cohesion. We found that cohesin co-localizes in mammalian genomes with the transcriptional insulator protein CTCF and showed that cohesin is required for gene regulation at the imprinted H19-IGF2 locus. Gene expression at this locus is believed to be controlled by formation of a chromatin loop which forms between CTCF sites specifically on the maternal allele. Our recent work indicates that cohesin is required for this chromatin interaction. We will describe experiments in which we are addressing how cohesin might contribute to the formation of chromatin loops and how cohesin might be converted into a “cohesive” state during DNA replication.

CYCLING WITHOUT CYCLINS: CDKs ARE MODULATORS AND EFFECTORS OF A TRANSCRIPTION NETWORK OSCILLATOR

Steven B Haase[1], Laura A Simmons Kovacs[1], David A Orlando[4], Michael B Mayhew[2], Sayan Mukherjee[3]

[1]Duke University, Biology, Durham, NC, 27008, [2]Duke University, Computational Biology and Bioinformatics, Durham, NC, 27708, [3]Duke University, Statistical Science, Durham, NC, 27708, [4] Whitehead, Biology, Cambridge, MA, 02142

Although it is widely accepted that cyclin/CDK forms the central core of the cell-cycle oscillator, we have observed that periodic events continue in budding yeast cells lacking all S-phase and mitotic cyclin genes. We proposed that a transcription factor network sustains oscillations of bud emergence and activates the temporal program of transcription in these mutant cells that otherwise appear to be arrested at the G1/S border. These cyclin mutant cells still expressed G1 cyclins, and we proposed that G1 cyclins act as part of the network oscillator, but it is not clear whether they play an essential role in supporting oscillations of the transcription network. To address this question, we examined global gene expression over time in *cdc28-4* mutant cells that lack all cyclin/CDK activities at the restrictive temperature, and do not undergo re-budding cycles. We find that a significant number of genes are still periodically transcribed in the absence of all CDK activity. However, with decreasing levels of CDK activity, oscillations appear to be less synchronous within the population and the average period length is extended. CDKs are known to regulate several transcription factors within the network, providing both positive and negative feedback. Our data suggest that this feedback from CDK activity is important for robust oscillations in wild-type cells. Despite the fact that the cell-cycle transcription program continues in *cdc28-4* cells, hallmark cell cycle events (e.g., budding, DNA replication, mitosis) do not occur. Together, our findings support a model in which a transcription factor network forms the core cell-cycle oscillator, however, the behavior of this scillator is modulated by CDKs, and CDKs are the essential effectors of the oscillator required for triggering cell-cycle events.

CELL CYCLE COMMITMENT

Andreas Doncic, Jan M Skotheim

Stanford University, Department of Biology, 371 Serra Mall, Stanford, CA, 94305

In yeast, the G1 checkpoint Start determines if a cell enters the mitotic cycle or engages the mating program, but never both since the aims of the two programs are diametrically opposed: mating produces one cell from two, while mitosis produces two cells from one. This dichotomy is reflected in the antagonistic relationships between the underlying signaling molecules.

When a mitotic cell cycle is selected, the upstream cyclin-Cdk complex (Cln3-Cdc28) initiates a transcriptional positive feedback loop of two more G1 cyclins (Cln1 and Cln2). In the presence of mating factor, a MAPK pathway activates Far1, which inhibits G1 cyclins to prevent cell cycle progression. We show that the inhibition of both Far1 and the MAPK scaffold protein Ste5 by Cln1 and Cln2 completes a double negative feedback system that is bistable. In this context, bistability is an effective way to generate mutually exclusive cell fates: if G1 cyclin activity is below threshold, pheromone-induced signaling drives it lower and the mating program ensues; however, if G1 cyclin activity (through positive feedback) is above threshold, pheromone-induced signaling is inhibited.

We use quantitative fluorescence microscopy coupled with microfluidics to precisely determine the commitment point in individual cells that are abruptly exposed to a step increase in pheromone concentration. In WT cells, this occurs just after the initiation of the G1 cyclin positive feedback loop, where 50±10% of the initially nuclear transcriptional inhibitor Whi5 (fused to GFP) has been phosphorylated by the G1 cyclins and removed to the cytoplasm. While the removal of cyclin feedback (*cln1*Δ*cln2*Δ) reduces the Whi5 threshold to ~20%, the removal of the 8 CDK sites on the MAPK scaffold Ste5 has no effect on the commitment point. However, *STE5(8A)* cells that are exposed to pheromone late in G1 (post-Whi5 exit) exhibit a mixed cell fate (15% of an asynchronous population). These mixed-fate cells initiate shmoo formation while failing to complete cytokinesis resulting in a 2C arrest. Thus, our data suggests a separation of function between G1 cyclin targets in the MAPK pathway: while the commitment point is set by the Cln1,2-Far1 interaction, CDK sites on Ste5 must also be phosphorylated to ensure exclusive expression of the mitotic program.

A SEQUENCE OF COUNTERACTING PHOSPHORYLATIONS RESTRICTS AN ASYMMETRIC GENE EXPRESSION PROGRAM TO EARLY G1

Emily Mazanka, Eric L Weiss

Northwestern University, BMBCB, 2205 Tech Dr , Evanston, IL, 60208

Restricting gene expression to specific times in cell division and differentiation requires close control of both activation and inactivation of transcription. In budding yeast, strict spatiotemporal regulation of the transcription factor Ace2 ensures that it acts only once in a cell's lifetime: at the M to G1 transition in newborn daughter cells. Phosphorylation by the Ndr/LATS family kinase Cbk1 activates Ace2 and drives its accumulation in daughter cell nuclei but it remains unclear how the transcription factor is inactivated. We found that Ace2's nuclear localization requires continuous rephosphorylation by Cbk1, and that inhibition of the kinase leads to Ace2's immediate loss of phosphorylation and export to the cytoplasm. Once exported, Ace2 cannot re-enter nuclei. This cytoplasmic sequestration involves phosphorylation of sites that block Ace2's nuclear import by the G1 CDKs Pho85 and Cdc28/Cdk1. Additionally, Pho85 reinforces Ace2's cytoplasmic retention through a mechanism independent of direct phosphorylation of the transcription factor. These findings show how sequential opposing kinase activities limit a daughter cell specific transcriptional program to a brief period during the cell cycle.

DIFFERENT LEVELS OF CYCLIN-CDK ORDER MITOTIC EVENTS IN S. CEREVISIAE

Catherine Oikonomou, Frederick Cross

Rockefeller University, Laboratory of Yeast Molecular Genetics, 1230 York Ave., New York, NY, 10065

Cell cycle events are triggered by a regulatory cyclin-dependent kinase (CDK) in complex with an activating cyclin. Different cyclin-CDK complexes are present at different times in the cell cycle, conferring order on the events they control. However, a single cyclin-CDK complex often triggers multiple events. In this case, what ensures that these events occur in the correct order? Stern and Nurse (1996), in their 'quantitative model for cell cycle control,' proposed that different activity levels of the kinase might trigger different events, leading to temporal order. In budding yeast, mitotic cyclin levels normally ramp up over a significant period of time; if different cyclin-CDK levels trigger different events, such events would then be temporally separated. Here we test this hypothesis by measuring levels of mitotic cyclin-CDK activity required to trigger distinct mitotic events. We apply a titrated pulse of activating cyclin and use time-lapse microscopy to measure the mitotic cyclin-CDK level required to trigger three mitotic events: growth depolarization, spindle formation, and spindle elongation. We observed that spindle formation required significantly more cyclin than growth depolarization, and spindle elongation required still more cyclin than spindle formation. These results, consistent with the temporal separation observed between these events in cycling cells, support the ordering hypothesis of Stern and Nurse.

We next tested candidate Clb-CDK targets for mechanistic roles in spindle formation. We found that Clb-CDK phosphorylation of the spindle pole body (SPB) bridge protein Sfi1 promoted efficient SPB separation, a critical step in spindle assembly. In contrast, while cyclin-CDK activity stabilizes the Cin8 kinesin, which also promotes spindle assembly, this stabilization was not rate-limiting for spindle formation.

Our results validate the Stern and Nurse model, demonstrating that variation in cyclin-CDK activity levels through the cell cycle can order cell cycle events. Additionally, we have used our system to begin to dissect critical steps in cyclin-Cdk control of spindle formation.

BISTABILITY MAY ALLOW FOR WAVE-LIKE PROPAGATION OF MITOTIC ACTIVITY AND MAINTAIN CELL SYNCHRONIZATION

Jeremy B Chang, James E Ferrell Jr

Stanford University, Chemical and Systems Biology, 259 Campus Dr., Stanford, CA, 94305-5174

Diffusion imposes a natural speed limit by which signals, such as active protein kinase, can spread in a biological system. In addition, diffusion times increase with distance squared, implying that diffusion is much slower over relatively large length scales than small length scales. Large cells like *Xenopus laevis* eggs (1.2 mm diameter) might therefore be expected to have difficulty propagating intracellular signals quickly enough to maintain synchronization. However, the cleavage divisions of early embryos of *Xenopus* are nearly synchronous, suggesting that a mechanism other than diffusion coordinates mitosis throughout the embryo's large cells. Here, we propose a mechanism by which the bistability of the CDK1 system, which includes the kinase Wee1 and phosphatase CDC25, may allow for CDK1 activation to spread in a wave-like manner that is faster than diffusion. We use time-lapse fluorescence microscopy of Xenopus cell-free egg extracts in a simple microfluidic channel to show that propagation of mitotic activity, as visualized by nuclear envelope breakdown, indeed spreads rapidly and linearly in time. We speculate that this mechanism of signal propagation may be a widely-employed mechanism to spread information quickly through large cells and through tissues.

THE DNA REPLICATION CHECKPOINT PROMOTES E2F-DEPENDENT CELL-CYCLE TRANSCRIPTION IN RESPONSE TO A DNA REPLICATION BLOCK.

Cosetta Bertoli[1], Tatyana I Kalashnikova[2], Clare H McGowan[2], Curt Wittenberg[2], Robertus A de Bruin[1]

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Sequential waves of gene expression are responsible, in large part, for imposing order on the events of the cell cycle, which is critical for the well being of cells and the organisms they compose. Our recent work has shown that the cell cycle transcriptional program in fission yeast, Schizosaccharomyces pombe, is, in turn, a target for cell cycle checkpoints required to maintain genome integrity. In fission yeast MBF activates transcription during the G1/S transition. A negative feedback loop, involving Nrm1p, ensures repression of MBF-dependent genes once cells transit into S-phase. However, in response to DNA replication stress the checkpoint protein kinases block timely repression of MBF-dependent genes by inactivating Nrm1 via phosphorylation, which is critical for the survival of the cell.
Using the understanding derived from the studies with fission yeast, we have uncovered that the transcriptional response to DNA replication stress is conserved in mammals. In mammals G1/S transcriptional regulation is mediated by the E2F transcription factor family (E2F1-8) and the pocket proteins (pRb, p107 and p130), which are functionally analogous to MBF and Nrm1p respectively. We show that E2F-dependent transcription is derepressed in human cells in response to DNA replication arrest. Knocking-down or chemically inhibiting the DNA replication checkpoint protein kinase Chk1 abrogates that response. Our data suggests that sequential binding of different repressor E2Fs and pocket proteins are likely to ensures repression of E2F-dependent genes once cells transit into S-phase. We present evidence that activation of the DNA replication checkpoint is likely to interfere with this control to block timely repression of E2F-dependent genes in response to DNA replication stress.
Our work in fission yeast established a simple but elegant mechanism by which checkpoint activation can override the regular periodic transcriptional program by directly regulating a transcriptional repressor. We now show that similar mechanisms involving regulators of E2F-dependent transcription may be acting in human cells to control the cell cycle transcriptional program in response to perturbation of DNA replication. It will be important to establish the precise mechanism of this control in human cells and the importance of this regularegulation for genome integrity in Eukaryotes.

THE MUVB COMPLEX DIFFERENTIALLY REGULATES THE EXPRESSION OF EARLY AND LATE CELL CYCLE GENES

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Transcriptional profiling in mammalian cells reveals a periodic expression of genes that peak in different phases of the cell cycle. Indeed, many genes involved in DNA replication and mitosis are transcribed and expressed just prior to when they are needed in the cell cycle. For example, CDC6 and B-Myb expression begins during G1-S, while Cyclin B1 and PLK1 expression occurs during G2-M. Most, if not all genes with periodic expression during the cell cycle are repressed during quiescence. The DREAM complex (Dp, Rb, E2F and MuvB) containing p130, E2F4, DP1 and the LIN/MuvB proteins LIN9, LIN54, LIN37, LIN52 and RBBP4 occupies promoters of more than 800 cell cycle regulated genes and represses their transcription during quiescence. When quiescent cells are stimulated to re-enter the cell cycle, MuvB proteins dissociate from p130, E2F4 and DP1 and independently associate with B-Myb to form the Myb-MuvB complex. ChIP revealed that the Myb-MuvB complex bound exclusively to promoters of genes expressed late in the cell cycle, a specific subset of DREAM target promoters. Functional annotation of Myb-MuvB target genes revealed that they had well described functions in mitosis. Significantly, the Myb-MuvB complex bound to promoters of late cell cycle genes during S phase when these genes were not expressed. Furthermore, RNAi depletion of B-Myb and LIN54 revealed that B-Myb was required for MuvB binding to promoters and that MuvB was required for B-Myb binding. Using a combination of promoter reporter assays, ChIP and gene expression studies in synchronized cells, we determined that the MuvB complex served to repress B-Myb dependent transcription during S phase. The Myb-MuvB complex remained bound to late cell cycle gene promoters until G2, when phosphorylation dependent proteasomal turnover of B-Myb resulted in the transcriptional activation of late cell cycle genes ensuring their expression just before mitosis. Our study reveals that B-Myb serves an essential role in the periodic expression of genes during G2-M and that association of the MuvB complex alternately with p130/E2F4 in G0, and B-Myb in S regulates global periodic gene expression throughout the cell cycle.
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E2F REGULATION IS DISPENSABLE FOR GROWTH CONTROL MEDIATED BY PRB

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The retinoblastoma tumor suppressor (pRB) regulates the progression of cells into S-phase through at least the control of E2F transcription and regulation of p27 stability. pRB is also capable of forming complexes that contain both E2Fs and chromatin remodeling factors (CRFs) to alter the chromatin environment of E2F target genes. Interestingly, the contribution of these distinct interactions to the overall ability of pRB to control cell proliferation is not well understood because they have yet to be compared in a single study.

We report the development of new pRB mutants that selectively disrupt individual protein interactions with pRB that allow us to characterize the contribution of each mechanism to a single cell cycle arrest. When examining individual interactions, the loss of the E2F-binding site results in only a partial decrease in pRB activity. Combined disruption of the E2F site along with other binding sites further reduces the activity of pRB in cell cycle arrest, suggesting that the other mechanisms participate simultaneously. This suggests that pRB can engage multiple growth arrest pathways. To explore the physiological relevance of non-E2F mechanisms of cell cycle control, we introduced substitutions into the mouse *Rb1* gene to generate the pRb-G mutant protein that is unable to interact with and regulate E2Fs through its small pocket binding site. Fibroblasts derived from homozygous $Rb1^{G/G}$ mice have deregulation of E2F target gene expression yet remain capable of inducing a cell cycle arrest. Strikingly live $Rb1^{G/G}$ mice have been observed into adulthood and are indistinguishable from wild-type litter mates. In contrast, $Rb1^{-/-}$ mice are embryonic lethal. This suggests that non-E2F pathways can compensate for loss of E2F regulation to mediate cell cycle control. Cell culture data suggests that this compensation is mediated through the control of p27 levels by pRB-CdhI-Skp2 complexes and is currently being investigated through the creation of $Rb1^{G/G}p27^{-/-}$ animals. Taken together this work suggests that, pRB engages multiple mechanisms through distinct binding interfaces that can act redundantly to induce a cell cycle arrest.

DISSECTING THE UNIQUE ROLE OF THE RETINOBLASTOMA TUMOR SUPPRESSOR DURING CELLULAR SENESCENCE

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The RB protein family (RB, p107, p130) has overlapping and compensatory functions in cell cycle control. However, cancer-associated mutations are almost exclusively found in RB, implying that RB has a non-redundant role in tumor suppression. We demonstrate that RB preferentially associates with E2F target genes involved in DNA replication and is uniquely required to repress these genes during senescence but not other growth states. Consequently, RB loss leads to inappropriate DNA synthesis following a senescence trigger and, together with disruption of a p21-mediated cell cycle checkpoint, enables extensive proliferation and rampant genomic instability. Our results identify a non-redundant RB effector function that may contribute to tumor suppression and reveal how loss of RB and p53 cooperate to bypass senescence.

THE RETINOBLASTOMA PROTEIN REGULATES FATE CHOICE AND LINEAGE COMMITMENT

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The Rb tumor suppressor is mutated in approximately one third of human tumors. Three tumor types, retinoblastoma, osteosarcoma and small cell lung cancer, are particularly associated with Rb mutation. However, the underlying basis of this tissue specificity is not well understood. Numerous functions have been ascribed to the product of the Rb gene, pRB. The best known is pRB's ability to block cellular proliferation, at least in part, through inhibition of the E2F transcription factors. Less well appreciated is pRB's ability to regulate a several transcription factors that are master differentiation inducers. Notably, depending on the factor and the context, pRB can either suppress or promote their transcriptional activity. For example, pRB binds to Runx2 and this seems to stimulate its ability to launch the osteogenic differentiation program. In contrast, pRB and E2F1 act together to suppress the ability of PPARgamma to induce adipogenesis. These observations are extremely intriguing. However, to date, there is no evidence that pRB's role in these differentiation complexes affects fate choice in vivo and/or the spectrum of Rb-deficient tumors. We have used a variety of mouse models, and the resulting cell lines, to address these questions in the context of mesenchymal tumors and tissue development. Our data show that Rb status plays a key role in establishing fate choice and/or fate commitment between brown fat and bone in vivo.

CDC14-DEPENDENT DEPHOSPHORYLATION OF A KINETOCHORE PROTEIN PRIOR TO ANAPHASE

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Clb-CDKs drive entry into S phase and mitosis by phosphorylating hundreds of substrates. In budding yeast, the Cdc14 phosphatase reverses this CDK phosphorylation to allow mitotic exit. The activity of Cdc14 is therefore tightly controlled. Cdc14 is sequestered in nucleolus from G1 until the onset of anaphase when it is released into nucleus and cytoplasm to dephosphorylate Clb-CDK substrates. Although it has been thought that Cdc14 is inactive during metaphase, recent work suggests that there is an active pool of Cdc14 in the nucleolus prior to anaphase onset. However, it has not been known whether Cdc14 is active outside of the nucleolus in metaphase. We found that the Dsn1 kinetochore component is dephosphorylated in a Cdc14-dependent manner prior to anaphase. Dsn1 is a component of the Mis12 kinetochore subcomplex that is essential for kinetochore assembly, and the protein has not been detected in the nucleolus. Dsn1 exhibits a dynamic phosphorylation and dephosphorylation pattern during cell cycle that is CDK-dependent. We generated a phospho-specific antibody against a CDK site in Dsn1 and found that it is dephosphorylated in metaphase in a Cdc14-dependent manner. Our results suggest that in addition to global localization control (i.e. by sequestering in nucleolus), the activity of Cdc14 is also regulated at substrate specificity and processivity levels, enabling efficient dephosphorylation of specific targets even when Clb-CDK activity is high.

MAX1 LINKS MBF-DEPENDENT TRANSCRIPTION TO COMPLETION OF S PHASE

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The MBF complex controls the transcription of genes required for DNA synthesis in fission yeast, like *cdc22* (ribonucleotide reductase), *cdc18* (CDC6) and *cdt1*. Transcription of these genes is cell cycle regulated, being activated early on G1 and inactivated at the end of S phase [1]. We have purified the MBF complex and isolated proteins that co-purify with the complex. Among them, we have found Max1, which is a homeodomain-containing protein that binds to the MBF complex and activates MBF-dependent transcription at the M/G1 transition in a Cdc2/CDK dependent manner. Fission yeast cells in which *max1* has been deleted are resistant to toxic concentrations of the DNA synthesis inhibitor hydroxyurea and show genomic instability (chromosome loss). We have also determined that when the DNA synthesis checkpoint is activated, Max1 is phosphorylated by the kinase Cds1/CHK1 [2] resulting in the abrogation of its binding to the MBF complex. As a consequence, MBF-dependent transcription is maintained active until cells are able to overcome the cell cycle arrest. Thus, Max1 couples normal cell cycle regulation and the DNA synthesis checkpoint in a single transcriptional complex.

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ROLE OF ATR AND CHK1 HAPLOINSUFFICIENCY IN REPLICATION AND CHROMOSOMAL FRAGILITY

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Common fragile sites (CFS) are sites of recurrent breaks on chromosomes, which are "expressed" under various stresses, most of them known to disturb DNA replication, as Aphidicolin (APH) treatment. The working model to explain the fragility of these sites is based on pertubation of replication, secondary DNA structures and the role of repair and checkpoints proteins. Among the actors involved in fragility that have be identified, it has been demonstrated that cell transiently depleted in ATR, a kinase involved in stalled replication fork signaling, or in one of its target, CHK1, show an enhanced chromosome fragility. However, it is now well established that these proteins, need a precise regulation and that haploinsufficiency of genes involved in ATR signaling pathway have functional significance.
We developed an approach to establish a collection of isogenic clones, presenting naturally occurring mutations for these genes. We used the property of mismatch repair deficiency of the HCT116 cell line to spontaneously generate mutations in homopolymeric repeats. Indeed, when a homopolymere repetition is located in a coding sequence, it is prone to induce a frameshift in the gene, then leading to haploinsufficiency. Following this approach, we have isolated « natural » haploinsufficient mutant for ATR and CHK1 and combination of these mutations. As it could be expected from literature, we have first demonstrated that atr and chk1 mutant clones are more sensitive to APH than their wild type counterpart and are prone to chromosome breakage, especially at CFS FRA3B and FRA16D. We next show by cytometry that mutant cells display anomalies in activation of the intra-S checkpoint and, by HALO and molecular combing, that they exhibit defects in genome-wide replication dynamics. Finally, preliminary data appear to show that double heterozygote clones have less replication issues than simple mutants. In good agreement with this observation, we found more activated forms of these proteins in simple mutants. An attractive hypothesis to explain these results is that gene dosage between ATR and CHK1 is important and finely regulated to allow a normal replication completion.

GCP6, A COMPONENT OF THE GAMMA-TURC IS REQUIRED FOR CENTROSOME DUPLICATION.

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Centrosomes are the major microtubule-organizing centers of an animal cell. In G1-phase of the cell cycle cells contain only one centrosome. For the formation of a bipolar mitotic spindle centrosomes have to be duplicated which occurs at the G1/S-phase transition. It was previously shown that gamma-tubulin is required for centriole duplication. In vertebrate cells gamma-tubulin is organized in a large multi-protein complex, the gamma tubulin ring complex (gamma-TuRC), which forms an essential template for microtubule nucleation at the centrosomes. GCP6 is one of the core components of the gamma-TuRC. Its function in mammalian cells is only poorly characterized. We find that in HeLa cells stable expressing murine GCP6-GFP under the endogenous promoter, GCP6 localizes to the centrosomes all over the cell cycle. In addition to this a colocalization of GCP6 with the centriolar marker protein centrin as well as localization along the spindle microtubules is detected. Furthermore, we observed that GCP6 is required for the formation of a bipolar mitotic spindle as downregulation of GCP6 results in spindles with poorly separated poles, combined with an enrichment of mitotic cells in prometaphase. The monopolar spindles bear a reduced number of centrioles, indicating that GCP6 plays a role in centriole duplication. This was confirmed in centrosome overduplication experiments in which GCP6 depletion inhibits centrosome amplification in aphidicolin arrested U2OS cells. Future studies will be aimed at clarifying if GCP6 influences centriole duplication by effecting gamma-TuRC assembly, microtubule nucleation or gamma-tubulin recruitment.

THE MOLECULAR CHAPERONE HSP90 IS REQUIRED FOR CELL CYCLE EXIT IN *DROSOPHILA*.

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Proper development depends on the coordination of cell proliferation and differentiation to produce the correct number of cells in space and time. Robust mechanisms exist to ensure that cells enter a permanently non-proliferative state upon terminal cell differentiation. For this cell cycle exit to occur normally, it is important that both the E2F/DP transcription factor and Cyclin E/Cdk2 activities be suppressed. However, the precise processes that act to restrain E2F/DP and Cyclin E/Cdk2 in differentiating cells are not clear. To learn more about the regulation of cell cycle exit, we have performed a genetic screen in *Drosophila*, designed to identify genes involved in silencing cell cycle gene transcription upon differentiation in the eye. This screen utilized a *pcna-miniW*$^{+}$ reporter that is highly E2F-responsive and results in a darker red eye color when crossed into genetic backgrounds that delay cell cycle exit. We have isolated a line mutant for *Hsp83*, the *Drosophila* homolog of mammalian Hsp90, which experiences increased E2F-dependent transcription and ectopic cell proliferation in pupal tissues at a time when neighboring wild-type cells are postmitotic. Further, these *Hsp83* mutant cells contain increased Cyclin/Cdk activity and accumulate proteins normally targeted for proteolysis by the anaphase-promoting complex/cyclosome (APC/C), suggesting that APC/C function is inhibited in cells lacking *Hsp83*. Future studies will focus on defining the mechanisms by which a loss of function of the chaperone *Hsp83* results in increased Cyclin/Cdk activity and decreased APC/C function, as well as determining which effects are critical for delaying cell cycle exit. Characterization of this function of *Hsp83* should provide new insights into the processes coordinating cell proliferation and terminal differentiation.

PROTEIN-PROTEIN INTERACTIONS LINK TRANSCRIPTION FACTOR NETWORK TO CLB REGULATION IN THE YEAST CELL CYCLE

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The coupled activities of Cdk-Clb and transcription network oscillators are essential for cell cycle progression in eukaryotes. The budding yeast cell cycle is driven by periodic changes in the activity of the Cdk1 kinase, and different pools of cyclins (Clbs) associate with Cdk1 in sequential waves of expression. The strictly delimited expression of cyclin genes stems from both positive and negative transcriptional controls as well as by cell cycle-dependent protein activity. Cdk1 has been implicated in transcriptional regulation either directly, by associating and phosphorylating transcription factors, or indirectly, by interacting with suppressors and activators of transcription factors. This results in inhibition or increase of transcription, respectively. However, the molecular mechanisms linking the transcription network oscillator to the activation of Cdk1-Clb complexes is not fully understood.
It has been recently shown that transcription and phosphorylation are coupled by feed-forward loops for cell cycle regulation. Here, we suggest a specific transcriptional regulation driving phase-specific cell cycle events. By combining directed protein-protein interaction techniques, ChIP and expression profiling using Solexa technology, we show that the Cdk-Clb oscillator can be coupled with the transcriptional oscillator such that the sequential appearance of Clbs is controlled by specific transcription factors. In turn, Cdk-Clb could modulate the activity of the transcriptional oscillator by recruiting the same transcription factors via Clb-mediated interactions.

SIC1 PLAYS A ROLE IN TIMING AND OSCILLATORY BEHAVIOUR OF B-TYPE CYCLINS IN BUDDING YEAST

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Biochemical feedback and feed-forward loops coordinate cell cycle oscillations occurring over a wide range of time scales. The budding yeast cell cycle oscillates between states of low and high cyclin-dependent kinase activity, driven by the association of Cdk1 kinase with B-type (Clb) cyclins. Various Cdk1-Clb complexes are activated and inactivated in a fixed, temporally regulated sequence during cell cycle progression, inducing the characteristic behaviour known as “waves of cyclins”. The mechanism that regulates the appearance on schedule of these complexes is currently unknown.
Here, we analyse the molecular basis of the oscillations of the Clbs, with major focus on the role of their inhibitor Sic1. We compare mathematical networks differing in the regulatory interactions that Sic1 may establish with Cdk1-Clb complexes. Our analysis suggests that the wave-like cyclins pattern derives from the binding of Sic1 to all three Clb pairs rather than from the degradation of Clbs. We show that a specific sequence of molecular interactions reproduces the oscillation-like behaviour of the Clb cyclins waves and verify experimentally the model predictions by protein-protein interaction techniques and time course experiments. Our results highlight a feed-forward regulation of Sic1 to synchronize the Cdk1-Clb complexes, acting as a timer in their appearance.

THE SKP2 PROMOTER INTEGRATES SIGNALING THROUGH THE NF-KB/IKK, P53 AND AKT/GSK3B PATHWAYS TO REGULATE AUTOPHAGY AND APOPTOSIS IN RESPONSE TO DNA DAMAGE.

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NF-κB and p53 are important regulators of the cellular response to stress. Here we demonstrate that p52/p100 NF-κB2 processing is induced upon DNA damage and that, in cooperation with p53, can regulate cell fate. Moreover, after DNA damage, the Skp2 gene, which contains binding sites for both factors, is regulated by p52 containing NF-κB complexes and p53. Importantly, this provides a node through which both pathways, together with signaling through the IKK, ATM and Akt pathways, can be integrated to regulate cell cycle arrest and autophagy. Underlining the importance of this pathway as a decision making nexus determining the response to DNA damage, p52 and p53 can either repress or induce Skp2 expression and this is dependent upon signaling through the Akt/GSK3β pathway. We show that GSK3β phosphorylation of p52 at serine 222 not only determines coactivator and corepressor recruitment to the Skp2 promoter, but also regulates p52 heterodimerisation specificity.

REGULATION OF AURORA B KINASE BY CULLIN3 MEDIATED UBIQUITINATION.

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Tight control of mitotic events is required to ensure faithful chromosome segregation and cytokinesis. Recently, it was shown that the proper cellular localization of the chromosomal passenger complex (CPC) requires the activity of the ubiquitin ligase Cullin3 (Cul3). The kelch-repeat containing substrate specific adaptor proteins Klhl9, Klhl13 and Klhl21 were found to establish interaction of Cul3 with the passenger kinase Aurora B. When components of the Cul3 complexes are depleted by RNAi, the CPC fails to accumulate on spindle midzone microtubules after anaphase onset and remains associated with chromosomes. However, it is still unclear how ubiquitination of Aurora B affects CPC localization.

To address this question we generated a cell line stably expressing Aurora B fused to a photoactivatable variant of GFP (paGFP). Using this cell line we are studying whether Aurora B relocalization from centromeres to the spindle midzone requires the degradation or transport of the chromosome-associated pool of Aurora B. Furthermore, this cell line enables us to measure kinetics of distinct Aurora B populations in the presence or absence of Cul3 complexes by selectively photoactivating AuroraB-paGFP on different mitotic structures. Gathering this kinetic information should lead to a better understanding of how ubiquitination of Aurora B by Cul3 regulates the localization of the CPC.

TYROSINE PHOSPHORYLATION OF P27KIP1 SERVES AS A CYCLIN D-CDK4 ON/OFF SWITCH

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In addition to its well-characterized growth suppressive role, p27Kip1 appears to promote proliferation by activating cyclin D-cdk4/6 kinase activity. We demonstrated that tyrosine (Y) phosphorylation of p27 converts it from a cdk4/6 inhibitor to an activator, as the presence of the phosphate forces the tail of p27 from cdk4's active site, permitting both ATP access and the required CAK phosphorylation of the cdk subunit. Thus, p27 functions as a bona fide "switch" turning cdk4/6 activity on or off, promoting or preventing proliferation respectively. Using two dimensional isoelectric focusing and p27 Y phospho-specific antibodies, we have found that p27 Y phosphorylation is tightly cell cycle regulated, and in non-transformed epithelial cells and T cells, is detected exclusively at the G0-G1 phase border or following release from quiescence. This correlates with the burst of cdk4/6 kinase activity seen during the G0-G1 phase transition, after which both p27 Y phosphorylation and cdk4/6 kinase activity decreases. Cdk4/6 activity is restored while p27 is still detected at G0 phase levels, suggesting that Y phosphorylation activates preassembled p27-cyclin D-cdk4 complexes. Cdk2 activity is not detected until p27 levels decrease, suggesting that activation of cdk4 and cdk2 correspond to the G0-G1 and G1-S phase transitions respectively. We have also found using several different types of cancer cells that p27 Y phosphorylation is no longer cell cycle regulated and the pool of Y phosphorylated p27 is significantly increased. Due to increased Y kinase activity, the p27 Y phosphorylation switch may be "locked" into a constitutive activator mode. In many tumor types, cyclin D-cdk4/6 are oncogenes, and p27 itself is a tumor suppressor, whose activity appears to be reduced, due to increased degradation or cytoplasmic mislocalization. However, low p27 levels are almost always detected, suggesting there is a selective advantage to maintain residual p27, presumably to stabilize and activate cyclin D-cdk4/6 complexes. While the pool of nuclear p27-cyclin D-cdk4 appears lower due to reduced or mislocalized p27, the percentage of Y phosphorylated, activated p27-cyclin D-cdk4/6 appears greater. Thus, we believe that p27 has a growth-promoting role during normal cell cycle proliferation, which translates into an oncogenic role during tumor progression.

REGULATION OF *S. CEREVISIAE* GLC7 (PP1) CELL CYCLE FUNCTION BY SHP1

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Glc7 is the only catalytic subunit of protein phosphatase 1 in *S. cerevisiae*. It plays major roles in regulating diverse cellular processes, including glycogen metabolism, cell cycle progression, chromosome segregation, sporulation, glucose repression, transcription, and meiosis. During the cell cycle Glc7 has been shown to oppose Ipl1 (Aurora B) kinase activity as bi-polar chromosome attachments are established and to function in spindle checkpoint silencing. *shp1* (suppressor of high-copy PP1) null alleles were identified in a screen for mutants suppressing the lethality of *GLC7* overexpression, and Shp1 is thought to be a positive regulator of Glc7. However, it remains unclear how Shp1 affects Glc7.
Our detailed analysis of *shp1* phenotypes revealed that the knockout has cell cycle defects similar to certain conditional *glc7* mutants. At the permissive temperature *Δshp1* cells show a significant delay in cell cycle progression during G2/M and arrest with separated spindle pole bodies and short spindles at 14°C. Overexpression of *GLC7* in *Δshp1* is not only tolerated, but suppresses the G2/M delay, resulting in a cell cycle distribution similar to wildtype. Furthermore, we demonstrate for the first time that a physical interaction between Glc7 and Shp1 exists *in vivo*. Currently we are investigating the specific role of Shp1 in regulation of Glc7 cell cycle functions.

DEFECTS IN CELL CYCLE CHECKPOINT FUNCTIONS ARE SPECIFIC TO BREAST CANCER MOLECULAR SUBTYPES

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Cancer is a disease of disregulated cell growth: thus, modern chemotherapeutic drug treatments often target tumor cells by interfering with the cell cycle of rapidly dividing cells. Breast cancer chemotherapy heavily relies on the topoisomerase II poison doxorubicin (Adriamycin). There are currently six molecular subtypes of breast cancer, luminal A, luminal B, basal-like, claudin-low, normal-like, and $Her2^{+}$, each of which presents itself as an individual disease with different prognoses. These molecular subtypes are thought to share similar molecular signaling defects that may be targeted for personalized chemotherapy regimens. Here we describe the cell cycle checkpoint defects present in the luminal, basal-like, and claudin-low subtypes. A panel of breast cancer cell lines were examined to identify cell cycle checkpoint function using a set of flow cytometry based assays. The basal-like and claudin-low subtypes both displayed a defective spindle-damage checkpoint. These subtypes exhibited increased numbers of polyploid cells upon treatment with the spindle poisons colcemid and nocodazole and exhibited a decreased accumulation of mitotic cells in the presence of colcemid. In contrast, the luminal breast cancer cell lines retained an effective spindle damage checkpoint, but displayed a defective decatenation G_2 checkpoint response to the topoisomerase II catalytic inhibitor ICRF-193. Luminal breast cancer cell lines exhibited decreased expression of the protein kinase ATM as measured by western immunoblot, suggesting a mechanism by which the decatenation G_2 checkpoint response might be attenuated. Furthermore, cytogenetic preparations of luminal lines treated with ICRF-193 displayed under-condensed and entangled chromosomes, and preliminary colony formation data suggests that the luminal breast cancers may exhibit an increased sensitivity to ICRF-193, when compared to basal-like or claudin-low subtypes. These results indicate that the luminal breast cancers are more responsive to a topoisomerase II catalytic inhibitor than the basal-like breast cancers. This may represent a new vulnerability of the Luminal B subtype with poor response to hormone therapy.

MITOTIC EXIT CONTROL OF THE NDR/LATS KINASE CBK1 REGULATES THE FINAL ACT OF CELL DIVISION

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The final act of budding yeast cell division is destruction of a septum deposited during cytokinesis, a process analogous to abscission in metazoans. It is not clear how this is forestalled until division is complete. Genes involved in septum destruction are induced by the M/G1 transcription factor Ace2, which is driven into the daughter cell nucleus immediately before cytokinesis by the Ndr/LATS kinase Cbk1. Cbk1 is phosphorylated at a conserved hydrophobic motif (HM) regulatory site, a modification required for its *in vivo* function. This site becomes phosphorylated prior to mitotic exit: we found this requires activation of the FEAR (fourteen early anaphase release) pathway. However, using a gain-of-function *cbk1* allele that bypasses the need for phosphorylation of this site, we found that the kinase is still negatively regulated. Cbk1 cannot activate the Ace2 transcriptional program until final release of the mitotic phosphatase Cdc14, which is initiated downstream of the mitotic exit network (MEN). Treatment with the phosphatase does not increase the intrinsic enzymatic activity of the kinase *in vitro*, but activates the pathway *in vivo*. These findings indicate that Cdc14 activates Cbk1 and Ace2 simultaneously by releasing both proteins from inhibitory phosphorylation. In this way, the rapid activation of Cbk1 and Ace2 are coordinated with the progress of mitotic exit.

ESTROGEN-MEDIATED PROLIFERATION OF BREAST CANCER CELLS REQUIRES MDM2 TO BYPASS THE G1 CHECKPOINT.

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Estrogen plays a major role in breast cancer development and progression. Increased cellular proliferation in the presence of estrogen allows for a higher mutation rate that can sabotage cellular genomic integrity. Over-expression of the oncoprotein Mdm2, a major negative regulator of the p53 tumor suppressor, is often observed in estrogen receptor positive breast cancers. To study the role of Mdm2 in estrogen-mediated cellular proliferation and survival, we examined the effect of estrogen on the Mdm2-p53 pathway in the estrogen receptor positive and p53 wild-type MCF-7 breast cancer cells. We observed that the estrogen-mediated increase in cellular proliferation correlated with an increase in the Mdm2 protein level. Importantly, when we blocked Mdm2 expression in cells with inducible shRNA, estrogen could no longer up-regulate cell growth. Mdm2 knockdown increased p21 and the percent of cells in G1 phase, which resulted in decreased cellular proliferation, whereas estrogen treatment could not overcome this effect. Furthermore, we observed that estrogen increased the Mdm2 protein level in cells exposed to the DNA damaging agent etoposide and the Mdm2 inhibitor Nutlin-3. We found that under these conditions, estrogen inhibited transcription of the pro-apoptotic gene *puma* without decreasing the p53 protein level or influencing p53 recruitment to the chromatin. The estrogen-mediated decrease in *puma* transcription correlated with a decrease in Puma protein and an increase in Bcl-2. Overall, our findings suggest that Mdm2 is required for estrogen-mediated cellular proliferation and survival, and that estrogen together with, or via, Mdm2 is signaling for cell cycle progression and programmed cell death inhibition.

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THE DNA REPLICATION CHECKPOINT COUPLES THE CELL-CYCLE TRANSCRIPTION PROGRAM WITH CELL-CYCLE PROGRESSION

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In the budding yeast, *Saccharomyces cerevisiae*, approximately 20% of the genome is transcribed periodically during the cell cycle. Periodic transcription is thought to play an important role in ordering cell-cycle events. However, in cells lacking S-phase and mitotic cyclins, cell-cycle progression is halted but periodic transcription continues. This result demonstrates that the periodic transcription program can be uncoupled from cell-cycle progression. We have proposed that this periodic transcription program is controlled by a transcription factor network that may function as an independent cell-cycle oscillator. Our observation that the network oscillator can be uncoupled from cell-cycle progression was made in a non-physiological arrest (S-phase and mitotic cyclin mutants). What happens to the network oscillator during a more "physiological" cell-cycle arrest, such as a checkpoint arrest? If periodic transcription is important for ordering cell cycle events, asynchrony between cell-cycle progression and the transcription factor network could cause problems for cells recovering from a checkpoint arrest. Thus we hypothesized that checkpoints regulate the network oscillator in order to couple it to cell-cycle progression. To test this hypothesis, we measured global mRNA dynamics over time in a synchronous population of G1 cells released into conditions that trigger the DNA replication checkpoint. We observe that a majority of the periodic genes in normally-cycling cells are no longer periodic when the cell cycle is arrested by the checkpoint. Although we find that periodic transcription is halted, many of the genes thought to be involved in overcoming replicative stress are continually expressed throughout the time course. Our data suggest that the DNA replication checkpoint pathway regulates the network oscillator to coordinate its progression with cell-cycle progression and to aid in overcoming replicative stress.

CHARACTERISATION OF THE CENTROSOME PROTEIN CEP63

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XCep63 was identified in a screen for ATM targets following the observation that DNA damage causes disruption of the mitotic spindle in *Xenopus laevis* egg extracts (Smith *et al.* 2009).

Recombinant DNA double strand breaks introduced into egg extracts cause disruption of mitotic spindle assembly, which is dependent on ATM/ATR activation and phosphorylation of XCep63. Supplementing extract with XCep63 that cannot be phosphorylated by ATM/ATR rescues this spindle disruption phenotype. XCep63 is also important in unperturbed mitotic spindle assembly. Interestingly, it is only centrosome dependent spindle assembly that is disrupted by ATM and ATR activity. Anastral spindles, formed from microtubules nucleating from chromatin, are resistant to ATM/ATR signalling. These findings show that the centrosome is an important ATM and ATR target following DNA damage in mitosis. It appears that XCep63 is a major ATM and ATR centrosome target and its phosphorylation by ATM and ATR leads to inhibition of its function in bipolar spindle assembly.

The proper segregation of chromosomes during mitosis is crucial for maintenance of genomic stability and there is a large amount of data describing the involvement of the centrosome in the DNA damage response. Since the response to DNA damage during mitosis is not well characterised, we are interested in studying the role of Cep63 in mammalian cells and postulate that it will serve as a key link between DNA damage signalling and regulation of mitotic progression. Cep63 is conserved in vertebrates and was originally identified in a screen for centrosome proteins (Andersen *et al.* 2003). Here we will present our current understanding of human Cep63 function.

LACK OF CYCLIN D1 KINASE ACTIVITY LEADS TO REDUCED LEVELS OF ERBB2-INDUCED SENESCENCE AND ACTIVATION OF AUTOPHAGY IN THE MAMMARY GLAND

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Overexpression of cyclin D1, as observed in a large proportion of human breast cancers, is believed to confer proliferative advantage through inactivation of the retinoblastoma protein, pRB. We have previously shown that a single amino-acid change that specifically abrogates the kinase-dependent function of cyclin D1 is sufficient to render mice resistant to breast cancer initiated by ErbB2. In addition, we identified a population of mammary progenitors that requires cyclin D1 activity for its self-renewal and contains the cellular target for MMTV-ErbB2 mediated transformation. While these findings clearly support a role for cyclin D1 activity in the mammary stem-progenitor cell hierarchy, the mechanisms responsible for the reduced number of progenitors in mutant mammary glands remain unexplored. We hypothesize that reduction in cyclin D1-associated kinase activity triggers the activation of tumor suppressive mechanisms, which might impair the ability of mammary progenitors to self renew and to become transformed. Surprisingly, mammary glands derived from cyclin D1KE/KE/MMTV-ErbB2 mice exhibit high levels of proliferation that leads to prominent ductal hyperplasia despite the fact that proliferating cells do not progress to form tumors. Nevertheless, ectopic proliferation was accompanied by reduced levels of phosphorylation at Ser-780 of pRb, suggesting that other D-type cyclins were unable to compensate for the lack of cyclin D1-associated kinase activity observed in this tissue. Moreover, the proliferative defects observed in mutant mammary glands correlated with a failure to implement the senescence program along with an upregulation of autophagy. These latter findings are significant in light of recent studies indicating that autophagy may play an active role in caspase-independent programmed cell death. Taken together, our data suggest a previously unknown role for cyclin D1-associated kinase activity in the orchestration of the premature senescence program and the suppression of autophagy in the pre-malignant mammary epithelium.

MECHANISMS ENSURING ROBUST CELL CYCLE EXIT UPON TERMINAL DIFFERENTIATION IN *DROSOPHILA MELANOGASTER*

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Different types of cellular quiescence are controlled in distinct ways, both in the manner of cell cycle inhibition and in the signaling pathways used to initiate withdrawal from the cell cycle. We have examined the process of cell cycle withdrawal upon terminal differentiation in the eyes and wings of *Drosophila melanogaster*. Terminal differentiation signals initiate cell cycle exit by repressing either the E2F transcription factor complex or by repressing Cyclin E/Cdk2 activity, the G1 Cyclin/Cdk responsible for S-phase entry. While inhibition of only one of these factors is necessary to initiate cell cycle exit, we have found that long-term quiescence is stably maintained in terminally differentiated cells by simultaneously limiting both E2F and Cyclin E activities, an effect we call “double-assurance”. This disrupts the positive regulatory loop that normally exists between E2F and Cyclin E and ensures that cell cycle exit is robust to the de-regulation of a single cell cycle factor. Recently, we have found that E2F-Cyclin E positive feedback is limited after terminal differentiation by the degradation of E2F activator complexes and formation of Cyclin E-resistant E2F repressor complexes. In addition, Anaphase Promoting Complex (APC/C) activity increases upon terminal differentiation to promote cell cycle exit by degrading key cell cycle proteins. These mechanisms are critical for the robust exit of differentiated cells as bypassing them forces cell cycle re-entry and leads to tumor-like outgrowths, composed of dividing but terminally differentiated cells. Currently we are working to identify the developmental signaling pathways that act to coordinate terminal differentiation with the decision to exit from the cell cycle.

ENDOCYCLING CELLS DO NOT APOPTOSE IN RESPONSE TO REREPLICATION AND DNA DAMAGE.

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The endocycle is comprised of alternating G and S phases and is widespread in nature, yet this modified cell cycle is not well understood. In Drosophila, a number of tissues switch from mitotic to endocycles in response to developmental signals. To understand these developmentally modified cell cycles, we have used a combined genomic, genetic, and cell biological approach, which has revealed differences in the cell cycle oscillator, cell growth, and checkpoints. Here we focus on our finding that endocycles have a distinct response to re-replication stress.

In tissues comprised of mitotically proliferating cells, we find that an increased abundance of the pre-RC protein Cdt1 is sufficient to induce genotoxic re-replication stress, resulting in activation of ATM / ATR checkpoint kinases in numerous repair foci. Heterochromatic regions have more repair foci suggesting that they may represent re-replication fragile sites. The ultimate fate for most of these cells is an apoptotic cell death that is dependent on the fly orthologs of the Chk2 and p53 tumor suppressors, similar to the re-replication response in humans and other organisms. In endocycling cells from a variety of tissues, increased Cdt1 also results in re-replication, genotoxic stress, and activation of ATM / ATR, but these cells do not apoptose. Our combined data suggest that endocycling cells do not apoptose because p53 transcriptional activity is blunted through epigenetic silencing of its target genes. Investigating how re-replication checkpoints and fragile sites differ among cell types may uncover conserved mechanisms relevant to tissue-specific genome instability and cancer in humans.

USE OF FLUORESCENT TOOLS FOR MONITORING CELL CYCLE IN LIVE AND FIXED CELLS

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Current fluorescence measurements of cell cycle using either nucleic acid stains for mathematically modeling DNA content or fluorescently labeled antibodies against BrdU, Ki67, PCNA, or cyclins are essential for characterizing changes in cell cycle. In addition to these methods, several new methods have recently become available.

Characterization of cell cycle using two fluorescent protein fusions which are cyclically targeted for degradation through an ubiquitination pathway is an elegant method pioneered by Miyawaki *et al*, for live cell cycle visualization, known by the acronym FUCCI (Fluorescence Ubiquitination Cell Cycle Indicator). Green and red fluorescent proteins (GFP and RFP) fused to geminin and Ctd1, respectively, balance in intensity near the entry into S-phase causing the nucleus to appear yellow when the color channels are merged. The green from GFP-geminin predominates through S, G2 and M phase, while the red from RFP-Ctd1 predominates during G1. Delivery of the FP chimeras is facilitated by baculovirus delivery technology which has been demonstrated to label primary and stem cells and most mammalian cultured cell lines. MAP4 RFP (microtubulin-associated protein 4) used with geminin FP chimeras provides additional information of cell cycle dynamics and can be used to identify cells in M phase.

Cell cycle dynamics can be further dissected through the incorporation of a thymidine analog detectable with click chemistry labeling. This method offers an alterative method to using the traditional antibody detection of the thymidine analog BrdU (bromo-deoxyuridine) to measure proliferative potential. Cells actively replicating DNA incorporate the analog EdU (ethynyl deoxyuridine), when, after fixation and permeabilization they react with a dye azide to form a covalent fluorescent tag. Since standard antibody fixatives are used, the cells can then be antibody labeled with cell cycle relevant targets like cyclin B1, Ki67, and phosphohistone H3 and stained for DNA content using nucleic acid binding dyes. Use of both thymidine analogs, EdU and BrdU, for sequential pulse labeling of proliferating cells further expands the potential for measuring S-phase proliferation potential. Although requiring the more cumbersome methods for BrdU detection, dual pulse labeling offers insight to temporally based proliferation events.

THE SPINDLE CHECKPOINT STABILIZES GEMININ AND ALLOWS ACCUMULATION OF CDT1-GEMININ COMPLEXES TO COUPLE REPLICATION-LICENSING TO SUCCESSFUL MITOSIS

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Cycling cells must ensure that DNA replication is not initiated until after mitosis has completed. In mammalian cells, the Cdt1 protein, a pre-replication complex (preRC) constituent, is rate-limiting for the recruitment of MCM proteins to Origins of replications (ORIs), directing replication licensing.

Here we show that Cdt1, which is unstable in G2 phase, is stabilized in mitosis. This might require the spindle-checkpoint independent loss of cyclin A, leading to inactivation of SCF-dependent turn-over of Cdt1. Interestingly, Cdt1 protein synthesis escapes the general inhibition of translation in mitosis. Together, this results in accumulation of Cdt1 during prometaphase. Cdt1 that accumulates in mitosis is kept in check by binding to Geminin, which inhibits preRC-formation.

To initiate preRC-formation in preparation of S-phase, Geminin needs to be degraded after mitosis. However, we found that Geminin destruction starts already when the chromosomes align at metaphase and is dependent on APC/C-Cdc20. Initiation of Geminin destruction is controlled by the spindle checkpoint. This is surprising because so far only two other proteins, cyclin B1 and Securin, have been identified as spindle checkpoint targets.

In conclusion, our results indicate that mitosis and subsequent spindle checkpoint inactivation are required to direct S-phase licensing. We show that accumulation of Cdt1 in mitosis creates a distinct time window for the accumulation of Geminin-Cdt1 complexes. Spindle checkpoint-dependent Geminin destruction at metaphase releases Cdt1, allowing preRC-formation. This sequence of events creates a unidirectional mechanism controlled by APC/C-Cdc20 that couples preRC-formation to a successful round of mitosis.

ACTIVE METABOLISM IN QUIESCENT FIBROBLASTS

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Many cells in the human body exist in the cellular state of quiescence, which is characterized by reversible exit from the cell cycle. Quiescent cells are widely reported to exhibit reduced cellular size, nucleotide synthesis and metabolic activity. Previous studies in lymphocytes and thymocytes have reported much lower glycolytic rates in quiescent compared with proliferating cells. In contrast to these previous findings, we show here that primary human fibroblasts continue to exhibit high metabolic rates when induced into quiescence via contact inhibition. The metabolic activity of quiescent fibroblasts (i.e., glucose consumption, lactate excretion) is comparable to that of their proliferating counterparts, yet they direct this activity differently. Using a metabolomics-based approach to monitor flux through metabolic pathways coupled with a computational model of metabolism based on ordinary differential equations (ODE), we were able to show that quiescent fibroblasts utilize glucose in all branches of central carbon metabolism including, surprisingly, the pentose phosphate pathway (PPP) and the tricarboxylic acid (TCA) cycle. Proliferating fibroblasts, on the other hand, attenuate the flow of glucose carbons from citrate into the rest of the TCA cycle. By feeding the cells with labeled glutamine, we also detected a “backwards” flux in the TCA cycle from α-ketoglutarate to citrate that was faster in quiescent than proliferating fibroblasts. The high metabolic activity of the fibroblasts is reflected in significantly increased production of the extracellular matrix proteins. We tested the functional significance of the high levels of PPP usage in quiescent fibroblasts by treating with a PPP inhibitor. Quiescent fibroblasts exhibited more cell death in response to treatment with a PPP inhibitor than proliferating fibroblasts, possibly reflecting the functional importance of NADPH generated by this pathway for free radical detoxification in quiescent cells. Our findings demonstrate that reduced metabolic activity is not a hallmark of the quiescent state. Further, quiescent cells, relieved of the biosynthetic requirements associated with generating progeny, may maintain a high metabolic rate, actively detoxify free radicals, and divert their metabolic potential toward the synthesis of proteins that are beneficial for the organism as a whole.

GENERATION OF FLUORESCENTLY TAGGED ENDOGENOUS PROTEINS BY RAAV RECOMBINOMICS TO STUDY MITOSIS IN HUMAN CELLS

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Mitosis is a highly dynamic process that is essential to generate two genetically identical daughter cells. Correct progression through mitosis requires the concerted actions of many regulatory pathways, which include protein phosphorylation/dephosphorylation and proteolysis. Live-Cell Imaging has been instrumental to our understanding of mitosis and its regulation, as it has allowed us to perform quantitative measurements of these pathways at high spatial and temporal resolution.
An outstanding challenge in order to obtain an integrated model of mitosis (and of the cell cycle in general) will be to "connect" data coming from Cell Biology and Biochemistry in the most unbiased manner. To this end, we developed a strategy to generate human cell lines expressing fluorescently labeled endogenous proteins "in a blink of an eye" by homologous recombination. This is done through the use of recombinant Adeno-Associated Virus (rAAV)-mediated knock-ins. The resulting reporter cell lines can then be used for quantitative Cell Biology and Biochemistry studies of your favorite endogenous protein. We show examples of insightful fluorescence quantifications of known mitotic regulators.

ACTIN-DEPENDENT CELLULAR PROLIFERATION IS MEDIATED BY TROPOMYOSIN TM5NM1

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Progression through the cell cycle is associated with profound changes in the actin cytoskeleton. Disruption of the actin cytoskeleton with pharmacological agents causes arrest in G1 phase and failure to enter S-phase, indicating that an intact actin cytoskeleton is required for cell cycle progression. The lack of specificity of these agents has hindered the analysis of the mechanisms responsible for this regulation. We have previously identified functionally distinct populations of actin filaments, each containing different isoforms of the actin filament-associated protein, tropomyosin (Gunning, Hardeman, O'Neill, Physiol Revs 2008). In the current study, we hypothesised that a specific population of Tm isoform-containing actin filaments regulates cell proliferation. The role of Tm5NM1-containing actin filaments was investigated in knock-out, knock-down, and overexpression model systems. Our data show that embryonic fibroblasts (MEFs) isolated from Tm5NM1/2-knockout mice have an impaired rate of proliferation in response to growth factor stimulation. This is accompanied by a dysregulation of MAPK signalling (phospho-ERK1/2) and decreased expression of the key G1-phase mediator, Cyclin D1. Neuroblastoma SHEP cells treated with siRNA against Tm5NM1/2 also have reduced proliferation rates and decreased Cyclin D1 expression. In comparison, Tm5NM1-overexpressing neuroblastoma-derived B35 cells display accelerated proliferation, and reduced propensity to withdraw from the cell cycle when treated with differentiation factors. These data suggest that actin-filaments containing Tm5NM1 mediate G1 phase progression, potentially via controlling the fidelity of signalling through the ERK/MAPK pathway. This indicates that Tm5NM1 has potential as a novel anti-proliferative therapeutic target.

MITOTIC CHROMOSOME CONDENSATION MEDIATED BY THE RETINOBLASTOMA PROTEIN IS TUMOR SUPPRESSIVE

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The retinoblastoma protein (pRB) is a well-known regulator of progression through G1 of the cell cycle. Recently, we uncovered a role for pRB that is critical for mitotic chromosome condensation, independent of G1 to S-phase regulation. Condensation and segregation of mitotic chromosomes is a critical process for cellular propagation and in mammals, mitotic errors can contribute to cancer pathogenesis. Therefore, we investigated whether this novel role of pRB in mitosis contributes to its role as a tumor suppressor using mice with a defective pRB-LXCXE binding cleft ($Rb1^{\Delta L/\Delta L}$).

By means of video microscopy, we investigated the role of pRB in mitosis. $Rb1^{\Delta L/\Delta L}$ mouse embryonic fibroblasts (MEFs) exhibit a delay in initiating anaphase, a significantly wider metaphase plate, and an increased frequency of lagging chromosomes. Interestingly, similar phenotypes were observed in wild type MEFs with defective Condensin II. Accordingly, we demonstrate pRB association with Condensin II on chromatin. Thus chromosome condensation facilitated by pRB is important for faithful chromosome segregation.

To investigate whether $Rb1^{\Delta L/\Delta L}$ contributes to tumorigenesis, we crossed $Rb1^{\Delta L/\Delta L}$ mice with $Trp53^{+/-}$ and $^{-/-}$ mice. Cancer prone $Trp53^{-/-}$ mice succumb to more aggressive cancers, with an increase in the frequency of whole chromosome and localized gains and losses. As well, mutant pRB accelerates loss-of-heterozygosity, leading to earlier tumor formation, in $Trp53^{+/-}$ mice. Therefore pRB's ability to condense chromosomes is an important aspect of pRB-mediated tumor suppression.

By utilizing $Trp53^{-/-}$ mice, we bypassed G1 to S-phase regulation and were able to study pRB in mitosis alone. We were also able to demonstrate an association of pRB with Condensin II, which helps facilitate chromosome condensation. These data reveal a new mechanism of tumor suppression, facilitated by pRB, in which genome stability is maintained by proper condensation of mitotic chromosomes.

A DNA DAMAGE RESPONSE SCREEN IDENTIFIES RHINO: A 9-1-1 INTERACTING PROTEIN REQUIRED FOR HOMOLOGOUS RECOMBINATION

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The DNA damage response (DDR) is a protein kinase cascade that orchestrates DNA repair processes via transcriptional and post-translational mechanisms. Cell cycle arrest is a hallmark of the DDR. We performed a high throughput, genome-wide, image-based siRNA DDR screen and uncovered a critical role for homologous recombination (HR) proteins and of Fanconi Anemia proteins in the maintenance of prolonged cell cycle arrest and to prevent massive genomic instability. Over 100 high scoring DDR candidate genes were interrogated with >7 siRNA per gene for their role in the DDR. Of these we identified a novel protein RHINO that is recruited to DNA damage sites independently of H2AX. RHINO exists in complex with Rad18 and Ubc13 as well as the Rad9-Rad1-Hus1 clamp (9-1-1) and TopBP1. RHINO is recruited to sites of DNA damage by the 9-1-1 complex and is required for a subset of HR events in a pathway parallel to or downstream of Rad51. RHINO acts to promote cell cycle arrest independently of Chk1 and represents a new arm of DDR signaling emanating from the Rad17/9-1-1 sensor of DNA damage.
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THE MYC/MAX/MIZ PATHWAY REGULATES THE EXPRESSION OF THE AURORA-A KINASE FOLLOWING TOPOISOMERASE I INHIBITION

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During the G2 phase of the cell cycle, the Aurora-A kinase plays an important role in centrosome maturation and progression to mitosis. In this study, we show that Aurora-A expression is downregulated in response to topoisomerase I inhibition. Using chromatin immunoprecipitation assays, we have observed that the Myc transcription factor and its Max binding partner are associated with the Aurora-A promoter during the G2 phase of the cell cycle. RNA interference experiments indicated that Myc is necessary for the activation of the Aurora-A gene. Following topoisomerase I inhibition, the expression of Myc decreased whereas Mad was upregulated, and the association of Myc and Max with the promoter of the kinase was inhibited. In parallel, an increased association of Miz-1 was detected on DNA, associated with an inhibition of the recruitment of transcriptional coactivators. Further confirming the link between Myc and Aurora-A, these two proteins were found to be overexpressed in tumor samples isolated from colorectal cancers and this was associated with an increased genomic instability and a decreased expression of Miz, Mad and p21waf1 mRNAs.
These results suggest a model by which the Aurora-A gene is inactivated by the G2 checkpoint following topoisomerase I inhibition. We propose that the overexpression of the Myc-Aurora-A pathway together with a downregulation of Mad and Miz-1 could be used as a prognosis signature of poor responses to topoisomerase I inhibitors.

J.C and S.C contributed equally to this work.

NUCLEAR SIZE PROMOTES VARIABLE NUCLEAR DIVISION CYCLE TIMING IN A MULTINUCLEATE CELL

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Synthesis and accumulation of conserved regulators, such as cyclins, promote cell cycle progression in most eukaryotes. When cells at different stages of the cell cycle are fused to form heterokaryons, the complement of regulators, which shuttle between the nucleus and cytoplasm, induce the nuclei to become synchronized. Exceptions to this paradigm are multinucleate fungi or multinucleate cells triggering cell cycle checkpoints, which often display asynchronous nuclear division among nuclei in a shared cytoplasm. To study the cell biological basis for nuclear autonomous division in a common cytoplasm, we are using a model multinucleate fungus, *Ashbya gossypii*, which is highly related to *S. cerevisiae* (95% of genes are homologous and syntenic). Notably, each nucleus within an *Ashbya* cell divides asynchronously despite access to a shared cytoplasm. Using this model system, we are investigating if asynchrony in cell cycle progression within a syncytium is programmed by the cell cycle machinery or is a result of molecular noise. To address this, we tracked nuclear division timing for sister nuclei born from one mitosis event using 4D-fluorescence microscopy. We then used statistical modeling to determine the relationship between sister division times. We found a positive association in division times indicating that sister nuclear division times are more closely related to one another than to the overall population of nuclei within the cell. One explanation for this relationship is that some nuclear feature (size, regulatory protein concentration, etc.) varies substantially between mitotic nuclei. This leads to similar sister timing but highly variable timing differences in the whole population of nuclei. To begin to test this, we looked at nuclear size and its relationship to cell cycle progression. We observed that nuclear volume at birth is highly variable (2.6 ± 1.1 μm3) and that small nuclei have longer nuclear division times. In addition, as with nuclear division time, sister nuclei are more similar in size to each other than the overall population. This indicates that nuclear size can influence division time even among nuclei within a common cytoplasm. However, nuclear volume immediately preceding mitosis is highly variable (5.6 ± 1.9 μm3) suggesting there is not an absolute size threshold that triggers division. In this work, we have demonstrated that nuclear size influences cell cycle progression of nuclei within a common cytoplasm. Variable nuclear size may be a conserved feature that promotes heterogeneous cell cycle behavior in a population of genetically identical CELLS.

THE MECHANISM AND FUNCTION OF PIP BOX/CRL4^{CDT2}-MEDIATED DESTRUCTION OF E2F1 DURING S PHASE

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Accurate progression through the cell cycle depends on the timely synthesis and destruction of a myriad of proteins. Recently it was discovered that the proteolysis of a small set of proteins is coupled directly to DNA synthesis occurring during S phase or DNA repair. We have shown that one of these proteins is the *Drosophila melanogaster* transcription factor E2f1, a member of the E2F family of transcriptional activators that plays a pivotal role in the G1-S transition. Like mammalian E2Fs, E2f1 is inhibited prior to S phase via interaction with the retinoblastoma protein (pRb) homolog, Rbf1. Activation of cyclin-dependent kinases during G1 results in hyper-phosphorylation of Rbf1, thereby relieving E2f1 repression and activating a transcription program that promotes entry into S phase. E2f1 is then rapidly destroyed during S phase. We hypothesize that S phase-specific destruction provides an important Rbf1-independent negative regulation of E2f1 that is essential for normal cell cycle progression. S phase-specific destruction of E2f1 requires a PCNA interacting protein (PIP) box-containing degron in the E2f1 NH2-terminus and the CRL4^{Cdt2} E3 ubiquitin ligase complex. Current models suggest that PIP degrons direct proteins to replication fork associated PCNA and that this interaction promotes ubiquitylation by CRL4^{Cdt2} and subsequent proteolysis by the 26S proteasome. This results in an elegant mechanism for coupling protein destruction with ongoing DNA replication. We will present data investigating which biochemical activities of E2f1 (e.g. DNA binding, Dp binding) are required for this destruction mechanism as well as the biological consequences of improper regulation of E2f1 abundance during the cell cycle. We have shown that expression of a mutant version of E2f1 lacking a functional PIP box (E2f1^{PIP3A}) in proliferating cells results in E2f1 accumulation during S phase, reduction of G1 length, and apoptosis. Our data indicate that S phase-coupled destruction of E2f1 and the induction of apoptosis by E2f1^{PIP3A} occur independent of E2f1's DNA binding domain. This result suggests that destruction during S phase inhibits an activity of E2f1 that cannot be achieved by removal of E2f1 from chromatin. We are testing this hypothesis in part by inducing destruction of E2f1 during mitosis rather than S phase and determining if this will relieve the phenotypic consequences (e.g. apoptosis) of E2f1^{PIP3A} expression. We conclude that carefully regulated S phase-coupled proteolysis of E2f1 is essential for normal cell cycle progression.

MTG8 IS CRITICAL FOR PROPER CELL CYCLE PROGRESSION AND DNA DAMAGE CONTROL

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Chromosomal translocations are closely associated with the development of acute leukemia and analysis of these translocations has led to the discovery of molecules that play important roles in the regulation of both growth and differentiation. One of the most common targets of chromosomal translocations in acute myeloid leukemia is the Myeloid Translocation Gene on Chromosome 8 (MTG8, also known as ETO). MTG8 is targeted by the t(8;21), which results in the fusion of the DNA binding domain of RUNX1 to nearly all of MTG8. Germline deleted *Mtg8*-null mice displayed aberrant gut architecture, which was suggested to be due to a mesenchymal defect. Due to the mesenchymal defects in these null mice, we examined mouse embryonic fibroblasts (MEFs) derived lacking *Mtg8*. Using RT-PCR, we found that found that *Mtg8* was expressed in both the wild type and heterozygous MEFs, but there was no discernible expression of *Mtg8* in the null MEFs. We then measured cell proliferation and found that *Mtg8*-null cells grow at a slower rate than heterozygous or WT MEFs. FACS analysis using Propidium Iodide stained MEFs indicated a marked accumulation of cells with 4N DNA content, suggesting a checkpoint slowing G2/M progression in the *Mtg8*-null cells. These cell cycle defects prompted an assessment of the DNA damage response. 53BP1 and phosphorylated H2AX (γH2AX) are located at the sites of DNA double strand breaks as part of the DNA damage response. Anti-53BP1 and anti-γH2AX were used in immunofluorescence analysis on the MEFs. Significantly more 53BP1 and γH2AX foci were detected in *Mtg8*-null MEFs compared to WT MEFs. Interestingly, *Mtg8*-null MEFs were protected from DNA damage when quiescent due to serum starvation, suggesting that the DNA damage occurred during DNA replication. Our work points to a cell cycle defect in *Mtg8*-null MEFs, and identifies a role for Mtg8 in S-phase progression and DNA damage control.

THE NOCUT CHECKPOINT PREVENTS RHO2 GTPASE ACTIVATION AT THE BUD NECK IN ORDER TO INHIBIT ABSCISSION

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Abscission, the final step of cytokinesis, resolves the plasma membrane of the two daughter cells leading to their final separation. This process is regulated by the NoCut checkpoint in yeast and in mammalian cells. NoCut-dependent regulation of abscission prevents premature daughter cell separation and DNA damage. In yeast, the NoCut checkpoint depends on the activity of the Aurora B kinase Ipl1 and the two anillin-related proteins Boi1 and Boi2. Activation of Ipl1 at the midzone leads to Boi1 and Boi2 shuffling from the nucleus to the bud neck and results in abscission inhibition. Here we report the identification of the Rho-GEF Tus1 , the Rho-GTPase Rho2 and the Tus1-interacting protein Cyk5 as new players of the abscission pathway. Furthermore, we show that Tus1 and Cyk5 are displaced from the bud neck, the site of cytokinesis, upon NoCut activation. Out of the 5 yeast Rho GTPases, Rho1, Rho2 and Rho4 localize to the bud neck in late mitosis. Deletion of Rho2 specifically impaired abscission, mapping Rho2 to the abscission pathway. Our data indicate that the NoCut checkpoint inhibits abscission through displacement of Tus1 and Cyk5 from the site of cytokinesis and thereby keeping Rho2 inactive. Accordingly, expression of a Rho2-GTP locked protein bypasses NoCut activation and permits cells to undergo abscission even in the presence of chromosome segregation defects. Thus, our data show that the Rho2 pathway is a key regulator of abscission and the major target of the NoCut checkpoint.

STUDYING CENTROMERIC HISTONE DYNAMICS THROUGH THE USE OF FRET

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At the center of every chromosome is a region of immense importance and mystery, the centromere. This structure is both maintained and defined by a centromere-specific histone 3 variant (CenH3). CenH3s possess both a highly divergent sequences across different species, and are incorporated in a replication independent fashion. Replacing the canonical histone 3 in large alternating domains at the centromere, many questions still remain about the dynamics of CenH3 across the cell cycle, structure of the nucleosomes CenH3s are incorporated into, and how CenH3 serves as a platform to recruit kinetchore proteins. Here we propose through the use Acceptor-Photobleaching Förster Resonance Energy Transfer (AP-FRET) the studying the dynamics of CenH3 will serve as a useful tool in pursuing the answers to these and additional questions. Our results with AP-FRET in HeLa cells, show that CenH3 possesses a unique dynamics when compared to canonical H3 across a variety of conditions. These results also serve as a proof of principle for the further use of AP-FRET in the study of centromeric chromatin dynamics.

SPECIFIC ROLES OF TARGET OF RAPAMYCIN IN THE CONTROL OF STEM CELLS AND THEIR PROGENY IN THE DROSOPHILA OVARY

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Growth, proliferation and differentiation of cells depend on many inputs, including diet. In multicellular organisms, the effects of diet are mediated by circulating factors that impact many cells simultaneously. Yet, an intriguing question is how such systemic, nutrient-dependent factors elicit specific responses in different cells, even within the same tissue. We previously showed that germline stem cells (GSCs) and follicle stem cells (FSCs) and their progeny in the Drosophila ovary respond to diet via insulin signals. Insulin signals directly modulate the GSC cell cycle at the G2 phase, and they also control the division and growth of their progeny. Additional factors, however, also contribute to the dietary control of oogenesis. Target of Rapamycin, or TOR, is part of a highly conserved nutrient-sensing pathway affecting growth, proliferation, survival, and fertility. Our recent unpublished studies show that TOR promotes GSC proliferation via G2 but independently of insulin signaling, and that TOR is required for the proliferation, growth and survival of differentiating germ cells. We also found that TOR activity modulates the proliferation of FSCs but, surprisingly, not of their dividing progeny, indicating specificity in the control of the stem cell cycle by TOR in the ovarian somatic lineage. TOR controls follicle cell number instead by promoting survival, independently of either the apoptotic or autophagic pathways. In addition, TOR controls the switch of follicle cells from mitotic divisions to the endoreplication program. These results uncover specific TOR functions in the control of stem cells versus their differentiating progeny, and reveal parallels between Drosophila and mammalian follicle growth.

A PROTEOMIC SCREEN REVEALS NEW PROTEINS INVOLVED IN DNA DAMAGE RESPONSE IN FISSION YEAST

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Eukaryotic cells respond to DNA damage by recruiting a large number of DNA repair proteins and checkpoint signaling proteins to sites of DNA damage on chromatin. The local concentrations of these proteins often reach high enough level to form so-called DNA damage-induced nuclear foci that are visible by microscopy.

Based on the premise that proteins localizing to sites of DNA damage are likely to play a role in DNA damage response, we have set up a system to visually screen for proteins involved in DNA damage response. A plasmid library expressing fission yeast ORFs fused with the yellow fluorescent protein (YFP) allows us to inspect the localization of each individual fission yeast protein. By engineering a fission yeast strain with a LacO repeat inserted next to an HO endonuclease cleavage site, we can simultaneously visualize the sub-nuclear location of the HO-induced DNA double-strand break (DSB) and the spatial distribution of the protein expressed from the library plasmid. We have screened nearly all 5000 proteins in the fission yeast proteome and identified 55 proteins that colocalize with the DSB markers. To our satisfaction, proteins known to act in DNA damage response are highly enriched in our screen hits. About one third of the screen hits have not been previously characterized. Many of these novel DNA damage response proteins are conserved in humans. In-depth analyses of the relocalization patterns of these proteins and the phenotypes of the corresponding deletion mutants have provided useful clues to their functions at DSBs.

SIZE IS A MAJOR DETERMINANT OF CELLULAR LIFESPAN

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Cultured normal human cells have a limited lifespan of ~50 doublings. These cells gradually enlarge as they age and lose their replicative potential. However, the biological relevance of the relationship between cell size and replicative potential is not well understood, and a causal role for size in cellular aging remains to be established. Deletion of many genes involved in ribosome biogenesis and cell growth convey a long-life phenotype on cells. Because we fortuitously noticed that many longevity genes concomitantly affect cell size, we have re-examined the potential linkage between cell size and lifespan. In yeast, many genes that encode ribosome proteins are present in duplicate pairs. Strikingly, deletion of only one gene of the pair results in small, long-lived cells. Moreover, long life nearly always correlates with reduced cell size. Therefore, we have tested the hypothesis that size determines cellular lifespan. Herein, we report that, regardless of birth size, yeast cells grow steadily with age and enter senescence at relatively constant cell size. Subsequently, lifespan is abnormally short in large cells. Moreover, mutations that increase cell size, decrease lifespan and vice versa. In addition, interestingly, deletion of protein components of the telomerase enzyme (e.g. est1 and est2 mutants) leads to abnormally large short-lived cells. Furthermore, epistatic interactions reveal that size per se modulates cellular lifespan. For example, induction or selection of abnormally large cells dramatically reduces cellular lifespan even in small cell mutants. In addition, nutrient starvation reduces the size and extends the lifespan of large cell mutants. Moreover, this ability is wholly dependent upon cell size effects. In examining a large number of mutants, we find that cell size modulates growth rate. In general, small cells grow slowly and vice versa. Subsequently, the rate at which cells increase in size over their lifetime determines cellular lifespan. Because both nutrients and specific genetic pathways regulate this rate, we conclude that size has an integral role in the control of cellular lifespan.

INSIGHTS INTO THE TPR COMPLEX OF THE APC FROM A CDC26-APC6 CRYSTAL STRUCTURE

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The anaphase promoting complex (APC) is a multisubunit ubiquitin E3 ligase essential to cell cycle regulation. The CDC26 subunit was previously shown to have a role in APC assembly, but its precise molecular function remained unclear. Our studies revealed that CDC26 stabilizes the structure of APC6, a core TPR protein essential for APC integrity and function. The TPR domain of APC6 coils around the rod-like N-terminus of CDC26, as seen in the crystal structure of a proteolytically derived complex, $CDC26^{N}$-$APC6^{TPR}$. CD analyses demonstrated that a CDC26-APC6 complex is structurally more stable than APC6 alone. Moreover, the N-terminal region of CDC26 ($CDC26^{N}$) is sufficient to impart increased structural stability to APC6 in vitro and also rescues the temperature-sensitivity of a cdc26Δ yeast strain. Mutations predicted to disrupt the CDC26-APC6 interaction eliminate this rescue. Thus, the $CDC26^{N}$-$APC6^{TPR}$ crystal structure reveals a protein-protein interaction critical to APC architecture.

HIGHER LEVELS OF DNTPS SUPPRESS GENETIC INSTABILITY IN *SACCHAROMYCES CEREVISIAE* HYPOMORPHIC *MEC1* (ATR) MUTANTS.

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MEC1, the essential yeast ATR/ATM homolog, controls the S phase checkpoint and prevents replication fork collapse at slow zones of DNA replication. *MEC1* is required for increasing deoxynucleoside triphosphate (dNTP) levels in S phase, which involves the degradation of Sml1, a negative inhibitor of ribonucleotide reductase (Rnr). The growth of some hypomorphic *mec1* mutants, is severely compromised in *rad52* mutants, deficient in double-strand break repair, suggesting that replication fork collapse or incomplete replication generates recombinogenic lesions. We previously observed a six-fold, tenfold and 30-fold higher rate of spontaneous sister chromatid exchange (SCE) , heteroallelic recombination, and translocations, respectively, in *mec1-21* mutants compared to wild type. Here we report that *TEL1*, *MRE11*, and *RAD50* are also required for the hyper-recombination phenotype, suggesting that *TEL1* promotes recombinational repair when replication forks collapse. The hyper-recombination phenotype correlates with lower dNTP levels, compared to wild type. By introducing a *dun1* mutation and eliminating inducible expression of *RNR* in *mec1-21*, rates of spontaneous SCE were increased 15-fold above wild type. The hyper-recombination phenotypes of both *mec1-21* and *mec1-21 dun1* were suppressed by *SML1* deletions, which increased dNTP levels. Measurements of each dNTP indicted that, compared to wild type, there was a significant decrease in basal dNTP levels in *mec1-21* and *mec1-21 dun1*, while the basal dNTP levels of *mec1-21 sml1*, *mec1-21 dun1 sml1*, and *sml1* mutants were similar and about two-fold higher than wild type. Interestingly, higher levels of dNTPs in *mec1-21 dun1 sml1* correlate with two-fold higher levels of spontaneous mutagenesis, compared to *mec1-21 dun1*. Thus , compared to wild type, more homologous recombination occurs in *mec1-21*, which correlates with lower dNTP levels. We suggest that higher dNTP levels in specific checkpoint mutants suppress the formation of recombinogenic lesions and instead promote error-prone replication.

A REVISED QUANTITATIVE MODEL OF CDK-MEDIATED CONTROL OF THE TEMPORAL ORDER OF S-PHASE AND MITOSIS

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Nearly 15 years ago, we proposed a quantitative model explaining how variations in activity of a single CDK through the cell cycle maintain the dependency of mitosis on DNA replication, and vice versa (Fisher & Nurse, 1996). The molecular basis for the model is now known: low CDK activity is required for origin licensing, moderate CDK activity promotes formation of pre-initation complexes and replication initiation, and high activity triggers a feedback-dependent switch ensuring unidirectionality of the G2/M transition. Recently, using Xenopus egg extracts, we found that, unlike M-phase, there is no such switch-like behaviour in CDK-mediated control of S-phase (Krasinska et al, 2008). We hypothesized that PP2A activity might explain the different dynamic behaviour of CDK-mediated control of S-phase and M-phase, since active PP2A prevents mitosis onset (Mochida et al, 2009) but is required to replicate DNA by loading CDC45 onto replication origins (Chou et al, 2002) – the same step at which CDKs act. Here, we find that mitosis and S-phase onset are controlled by an equilibrium between CDK activity and PP2A activity. This equilibrium is modulated by a checkpoint stimulated by unreplicated DNA. If PP2A is inhibited, switch-like behaviour at mitosis onset is abolished, and CDK activity is integrated over time to promote mitosis onset. However, PP2A is required for DNA replication independently of its role in preventing mitosis onset. These results show that the dynamic cell-cycle response to CDK activity is interpreted in the context of opposing phosphatases, which ultimately maintain the temporal order of S-phase and mitosis.

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MONOPOLAR SPINDLES INDUCED BY EG5 INHIBITION CAN BE RESCUED BY ALTERING MICROTUBULE DYNAMICS

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Studies on monopolar spindles have taught us a lot about the forces involved in bipolar spindle formation during mitosis. Eg5 is a kinesin which is essential for bipolar spindle formation. Its inhibition results in star-shaped monopolar spindles. Recently, it was shown that depletion of the molecular motor dynein, an antagonist of Eg5, rescues the formation of bipolar spindles in cells with reduced Eg5 activity.
We performed an RNAi screen and found that depletion of chTOG, a known microtubule (MT) associated protein, can rescue inhibition of Eg5 as well. Consistent with its role as a microtubule stabilizer, the depletion of chTOG results in the formation of shorter than normal, often multipolar spindles. To understand how depletion of chTOG is able to separate spindle poles when Eg5 is inactive we followed spindle formation in such cells. Eg5-inhibited cells normally enter mitosis with two unseparated centrosomes which correspond to the microtubule organizing centers (MTOCs). Intriguingly, upon chTOG depletion, we found that (i) cells entered mitosis with more than two separated MTOCs which (ii) were organized into bipolar spindles in an Eg5-dependent manner. To understand if other perturbations of MT dynamics can have the same effect, we tried to rescue Eg5 inhibition with MT drugs nocodazole and taxol. Treatment with nocodazole was able to rescue bipolar spindle formation in HeLa and BSC-1 cells. The rescue worked only if nocodazole was added before entry into mitosis. Thus, our data demonstrate that altered microtubule dynamics can rescue loss of Eg5.
To our knowledge, we show for the first time that depletion of nonmotor proteins and changes in MT dynamics can rescue inhibition of Eg5. Changes in MT dynamics might lead to fragmentation of MTOCs shortly before mitosis or they might favor spontaneous MT nucleation at multiple sites and thus substitute for separated spindle poles. We are currently trying to understand the precise mechanism by which changes in MT dynamics can lead to formation of multiple MTOCs by analyzing how these are related to centrosomes.

SIGNIFICANCE OF CDC20 DESTRUCTION FOR CELL CYCLE FIDELITY

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The critical event of mitosis is the synchronous and equal segregation of sister chromatids to opposite poles of the cell. The Anaphase-Promoting Complex or Cyclosome (APC) is an E3 ubiquitin ligase that initiates anaphase by catalyzing the attachment of ubiquitin to key mitotic regulators, targeting them for destruction by the proteasome. APC activity requires association with the activator subunit Cdc20 at the metaphase-to-anaphase transition and Cdh1 in late mitosis and G1. Cdc20 function is regulated through its abundance, with a transcriptional peak in early mitosis and APC-dependent destruction in late mitosis by two mechanisms: Cdc20-dependent autoubiquitination and Cdh1-dependent ubiquitination. In this work, we addressed the significance of Cdc20 destruction in budding yeast by making Cdc20 mutants that cannot be ubiquitinated. We began by making a yeast Cdc20 mutant in which all 39 lysines are changed to arginines (K0 mutant). The K0 mutant is viable at 22°C, but not at 37°C, and is nonfunctional in vitro. To generate a more conservative non-ubiquitinatable version of Cdc20, we analyzed the importance of lysines in different regions of Cdc20. We found that most Cdc20 ubiquitination in vitro is abolished by mutation of 9 lysines in an N-terminal 60 amino acid region, with some contribution from a single lysine in the C-terminal WD40 domain. Mutation of these ten lysines (K29 mutant) blocks all ubiquitination in vitro. We also identified a non-ubiquitinatable mutant (K5 mutant) that contains only five lysines in the WD40. Replacement of the wildtype copy of Cdc20 with the K29 or K5 mutant is not lethal, and both proteins are fully functional as activators in vitro. Overexpression of these mutants is lethal, and we are currently analyzing specific cell cycle defects in cells expressing these mutants under normal *CDC20* transcriptional control.

CELL CYCLE PROGRESSION REQUIRES THE CDC-48$^{UFD-1/NPL-4}$ COMPLEX FOR EFFICIENT DNA REPLICATION

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Dividing cells have to ensure correct duplication and propagation of their genomic information to the daughter cells. Therefore, DNA replication and separation of sister chromatids are tightly controlled processes central for all eukaryotes. Our laboratory identified CDC-48 to play a crucial role during cell cycle progression in *Caenorhabditis elegans*. CDC-48 is a ubiquitin-selective chaperone with fundamental functions in diverse cellular pathways. Association with the cofactors UFD-1 and NPL-4 is essential for the cell cycle-related function of CDC-48. Consequently, depletion of the CDC-48$^{UFD-1/NPL-4}$ complex by RNAi causes defects in the cell division cycle and chromatin structure. The cell cycle-delay of embryos lacking the CDC-48$^{UFD-1/NPL-4}$ complex is suppressed by co-depletion of the S-Phase checkpoint kinases ATL-1/CHK-1. Moreover, chromosomal bridges between separating sister chromatids in anaphase and focal accumulation of the DNA repair protein RAD-51 indicate a crucial function of CDC-48 in the regulation of DNA replication.
Currently, we analyze reporter strains expressing GFP-fusions of replication factors recapitulating distinct steps of DNA replication. The comparison of replication factor dynamics of RNAi-depleted to wild type embryos will provide detailed insight into the role of the CDC-48$^{UFD-1/NPL-4}$ complex in cell cycle regulation. Furthermore, biochemical and genetic approaches have been initiated to identify additional targets.

CONTRIBUTION OF THE *MIR-11* MICRORNA TO THE *DE2F1* FUNCTION

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MicroRNAs play roles in various biological processes, including cell proliferation, differentiation, and apoptosis. As these processes are implicated in a wide range of diseases, microRNAs are being studied to both understand the disease process, and as potential tools and targets for treatments. The E2F transcription factors are important regulators of cell proliferation and apoptosis while their deregulated activity is thought to drive proliferation in tumor cells. Curiously, the *miR-1*1 microRNA is located within an intron of the *Drosophila de2f1* gene. In order to understand a relationship between *miR-11* and its host gene *de2f1*, we have generated a *miR-11* mutant using homologous recombination. The *miR-11* mutant allowed us to study contribution of the microRNA to cellular function of *de2f1*.

STUDIES OF THE SPINDLE ASSEMBLY CHECKPOINT IN *XENOPUS*

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The spindle assembly checkpoint (SAC) regulates passage through mitosis and represents a critical point of regulation that ensures the accurate segregation of the newly replicated genome. The kinetochore and centromeric regions of chromosomes govern this process in a complex structural and signalling hierarchy. An active SAC will arrest cells in mitosis and involves the recruitment of various factors to the chromatin and the downstream post-translational modification of SAC signalling factors.

The *Xenopus leavis* cell free extract system is an ideal platform from which to identify novel players in SAC signalling and mitotic regulation since chromatin and proteins can be easily purified in large quantities. More importantly replicating chromatin can be added to actively cycling extracts, which can be manipulated by DNA damaging agents or drugs that cause defined perturbations to cell cycle progression. Using mass spectrometry analysis we examined the dynamics of proteins recruited to chromatin in the event of SAC activation. Here we present data from this study that reveals a mechanism by which the ATM dependent DNA damage checkpoint may converge with the spindle assembly checkpoint to regulate mitosis.

FAM64A IS A NOVEL CELL CYCLE-REGULATED PROTEIN INVOLVED IN THE ESTABLISHMENT OF A BIPOLAR SPINDLE

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Successful segregation of chromosomes during mitosis requires the establishment of a bipolar spindle. Bipolar spindle formation relies on both microtubule dynamics and sliding, as depletion or inactivation of kinesin motor proteins Eg5 or Kif2A leads to monopolar spindles. To identify novel genes involved in spindle morphology, we utilized the gene expression profile of cycling HeLa cells and hierarchical clustering. Several uncharacterized genes were identified that show peak expression during G2/M phase and are co-expressed with genes known to function in establishing the mitotic spindle. Here, we use siRNA, epitope-tagging, fixed and live cell fluorescence imaging, and co-immunoprecipitations followed by mass spectrometry to examine the function of one candidate gene, Fam64A, in HeLa cells. Database queries for Fam64A show no known protein domains in the predicted protein sequence, and no sequence similarity to other human genes. The protein has uncharacterized orthologs in placental mammals. FAM64A is cell cycle-regulated at multiple levels. The mRNA shows periodic expression during the cell cycle with peak expression in G2/M phase. FAM64A protein is degraded by the proteasome at anaphase onset and during G1 phase, which is inhibited by the addition of MG132. Depletion of FAM64A using three independent siRNAs results in a 10-fold increase in the number of mitotic cells exhibiting monopolar spindles. Expression of a non-siRNA targetable FAM64A-GFP construct results in a partial rescue of the monopolar defect, suggesting the phenotype is not an off-target effect. The FAM64A-GFP fusion protein localizes to the nucleus during interphase and to the cell cortex in mitosis. The inability to detect spindle localization suggests FAM64A contributes to spindle bipolarity through mechanisms that are independent of other motors like Eg5 and Kif2A. Analysis of GFP-FAM64A binding partners by co-immunoprecipitation shows that it interacts with Borealin, AurKB, Survivin, INCENP and recovers endogenous FAM64A. Therefore, FAM64A may be a novel member of the Chromosomal Passenger Complex.

ANAPHASE REGULATION BY SECURIN IN NORMAL HUMAN SOMATIC CELLS

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The irreversible step of sister chromatid separation at anaphase is regulated by the spindle assembly checkpoint (SAC) which monitors microtubule attachments to each and every kinetochore. Once all sister chromatids have formed bipolar attachments to the mitotic spindle, the anaphase promoting complex (APC) ubiquitylates substrates including cyclin B and securin, triggering their degradation and promoting sister chromatid separation by the activation of the cysteine protease separase. Securin is the inhibitory chaperone for separase which cleaves a subunit of the cohesin complex, dissolving the molecular glue holding sister chromatids together and thus allowing for their separation at anaphase. The inactivation of securin in budding yeast results in the early activation of separase and the separation of sister chromatids prior to bipolar attachment to the spindle. Additionally, securin is involved in a positive feedback loop involving its phosphorylation by cdc2/cyclin B providing for its rapid degradation upon satisfaction of the SAC. This feedback loop in budding yeast results in the switch-like behavior observed as the synchronous separation of sister chromatids at the onset of anaphase. In metazoans however, separase activity is also inhibited by phosphorylation by, and direct binding of, cdc2/cyclin B exclusive of securin binding. To explore securin regulation of separase activity at anaphase, a system in which securin is essential for efficient anaphase has been established. Retinal pigment epithelial cells immortalized with human telomerase (RPE1-hTERT) in which *hSecurin* can be conditionally inactivated have been generated. Within 48 hours of cre-mediated recombination, securin protein is lost, followed by the depletion of separase protein. These cells are unable to undergo multiple cell divisions and instead display a senescent phenotype. Efficient sister chromatid separation is largely absent as demonstrated by time-lapse microscopy and immunofluorescence of molecular markers of anaphase. Securin is therefore necessary for faithful chromosome segregation in non-transformed human somatic cells and this provides a sensitized system in which to probe the function and post-translational regulation of securin during mitosis.

CDK-DEPENDENT PHOSPHORYLATION OF LTE1 RESTRICTS CELL POLARIZATION THROUGH INHIBITION OF RAS AND BUD1

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In the budding yeast S. cerevisiae completion of mitosis is controlled by a signal transduction cascade called the Mitotic Exit Network (MEN) that ultimately activates Cdc14 and inhibits CDK. Tem1, a small G-protein, is at the top of MEN and is negatively regulated by the two-component GAP Bfa1/Bub2. Lte1 promotes MEN activity but its molecular role remains unclear. Lte1 is a daughter-specific protein with a complex pattern of cell cycle-dependent phosphorylation. It contains a GEF-like domain and it was proposed to act directly on Tem1 as GEF but no biochemical evidence supports this model. Recently we described a role of Lte1 in controlling polarisome behaviour. Here we show that Lte1 controls cell polarization by inhibiting Ras and Bud1. Binding to and inhibition of Ras and Bud1 are cell cycle - dependent and rely on a complex Lte1 phosphorylation pattern determined by sequential steps of Cla4 and Clb/CDK activities. Cla4-dependent phosphorylation is essential to increase Lte1 affinity for the polarisome and is a prerequisite for the subsequent Clb/CDK-dependent phosphorylation that increases the affinity for Ras. The importance of the regulation of Ras binding is demonstrated by the localization of the Lte1-8N allele that has lost the daughter specificity because is constantly bound to Ras. Importantly, over-expression of Lte1-8N leads to a Ras-dependent cell cycle arrest in G1, suggesting that once bound to Ras, Lte1 inhibits it. Mitotically arrested lte1Δ cells develop hyper-polarized buds, this phenotype can be suppressed by deletion of BUD1. Bud1, a small G-protein with high homology with Ras, is implicated in bud site selection. We show that Bud1 can also interact with Lte1 and we propose that, like Ras, Lte1 binds and inhibits Bud1, contributing to the restriction of cell polarization in G2/M.

PROTEIN TYROSINE KINASE 6 EXPRESSION IS REGULATED BY P53-DEPENDENT AND INDEPENDENT MECHANISMS IN RESPONSE TO DNA-DAMAGE.

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BACKGROUND & AIMS: Protein Tyrosine Kinase 6 (PTK6) is an intracellular tyrosine kinase that promotes epithelial cell differentiation and cell cycle exit in the normal intestine. However, following DNA damage, PTK6 is induced in proliferating crypt epithelial cells where it promotes apoptosis by inhibiting prosurvival signaling pathways. The tumor suppressor protein p53, which is frequently mutated in colon cancer, also plays a key role in DNA-damage induced apoptosis in the intestine. The aim of our study was to examine cross talk between PTK6 and p53 and determine contributions of PTK6 to p53-dependent and independent DNA-damage induced apoptosis. METHODS: Isogenic HCT116 p53+/+ and p53-/- cells were subjected to 20 Gy of gamma-irradiation and harvested at 0, 3, 6, 24, 48, and 72 hours. Expression of PTK6, p53, p21, cleaved caspase-3, and beta-actin was examined by immunoblotting. Expression of mRNAs encoding PTK6 and the well-characterized p53 target gene p21 was examined by qRT-PCR. RESULTS: Irradiation led to rapid accumulation of wild type p53 protein in HCT116 p53+/+ cells and activation of the p53 target gene encoding p21 by three hours post irradiation. Induction of PTK6 was observed by 24 hours, with highest levels at 48 and 72 hours post irradiation. While PTK6 was induced in both HCT116 p53+/+ and p53-/- cell lines, higher levels of PTK6 protein and mRNA were detected in p53+/+ HCT116 cells suggesting a p53-dependent component in PTK6 induction. However the PTK6 gene does not appear to be a direct target of p53. Highest levels of cleaved caspase-3 and apoptosis were detected in PTK6 positive p53-/- cells. Stable knockdown of PTK6 in HCT116 p53+/+ and p53-/- cells using two different shRNAs led to impaired apoptosis in HCT116 p53-/- cells. CONCLUSIONS: PTK6 expression is regulated by p53-dependent and independent mechanisms, and PTK6 is positively regulated by wild type p53. Expression of PTK6 promotes apoptosis of HCT116 p53-/- cells and enhances the response of p53-/- colon tumor cells to irradiation.

IDENTIFICATION OF FORKHEAD TRANSCRIPTION FACTORS NECESSARY FOR CELL CYCLE PROGRESSION IN U2OS CELLS BY REAL TIME MONITORING OF PERIODIC GENE EXPRESSION.

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Deregulation of the cell cycle underlies nearly all cancers. To identify novel regulators of the cell cycle, we developed a system to monitor cell cycle-regulated gene expression in real time. We used the LumiCycle system, initially developed for analysis of circadian gene expression, to monitor luciferase activity driven by either a minimal E2F1 promoter, which has peak expression in G1/S, or a basal promoter with six Forkhead DNA binding sites (6xDB), which has peak expression at G2/M. After cell synchronization, luciferase activity was measured every 10 minutes for up to four days, monitoring 3 – 4 synchronous cell cycles in a non-destructive manner. Luciferase activity driven by a constitutive SV40 promoter did not show periodic expression, whereas the 6xDB reporter showed peak expression at G2/M, which coincides with peak protein levels and phosphorylation of Forkhead box M1 (FOXM1). The 6xDB construct is responsive to FOXM1 induction, and FOXM1 binds to the reporter promoter as determined by chromatin IP. To identify other Forkhead box transcription factors that regulate the cell cycle, we used siRNAs to screen seven Forkhead transcription factors implicated in cell cycle control and assayed their effect on periodic gene expression. We identified two Forkhead genes FOXJ3, and FOXK1, that ablated cell cycle dependent oscillations when knocked down. Knockdown of these genes eliminated periodic expression from both the E2F1 promoter and the 6xDB construct, suggesting a general effect on the cell cycle. FACS analysis of cells treated with siRNAs against these two genes showed an increase in the number of cells in G1. Analysis of U2OS cells treated with FOXJ3 and FOXK1 siRNAs shows decreased cell growth. Therefore, multiple FOX transcription factors, in addition to FOXM1, are necessary for proper cell cycle progression in human U20S cells.

MOLECULAR CROSSTALK BETWEEN THE APC/C AND JNK

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The Anaphase-Promoting Complex or Cyclosome (APC/C) is a major ubiquitin ligase complex responsible for cell cycle transitions through timely and coordinated degradation of several key cell cycle regulators. APC/C activation is mainly ensured by binding with either Cdc20 or Cdh1 subunits. c-Jun-NH2-terminal Kinase (JNK) is a stress-activated protein kinase that plays a central role in the regulation of cellular stress responses by controlling cell death and survival pathways. We report here that nuclear-localized JNK is degraded by APC/C^{Cdh1} during exit from mitosis and G1 phase of the cell cycle. Expression of a non-degradable JNK in cells induces prometaphase-like arrest and aberrant mitotic spindle dynamics. Strikingly, we also found that JNK directly phosphorylates Cdh1 during G2 and early mitosis. JNK-mediated phosphorylation of Cdh1 changed its subcellular localization, reducing its ability to associate with APC/C core components and attenuating the APC/C^{Cdh1}-mediated substrate ubiquitination during G2-M. The newly identified regulatory mechanism between JNK and Cdh1 reveals the role and function of JNK during the cell cycle.

JNK-MEDIATED PHOSPHORYLATION OF CDC25C REGULATES CELL CYCLE ENTRY AND G2/M DNA DAMAGE CHECKPOINT

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c-Jun NH2-terminal Kinases (JNKs) play a central role in the cellular response to a wide variety of stress signals. Following their activation, JNKs induce phosphorylation of substrates which control proliferation, migration, survival, and differentiation. Recent studies suggest that JNKs may also play a role in cell cycle control, although the underlying mechanisms are largely unexplored. Here we show that JNK directly phosphorylates Cdc25C at Serine 168 during G2 phase of the cell cycle. Cdc25C's phosphorylation by JNK negatively regulates its phosphatase activity and thereby Cdk1 activation, enabling a timely control of mitosis onset. Unrestrained phosphorylation by JNK, as obtained by a cell cycle-stabilized form of JNK or as seen in some human tumors, results in aberrant cell cycle progression. Additionally, UV-irradiation-induced G2/M checkpoint requires inactivation of Cdc25C by JNK phosphorylation. JNK phosphorylation of Cdc25C, as well as Cdc25A, establishes a novel link between stress signaling and unperturbed cell cycle and checkpoint pathways.

PLK 1-MEDIATED PHOSPHORYLATION OF TANKYLASE 1 MAY REGULATE ITS STABILITY AND POLY(ADP-RIBOSYL)ATION ACTIVITY DURING MITOSIS.

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Telomeres are essential for genome stability in all eukaryotes. Changes in telomere functions and the associated chromosomal abnormalities have been implicated in human diseases, such as aging and cancer. Human telomeres are regulated by telomerase, a reverse transcriptase that adds telomeric TTAGGG repeats onto the 3' end of chromosomes, and telomeric repeat-binding proteins such as TRF1 and TRF2, two double-stranded DNA binding proteins.

Tankylase 1 was identified as a TRF1-binding protein, and is a member of the PARP family of enzyme. Tankyrase 1 PARsylates its binding partner TRF1, and in doing so inhibits TRF1 binding to telomeres, allowing access of telomere to telomerase. Thus, tankylase 1 plays as a positive regulator of telomere length by antagonizing TRF1.

Our recent approach to identify the interaction partners of polo-like kinase 1 (Plk1) facilitated by proteomics and subsequent matrix-assisted laser desorption ionization-time of flight analyses reveals that tankylase 1 is a novel Plk1-binding protein. We are currently pursuing the molecular and cell biological functions of this candidate in regulating the telomere length and its potential relevance to human disease.

THE DYNAMICS OF EXIT FROM MITOSIS IN BUDDING YEAST

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Cell cycle events in eukaryotes are regulated by periodic activation and inactivation of a family of cyclin–dependent kinases (Cdks). Entry into mitosis is initiated by accumulation of Cdk in complexes with B-type cyclins, and exit from mitosis requires inactivation of these Cdk-cyclin complexes and dephosphorylation of Cdk targets. In budding yeast, the Cdks are inactivated by Cdc20- and Cdh1-dependent proteolysis of Clb1 and Clb2 and by binding with inhibitors Sic1 and Cdc6. Cdc14 is an essential phosphatase promoting mitotic exit, it activates Cdh1 and it dephosphorylates Cdk targets. Cdc14 is kept inactive by forming a complex with Net1 protein in the nucleolus, and it is released from the complex when Net1 becomes phosphorylated upon mitotic exit. We have developed a deterministic ODE model for the control of Cdc14 release as budding yeast cells exit from mitosis. Our model provides a rigorous account of the factors affecting the dual exit pathways, called FEAR (Cdc14 early anaphase release) and MEN (mitotic exit network). The model captures the dynamics of mitotic exit in wild-type and in all 100+ mutant yeast cells studied up to date. We propose a novel mechanism for multiphosphorylation of Net1 by several kinases: Cdk, Cdc5 (Polo) and Dbf2/Mob1 (through activation by Cdc15). Understanding how Polo-like kinase fit into the exit pathway is important because Polo-like kinase is being actively pursued as a therapeutic target in the treatment of human cancer. The model also clarifies the mitotic exit functions of separase (Esp1): its non-catalytic function in inhibiting PP2A and thereby promoting activation of the FEAR pathway, and its catalytic function in degrading cohesins and thereby promoting sister chromatid separation at anaphase I, spindle elongation at anaphase II, and MEN activation in telophase.

STRUCTURAL BASIS OF DIMERIZATION-DEPENDENT UBIQUITINATION BY THE SCF^{FBX4} UBIQUITIN LIGASE

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SCF (Skp1-Cul1-Rbx1-F box protein) ubiquitin ligases are a diverse family of enzymes that are critical for controlling the stability of a broad spectrum of cellular proteins in eukaryotes. The F-box proteins are the substrate-recognition subunits of the SCF complexes and recruit substrates through their variable C-terminal protein-protein interaction domains. Recently, a large number of SCF ligases have been shown to dimerize through their F-box subunits to mediate ubiquitination activity, but the underlying mechanism and regulation remain incompletely understood. In this project we carry out a structural and functional investigation into the dimeric F-box protein Fbx4 and elucidate the molecular basis of Fbx4 F-box protein dimerization in promoting the ubiquitination activity of the SCF^{Fbx4} ligase. The SCF^{Fbx4} ligase was recently identified as the E3 responsible for the ubiquitination and subsequent degradation of the cell-cycle regulator cyclin D1 and telomeric DNA-binding protein Pin2 (also known as TRF1). Inactivation of Fbx4 is associated with cyclin D1 accumulation in cancer and overexpression of Fbx4 results in enhanced Pin2 degradation and progressive telomere elongation. Using Pin2 as a model substrate, we demonstrated that Fbx4 dimerization is required for efficiently stimulating polyubiquitination of Pin2 in vitro. The crystal structure of the Skp1-Fbx4 complex reveals an unusual antiparallel dimer configuration in which the linker domain of Fbx4 interacts with the C-terminal substrate-binding domain of the other protomer. Moreover, the C-terminal substrate-binding domain of the Fbx4 protein adopts a compact α/β fold that is distinct from those of the known F-box proteins, reinforcing the notion that Fbx4 represents a new subfamily of substrate-recognition proteins in SCF ligases. Biochemical studies indicate that two regions in Fbx4 are critical for its dimerization, substrate binding and activation. These findings provide the first structural view of a dimeric F-box protein and suggest a role for F-box dimerization in the SCF-mediated ubiquitination reaction.

PCNA: NOT JUST A BYSTANDER IN CRL4^{CDT2}-MEDIATED UBIQUITYLATION

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The E3 ligase Cul4-Ddb1-Cdt2 (CRL4^{Cdt2}) is emerging as an important cell cycle regulator that ubiquitylates numerous proteins in S phase and after DNA damage. The substrates of CRL4^{Cdt2} include Cdt1, p21, CKI-1, fly E2F, and worm pol Eta. Recently, we have identified the histone methyl transferase Set8 as a new CRL4^{Cdt2} substrate (see Abstract by Centore et al.). All known substrates of CRL4^{Cdt2} contain a 'PIP degron', which consists of a classical PCNA interacting protein motif (PIP box) and a basic amino acid located four residues downstream of the PIP box ("B+4"). When a CRL4^{Cdt2} substrate uses its PIP degron to dock onto PCNA that is engaged in DNA replication or repair, CRL4^{Cdt2} is recruited to the complex dependent on the B+4 residue, whereupon the substrate is ubiquitylated. These observations suggest that CRL4^{Cdt2} might be the first example of a ubiquitin ligase that recognizes a composite degron motif consisting of two polypeptides (the PIP degron and PCNA). To test this model, we addressed what effect PCNA mutations have on CRL4^{Cdt2}-mediated proteolysis. Mutation to alanine of two PCNA residues, E124 and D122, that cradle residue B+4 of the PIP degron completely abolished Cdt1 destruction in Xenopus egg extracts. Importantly, while these mutations had no effect on DNA replication or the ability of Cdt1 to dock onto PCNA, they abolished recruitment of CRL4^{Cdt2} to the PCNA-degron complex. Similar results were observed with Set8. Our results indicate that CRL4^{Cdt2} makes direct contacts not only with the PIP degron but also with PCNA, strongly supporting the model that CRL4^{Cdt2} recognizes a bipartite degron consisting of two polypeptides.

INVESTIGATION INTO SPINDLE ASSEMBLY CHECKPOINT FUNCTION IN *DROSOPHILA MELANOGASTER*

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The spindle assembly checkpoint (SAC) delays the metaphase to anaphase transition in response to lack of kinetochore-microtubule attachment and tension, until bipolar attachment of all chromosomes has been achieved. Kinetochore dynamic checkpoint proteins including Cdc20, Mad2 and BubR1 (along with Bub3), are the key components of a mitotic checkpoint complex (MCC), which inhibits activation of the Anaphase Promoting Complex/Cyclosome (APC/C) to prevent cell progression until the checkpoint is satisfied. Although it is hypothesised that the sequential formation of this mitotic checkpoint inhibitory complex is the mechanism by which the activity of Cdc20 (as the activator of the APC/C) is sequestered, the necessary conditions for the formation of the MCC remains subject to speculation. This includes aspects such as the order of formation and direct requirement of the kinetochore in protein-protein interactions. With the apparent recruitment of key checkpoint proteins upon checkpoint activation, clearly the kinetochore plays an important role. Recent studies suggest that unattached kinetochores not only catalyze the production of the MCC, but may also amplify Cdc20 inhibition and initiate the inhibition of Cdc20 which is already bound to APC/C. But with MCC found to be present outside of metaphase in HeLa cells, and complex formation in yeast previously shown to be independent of intact kinetochores, it could be argued that direct kinetochore interaction may not always be required as the template for the generation of this complex. Using Drosophila as model, we hope to expand upon current knowledge of the requirements involved in the interactions between key MCC proteins, by looking at how the SAC may be affected should the activity/behaviour of components of the MCC be disrupted.

PHOSPHOPROTEOMIC ANALYSIS OF THE EFFECT OF CYTOPLASMIC DEPLETION OF MIOTOTIC CYCLINS IN BUDDING YEAST

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Sub-cellular compartmentalization is an important factor in determining the spectrum of substrate proteins that are phosphorylated by the various cyclin-cyclin-dependent kinase pairs that regulate cell cycle transitions. The four budding yeast mitotic cyclins, Clb1-4, all concentrate in the nucleus but are present at lower concentrations in the cytoplasm. Clb2 also exhibits specific localization to the septin ring at the mother-bud neck. We have previously examined the cytoplasmic function of Clb2 by expressing a clb2 protein with inactivating mutations in two nuclear export signals (clb2-NESm) in a strain background lacking the other three mitotic cyclins (*clb1,3,4*Δ). The resulting strain exhibited a Swe1-dependent elongated bud morphology and a defect in cell wall integrity. To further investigate the basis for these mutant phenotypes, we have performed a phosphoproteomic study employing stable isotope labeling with amino acids in cell culture (SILAC) and phosphopeptide enrichment by immobilized metal affinity chromatography (IMAC) to compare the spectrum of phosphorylated proteins in *clb1,3,4*Δ and *clb1,3,4*Δ *clb2-NESm* cells. Phosphorylated peptides that showed significant differences in abundance between the two strains included those derived from cell wall components, proteins of the cell wall integrity signaling pathway, and proteins involved in vesicle trafficking and the ubiquitin-proteasome pathway.

THE ROLE OF KRÜPPEL-LIKE FACTOR 4 (KLF4) PROTEOLYSIS IN TGF-BETA SIGNALING

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Krüppel-like factor 4 (KLF4) is a transcriptional factor, whose physiological role has been implicated in the regulation of a variety of biological processes, including cell proliferation, differentiation, apoptosis and stem cell reprogramming. How KLF4 is regulated has attracted considerable interest because of its putative role in carcinogenesis and stem cell biology. Recent studies have revealed that regulation of KLF4 is through epigenetic machineries, such as methylation and acetylation, that modulate its function in transcriptional activation. Besides the aforementioned epigenetic mechanisms, ubiquitin-dependent proteolysis has also been suggested to be involved in the regulation of KLF4, especially during cell cycle progression and in response to genotoxic stress. However, the exact mechanism by which KLF4 is regulated by the ubiquitin-proteasome system remains largely unknown. In this study, we have analyzed the regulatory mechanism of KLF4 as related to protein degradation and further identified a novel role for KLF4 in mediating TGF-beta signaling. We have shown that KLF4 undergoes proteolysis in response to TGF-beta signaling. TGF-beta-induced KLF4 destruction results in enhanced TGF-beta-responsive transactivation. We have further demonstrated that KLF4 interacts with Cdh1, a substrate factor for APC/C. Silencing Cdh1/APC by the RNA interference leads to stabilization of KLF4 and attenuation of TGF-beta response. Results from the mutagenesis study coupled with the ubiquitylation analyses suggest that a destruction box near the amino terminus of KLF4 (amino acid residues 45-48) mediates the catalysis of KLF4 ubiquitylation by Cdh1/APC in response to TGF-beta signaling. Stabilization of KLF4 inhibits TGF-beta-mediated PAI-1 activity and further abrogates TGF-beta-induced growth inhibition. In summary, the present results suggest that KLF4 plays an important role in TGF-beta signaling pathway and Cdh1/APC is the putative ubiuqitin-protein ligase that governs TGF-beta-induced proteolysis of KLF4.

A NOVEL FUNCTION OF YEAST GSK-3 KINASE HOMOLOGUE, MCK1P, IN THE CONTROL OF DNA REPLICATION

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Orc6p is a part of the pre-replicative complex (pre-RC) which binds to origin of DNA replication to promote initiation of DNA replication. DNA replication has to take place only once per cell cycle. After the initiation of DNA replication, S-phase cyclin (Clb5p) binds to Orc6p to inhibit the second round of DNA re-replication. To better understand the Orc6p function in S. cerevisiae, synthetic genetic array (SGA analysis) was performed using Orc6 mutant, ORC6-rxl. The ORC6-rxl mutation eliminates Clb5-Orc6 binding, but these strains are viable. We looked for haploid gene deletion strains in which ORC6-rxl was synthetically lethal; in other words, haploid deletion strains which require Clb5p-Orc6p protein binding for their survival. Interestingly, we found that mck1 deletion cells showed synthetic lethality with the cells containing the ORC6-rxl mutation. The mck1 deletion cells also caused synthetic lethality with cells with ORC6 or ORC2 phosphorylation mutations (ORC6-ps or ORC2-ps), but not with other pre-RC mutations such as MCM7-NLS or CDC6delNT. The phenotype of the synthetic lethality in mck1 ORC6-rxl using a temperature sensitive conditional mutant of mck1 (mck1-16) in combination with ORC6-rxl mutant was analyzed. The mck1-16 ORC6-rxl cells showed DNA content more than 2C DNA at non-permissive temperature. We previously reported that DNA re-replication is monitored by DNA damage checkpoint surveillance mechanism. Deletion of these checkpoint genes enhances DNA re-replication phenotype. The lethality in the mck1-16 ORC6-rxl cells was enhanced when DNA damage checkpoint gene, such as MEC1, was deleted. Furthermore the lethality in the mck1-16 ORC6-rxl was dependent on CLB6. We will discuss the possibility that Mck1p has a role in the control of DNA replication.

THE MPS1 PROTEIN KINASE CONTROLS THE CYTOSOLIC AND KINETOCHORE-BASED BRANCHES OF THE SPINDLE CHECKPOINT IN HUMAN CELLS

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The spindle assembly checkpoint (SAC) in mammals comprises both a cytosolic "timer" and a kinetochore-based "catalyst" that together mediate the production of an anaphase-inhibitory signal. Here we use chemical genetics to show that the protein kinase Mps1 is a crucial regulator of both branches of the SAC. Human *MPS1*-null cells were generated via gene targeting and reconstituted with either the wildtype kinase ($Mps1^{wt}$) or a mutant version ($Mps1^{as}$) whose ATP-binding pocket was enlarged to accommodate bulky purine analogs. Mps1 inhibition caused two major defects in M phase progression: first, it dramatically accelerated mitotic timing, such that cells completed the NEB-to-anaphase interval in 12 minutes, and prevented Cdc20's association with either Mad2 or BubR1 during interphase, i.e., before the appearance of functional kinetochores. Second, Mps1 inhibition during mitosis evicted all known SAC transducers (including Bub1, BubR1, Mad1, Mad2, and Zw10) from the outer kinetochore and provoked severe chromosome mal-orientation. However, contrary to a recent report, phosphorylation of Aurora B-dependent substrates was unaffected. Remarkably, cytosolic restoration of Mps1 kinase activity circumvented these kinetochore defects and rescued M phase timing and SAC proficiency in the presence of spindle poisons. We conclude that Mps1 has two distinct but complementary roles in SAC regulation: (i) transient cytoplasmic activation of Cdc20 inhibitor(s), and (ii) recruitment of factors that promote sustained anaphase inhibition and chromosome bi-orientation to unattached kinetochores.

CYTOKINESIS IN THE PRESENCE OF LAGGING CHROMOSOMES INDUCES DNA DAMAGE

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Aneuploidy is a common characteristic of tumor cells and a substantial amount of evidence has accumulated that suggests chromosome instability contributes to tumorigenesis. However, in apparent contradiction to this, aneuploidy has mainly been correlated with decreased viability of organisms and (untransformed) cells in culture. Aneuploidy is thought to arise as a consequence of chromosome segregation errors. The decrease in viability is thought to be caused by overall stress responses due to gene dosage imbalances. Recently it has been shown that single chromosome missegregation events can induce a p53-p21 dependent cell cycle arrest, which is thought to be caused mainly by activation of the p38-MAPK pathway (Thompson et al., 2010). However, it is currently unknown whether this is the only pathway that can lead to p53 activation after chromosome missegregations.
In this study we show that within several hours after a chromosome missegregation event the ATM/ATR-dependent DNA damage pathway is activated. We observe phosphorylation of H2AX and localization of 53BP1 to DNA positioned in the cleavage furrow in both U2OS and untransformed RPE cells, suggesting that cleavage furrow ingression could induce DNA damage. Consistent with this, inhibition of cytokinesis in the presence of chromosome missegregations reduces the DNA damage in the daughter cells. From these results we hypothesize that during a chromosome mis-segregation event DNA can get trapped in the cleavage furrow of the dividing cell. This then damages the DNA, which activates the DNA damage response pathway, which in turn induces a cell cycle arrest. This DNA damage pathway activation could, in addition to p38 MAPK activation, play a role in the observed p53-p21 induction after chromosome segregation errors.

A ROLE OF THE TRANSCRIPTION FACTOR NETWORK OSCILLATOR IN CONTROLLING CELL-CYCLE PERIOD

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Cyclin/Cdk complexes have long been regarded as the principal component of oscillator that drives cell-cycle progression in eukaryotes. However, previous work by our lab found that in Saccharomyces cerevisiae, oscillations in budding and periodic gene expression persist in the absence of B-Cyclin/Cdks, which normally regulate S phase and mitosis. We proposed that a transcription factor network, constructed from periodically expressed transcription factors, maintains these oscillations (Orlando et al. 2008). If the network truly controls the cell-cycle period, we expect the oscillation period could be changed by manipulating the expression of transcription factors within the network. Therefore, we introduced two types of perturbations within the network: deletion of transcriptional repressors and constitutive expression of transcriptional activators. Indeed, we observe that some perturbations decrease the period of B-cyclin-independent oscillations, while others extend the period length. Together, our data suggest the transcription factor network oscillator plays a fundamental role in controlling cell cycle period.

Orlando, D. A., C. Y. Lin, et al. (2008). "Global control of cell-cycle transcription by coupled CDK and network oscillators." Nature 453(7197): 944-U78.

CDC2-NEDD1-PLK1 AXIS REGULATES BIPOLAR SPINDLE FORMATION VIA TWO DISTINCT MECHANISMS

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Establishment of spindle bipolarity is pivotal for proper chromosome segregation and mitotic progression. Mammalian polo-like kinase 1 (Plk1) has been thought to play a central role in this process. However, how Plk1 contributes to this event remains unknown. Here we demonstrated that Cdc2-dependent phosphorylation on a γ-TuRC recruitment protein, Nedd1/GCP-WD, at two distinct sites provides both temporal and spatial cues to timely bring about Plk1 functions to the centrosomes and spindles via phosphorylation-dependent interaction with the polo-box domain of Plk1. At centrosomes, Plk1 interacted with a C-terminal phosphorylated motif of Nedd1 to phosphorylate the latter, a step suggested to be important for centrosome-based microtubule nucleation. Immediately following this event, Plk1 also interacted with an N-terminal phosphorylated motif of Nedd1 along the spindles and phosphorylated an Augmin subunit, Hice1, to promote microtubule-based microtubule nucleation. Loss of Nedd1-mediated Hice1 phosphorylation by Plk1 resulted in impaired Augmin interaction with microtubules and diminished γ -tubulin recruitment to the spindles that ultimately led to defects in proper bipolar spindle formation and chromosome segregation. Taken together, the data provided here demonstrate the underlying mechanisms of how the two distinct Plk1-Nedd1 interactions at the centrosomes and spindles are deciphered into different biochemical and cellular outcomes to achieve normal bipolar spindle formation and mitotic progression.

DCDC5, A NOVEL MIDBODY LINKER BETWEEN DYNEIN, MICROTUBULES AND RAB-8/RAB-6 VESICLES

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DCDC5 is a member of the Doublecortin (DCX) domain protein superfamily, in addition to a tandem DCX domain it possesses a carbohydrate-binding domain (Ricin-type beta-trefoil). DCX domains were first identified in the DCX protein, a neuronal microtubule-associated protein (MAP). Here we show that DCDC5 is specifically expressed during mitosis. The expression initiates at prophase and during metaphase DCDC5 localizes to spindle microtubules and cortical sites. Later, DCDC5 expression confined to the midbody and cytoplasm in telophase and cytokinesis. Following reduction of DCDC5 by shRNA, the function of the midbody was compromised by several criteria. Rab8- /Rab6-GFP vesicles failed to transport to the midbody, the position of the midbody deviated from the median of the cell, and cytokinesis time increased. Consequently, the number of multinucleated cells and apoptotic cells increased. Our biochemical results indicated that DCDC5 binds to microtubules and interacts with several key proteins involved in cell cycle regulation. DCDC5 interacts with Polo kinase-1, dynein intermediate chain (DIC), NudC, LIS1 and with an activated form of Rab6. Collectively, these data suggest that DCDC5 is a Microtubule Associated Protein, which acts as a potential linker protein, participating in microtubule associated dynein-mediated transport of Rab8-/6-vesicles to the midbody.

MODIFICATION OF NEURAL LINEAGE SIZE ALONG THE ANTEROPOSTERIOR AXIS

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The nervous system contains vast numbers of neuronal sub-types, generated at the proper time, in the proper location, and in proper numbers. While tremendous progress has been made with respect to the underlying molecular genetic mechanisms controlling neuronal diversification per se, much less is known regarding how precise cell numbers of each neuronal sub-type are generated. Although poorly understood, this likely requires a complex interplay between positional, temporal and lineage-specific cues on the one hand, and the cell cycle machinery on the other.

To address this issue, we are studying a specific neural progenitor cell and its lineage, the neuroblast 5-6, located in the Drosophila embryonic central nervous system (CNS). Previous studies have identified a number of regulatory genes and pathways acting to specify this progenitor cell and to control lineage progression, as well as to specify unique cell fates within this lineage. Many of these regulatory genes appear to play dual roles, both specifying intermediate and terminal cell fates, as well as controlling cell numbers of each cell type and overall lineage size. The combination of high resolution and the many regulatory genes identified makes this lineage a powerful model for addressing the interplay between regulatory genes and the cell cycle.

The fly embryonic CNS contains 18 segments and one neuroblast 5-6 is generated in each hemi-segment. Neuroblasts stereotypically generate fixed lineage sizes, and in thoracic segments, the 5-6 neuroblast generates a lineage of 20 cells. However, lineage size is modified along the anterior-posterior axis, indicating that positional cues act to modify this lineage, presumably by acting on the cell cycle machinery. We find that Hox genes, as well as Hox co-factors (Pbx/Meis) play critical roles in controlling lineage size of the 5-6 lineage. Surprisingly, Hox/Pbx/Meis act in different ways to control lineage size along the AP axis, indicating that these positional cues act in a context-dependent manner.

PHOSPHO-REGULATION OF THE Γ-TUBULIN COMPLEX: CLUES FROM THE YEAST CENTROSOME PHOSPHOPROTEOME

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The yeast centrosome (known as the spindle pole body, SPB) serves as a microtubule-organizing center to nucleate microtubules in interphase and establish the mitotic spindle. Proper mitotic spindle function is important to maintain a diploid genome, as structural and numerical centrosomal defects are correlated with chromosome instability in many types of tumors. All of the SPB components have been identified; however, the regulation of SPB duplication and function is yet to be well described. A number of phosphorylation sites have been identified on SPB proteins, a few of which have been shown to contribute to assembly and function of the SPB. Here we provide an extensive inventory of phosphorylation events found on the 18 core SPB proteins based on mass spectrometric analysis of intact SPB complexes. We have excellent peptide coverage across the proteins of the SPB and we have identified a total of 296 phosphorylation sites, including 26 tyrosine phosphorylation sites and 49 potential Cdk sites. We have also identified phosphorylation on numerous SPB-associated proteins, including SPB-associated kinases and the mitotic exit complex. To correlate cell cycle timing with the function of phosphorylation, we also have mapped cell cycle specific sites found in SPBs from cells arrested in G1 (61 unique sites) or mitosis (111 unique sites). We have examined the conservation of phosphorylation sites in the orthologs of the 18 SPB proteins. The analysis of γ-tubulin reveals phosphorylation on the very conserved S360 residue. Mutation of S360 to alanine yields no phenotype; however, a phosphomimetic mutation of this site leads to a mitotic delay and a defect in SPB function. We are further characterizing this phenotype. Overall, our work greatly expands the understanding of centrosome structure by exploring the extent of phosphorylation within the protein complex; this information will allow analysis of the contribution of this prominent post-translational modification in centrosome structure and function.

HOW CELLS MEASURE SIZE: RELATIVE WEE1 AND CDC25 LEVELS ARE SURROGATES FOR THE NUCLEAR:CYTOPLASMIC RATIO

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All cells have a characteristic size, which is regulated by the coordination of growth and cell division. Despite a wealth of knowledge about cell growth and division, little is understood of the direct mechanisms that control cell size. However, it is well accepted that nuclear content and cytoplasmic content play a role in cell size determination and that the nuclear:cytoplasmic ratio is a parameter that determines cell size. In the fission yeast *Schizosaccharomyces pombe* there is evidence for size checkpoints at the G1/S and the G2/M boundaries.

We hypothesize that Wee1 and Cdc25 represent a self contained size-control module that is a surrogate for the nuclear:cytoplasmic ratio at the G2/M boundary. In our hypothesis, the Wee1 mitotic inhibitor is expressed at a rate proportional to nuclear content whereas the Cdc25 mitotic inducer is expressed at a rate proportional to cytoplasmic volume. The amount of Wee1 and Cdc25 represent the nuclear:cytoplasmic ratio and determines cell size at division. In other words, during most of G2 the amount of Wee1 is greater than the amount of Cdc25 and thus inhibits mitosis by inhibiting Cdk1. However, as the cell size increases the amount of Cdc25 increases and when the cell reaches a size threshold sufficient for mitosis to occur the amount of Cdc25 will surpass the amount of Wee1 and induce mitosis.

We have tested our hypothesis by accurately measuring expression of Wee1 and Cdc25 via luciferase fusions. Our initial results support a model where Wee1 expression stays relatively constant while Cdc25 expression increases as cell volume increases in G2. We are currently testing additional predictions of our hypothesis.

SSU72 IS A COHESIN-BINDING PROTEIN THAT REGULATES THE RESOLUTION OF SISTER CHROMATID ARM COHESION

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Cohesin is a multiprotein complex that establishes sister chromatid cohesion from S phase until mitosis or meiosis. In vertebrates, sister chromatid cohesion is dissolved in a stepwise manner: Most cohesins are removed from chromosome arms via a process that requires Plk1, aurora B and Wapl, while a minor amount of cohesin, found preferentially at the centromere, is cleaved by separase following its activation by the anaphase-promoting complex/cyclosome. In the current study, we report that our fission yeast two-hybrid assay identified hsSsu72 as a Rad21-binding protein. Additional experiments revealed that Ssu72 directly interacts with Rad21 and SA2 in vitro and in vivo, and associates with mitotic sister chromatids. Interestingly, depletion or mutational inactivation of Ssu72 phosphatase activity caused premature sister chromatid separation, whereas the overexpression of Ssu72 yielded high resistance to the resolution of sister chromatid cohesion. Further studies showed that Ssu72 regulates the cohesion of chromosome arms but not centromeres. Thus, our study provides important new evidence suggesting that Ssu72 is a novel cohesin-regulatory protein capable of regulating cohesion between sister chromatid arms.

P53 MODIFICATION MODULATION DETERMINED CELL FATE BY MICROTUBULE INHIBITOR DRUGS

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Tumour suppressor p53 plays important roles in cellular responses such as cell cycle arrest, senescence, DNA repair, apoptosis and autophagy by various stimuli. Previous reports showed DNA damage agent response correlated with p53 wild type existence, however, antimicrotubule drugs response did not correlated with p53 status even though its protein induced. The way of p53 responses distinguish different stimulus and has selectivity for cell death or survival still remains to be clarified. One possible layer of p53 regulations is stabilization and target gene selective activations through different modifications. To investigate antimicrotubule drugs induced various cellular response by p53 may involved different modification, we treated topoisomerase inhibitor etoposide, and two antimitotic drug, taxol/nocodazole in normal mammary epithelial cell (HMEC), human diploid fibroblast (HDF) and HCT116 cell, which all are p53 wild type cells. Etoposide appeared similar response of cell survival, but, taxol and nocodazole showed various cell survivals in three different cell types. Besides, mitotic check point proteins induction was similar, even activation time point was little different. Interestingly, p53 protein modifications showed different patterns; still p53 was up regulated in all three cells by both anti-microtubule drugs. Acetylations of p53 are more dramatic changes in death prone cell rather than phosphorylation. Inhibition of p53 deacetylation by Sirt1 siRNA enhanced apoptosis, and HDAC inhibitor increased p53 acetylation, followed by apoptosis target proteins activation and increased apoptosis compare to taxol alone. These results demonstrate that p53 acetylation is the possible antimicrotubule drug induced apoptosis regulatory points and its regulation might enhance antimicrotubule chemodrug responsibility.

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ACTIVATION OF THE S-PHASE CHECKPOINT INHIBITS DEGRADATION OF THE F-BOX PROTEIN DIA2

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A stable genome is critical to cellular viability and proliferation. The progression of DNA synthesis may be hindered by the presence of damaged DNA or genotoxic stresses, which can lead to replication fork stalling or fork collapse. Such events have the potential to induce genomic instability, one of the hallmarks of cancer cells. In response to replication stress, the S-phase checkpoint pathway is activated to delay cell cycle progression and inhibit late origin firing. In budding yeast, the chromatin-bound F-box protein Dia2 is required to maintain genomic stability and may help replication complexes overcome sites of damaged DNA and natural fragile regions. SCF (Skp1/Cul1/F-box protein) complexes are modular ubiquitin ligases. We find that Dia2 is itself targeted for ubiquitin-mediated proteolysis and that activation of the S-phase checkpoint pathway inhibits Dia2 protein degradation. S-phase checkpoint mutants fail to stabilize Dia2 in response to replication stress. Deletion of *DIA2* from these checkpoint mutants exacerbates their sensitivity to hydroxyurea, suggesting that stabilization of Dia2 contributes to the replication stress response. Unlike other F-box proteins, deletion of the F-box domain in Dia2 does not stabilize the protein. Instead, a twenty amino acid motif in the N-terminus that is also required for nuclear localization is necessary for degradation. When a strong NLS is added to *dia2* mutants lacking this motif, the Dia2 protein is both stable and nuclear. Finally, expression of stable, nuclear Dia2 leads to changes in cell cycle dynamics. Together, our results indicate that Dia2 protein turnover does not involve an autocatalytic mechanism and that Dia2 proteolysis is inhibited by activation of the replication stress response. We propose that the inhibition of Dia2 proteolysis may be a novel target of the S-phase checkpoint pathway.

CELL CYCLE RE-ENTRY OF TERMINALLY DIFFERENTIATED MUSCLE CELLS IN *C. ELEGANS*

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Permanent arrest of proliferation associated with terminal differentiation is thought to depend on CDK-inhibitory proteins, transcriptional repression by pRb family members and stable gene silencing. We study the differentiation-associated cell-cycle arrest of the *C. elegans* body wall muscle *in vivo*. Muscle cells exit the cell cycle and differentiate even in the apparent absence of *lin-35* Rb and *cki-1* Cip/Kip function. However, expression of G1 Cyclin/CDK combinations triggered gradual cell-cycle re-entry of body wall muscle during larval development. Specifically, we observed S-phase reporter expression, DNA replication, chromosome condensation and nuclear division in post-embryonic muscle cells. Quiescent cells were much more efficiently induced to re-enter the cell cycle than differentiated cells, which indicates multiple levels of cell-cycle inhibition in differentiated cells. Importantly, induction of cell-cycle entry did not interfere with the differentiated state. Muscle-specific gene expression profiling revealed that *cye-1* Cyclin E/*cdk-2* CDK2 expression activates a specific cell-cycle transcriptional program enriched in E2F target genes. Expression of *cyd-1* Cyclin D/*cdk-4* CDK4/6 induced an overlapping but larger number of genes, triggered DNA replication more efficiently than *cye-1/cdk-2*, and down-regulated transcription of genes with catabolic functions while activating several anabolic genes. Our results highlight differential functions of the Cyclin D/CDK4 versus Cyclin E/CDK2 CDK complexes, and show that a cell-cycle transcriptional program can be activated coincident with the differentiated state and without general reprogramming of chromatin.

LOADING OF SORORIN ONTO CHROMATIN REQUIRES ECO IN *XENOPUS LAEVIS* EXTRACTS

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DNA replication creates identical sister chromatids that are held together until cell division by a large multi-subunit protein complex called cohesin. Cohesin is loaded onto chromatin prior to DNA replication. Several other activities ensure that cohesion is accurately established and maintained. In vertebrates, these include two acetyltransferases, Eco1 and Eco2, and a recently identified protein, sororin. The mechanism by which cohesion is established, and the nature and topology of the cohesin-DNA interaction are not well understood. We have been investigating the relationships among cohesion factors, with particular emphasis on vertebrate-specific elaborations, using extracts from eggs of *Xenopus laevis*.

We have cloned and sequenced full length *Xenopus* orthologs of Eco1 and Eco2. Both proteins contain the functional motifs found in all other known members of this gene family; they also possess unrelated and poorly conserved N-terminal extensions, as have been seen in other vertebrates. Functional dependencies between these Eco family proteins and other cohesion factors were examined by depletion of the endogenous proteins from egg extracts. In particular, the relationships between Eco proteins, DNA replication and recruitment of various cohesion factors to the chromatin were analyzed. Our data show that Eco1 and Eco2 functionally interact with sororin to regulate cohesion, and that Eco2 levels are controlled by cell cycle progression. Experiments are currently underway to identify regions of the Eco proteins that are critical to this interaction.

AURORA B CONTROL OF THE SPINDLE ASSEMBLY CHECKPOINT PATHWAY IN MOUSE OOCYTES

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The Spindle Assembly Checkpoint (SAC) acts to prevent mis-segregation of homologous chromosomes during the first meiotic division of mammalian oocytes. In mitosis, Aurora B kinase plays an essential role in the SAC response by affecting microtubule stability across sister kinetochores. Here we show, using the Aurora B inhibitor ZM447439 a similar function for Aurora B in mouse female meiosis. Thus ZM447439 accelerated passage through meiosis I by about 1 hour, generated lagging chromosomes at anaphase, and caused a five fold increase in homolog non-disjunction rates (from ~3% to 15%). It was able to overcome a SAC arrest imposed by the addition of nocodazole and this effect was shown, by cyclin B1-GFP imaging, to be due to reactivation of the Anaphase-Promoting Complex. Thus we were able to block cyclin B1 degradation by addition of nocodazole to maturing oocytes, and the further addition of ZM447439 caused re-initiation of its degradation. We conclude that Aurora B serves similar meiotic SAC functions in mammalian oocytes to those observed in somatic cells during mitosis. This is despite differences in spindle architecture brought about the meiotic mono-orientation of sister kinetochores.

GENETIC ANALYSIS OF THE ESSENTIAL NUCLEAR PORE COMPLEX PROTEIN SONB^{NUP98} TO DETERMINE ITS ROLE IN THE DNA DAMAGE RESPONSE

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The nuclear pore complex proteins (Nups) have increasingly become implicated in various aspects of the response to DNA damage including the actual repair of damaged DNA. SONB^{Nup98} is a conserved component of nuclear pore complexes and is essential in *Aspergillus nidulans*. The *sonB1* allele was identified in a screen for suppressors of the temperature sensitive *nimA1* allele of the mitotic NIMA kinase. Importantly, subsequent genetic analyses using the *sonB1* allele indicated SONB^{Nup98} has a role in a novel response to DNA damage. *sonB1* mutant cells are hypersensitive to DNA damaging agents and are lethal when combined with a deletion of *scaA*^{Nbs1}, a breast cancer susceptibility gene in humans. We have extended the previous studies of SONB^{Nup98} and SCAA^{Nbs1} to further characterize the role of SONB^{Nup98} in the response to DNA damage using several approaches. We have performed a screen to isolate other genes that suppress the temperature sensitivity of the *nimA1* allele that also confer DNA damage sensitivity, similar to the *sonB1* allele. In addition to re-isolating *sonB1*, we have isolated several suppressors extragenic to *nimA* and *sonB*, and are in the process of identifying these genes. We are also taking a candidate approach to identify genes involved in the DNA damage response pathway with SONB^{Nup98}. Many proteins involved in the DNA damage response are also involved in telomere maintenance. Interestingly, *sonB1* displays genetic interactions with genes that have roles both in telomere maintenance and the DNA damage response but not with genes that play a role in only the DNA damage response. Deletion of RADC^{Rad52}, a protein essential for maintenance of telomere length in the Alternative Lengthening of Telomeres (ALT) pathway, is lethal when combined with the *sonB1* allele. Deletion of the putative *S. pombe* Rad9 homolog results in enhanced DNA damage sensitivity when combined with the *sonB1* allele but not when combined with the *scaA1* mutant allele. These results suggest that Rad9 is acting in the same pathway as SCAA^{Nbs1} and in parallel with SONB^{Nup98} to maintain genomic integrity, possibly by maintaining telomere length via an ALT pathway.

MAMMALIAN FBH1 IS IMPORTANT TO RESTORE NORMAL MITOTIC PROGRESSION FOLLOWING DECATENATION STRESS.

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Chromosome decatenation is essential for chromosome condensation and sister chromatid separation during mitosis. Accordingly, disruption of decatenation can lead to abnormal chromosome segregation and aneuploidy, which can lead to cancer development. Topoisomerase 2 (Top2) is a key enzyme in chromosome decatenation, such that Top2 catalytic inhibitors (e.g. ICRF-193) disrupt chromosome decatenation without inducing double-strand breaks, thereby causing a decatenation stress. Since these Top2 catalytic inhibitors are used during cancer therapy to limit cardiac toxicity of chemotherapeutic agents, it is important to characterize the range of mechanisms that protect genomic integrity from such decatenation stress. For example, activation of a decatenation checkpoint to delay mitosis onset appears to be important for proper chromosome segregation. However, this decatenation checkpoint appears defective in stem cells. Thus, stem cells likely rely on additional pathways to ensure normal mitotic progression following decatenation failure. In this study, we have identified a factor (F-box helicase 1, or Fbh1) important for the recovery from a decatenation stress in mouse embryonic stem cells (ES). We have found that Fbh1-deficient ES cells showed an increased sensitivity to ICRF-193 treatment. We also observed an accumulation micronuclei and multilobed nuclei in Fbh1-deficient cells after decatenation stress, which indicates increased genomic instability. Moreover, from time-lapse analysis of chromosomes in Fbh1-deficient mitotic cells, we find that Fbh1 is important to promote normal anaphase separation of chromosomes after decatenation stress. These findings provide further evidence for additional pathways that limit the genotoxic effects of decatenation stress in stem cells, which likely involve Fbh1.

AUTO-REGULATORY MECHANISM OF PLK1 RECRUITMENT TO AND DISSOCIATION FROM THE KINETOCHORES VIA THE FORMATION OF THE PLK1-PBIP1-CENP-Q COMPLEX

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Mammalian polo-like kinase 1 (Plk1) plays a pivotal role during M-phase progression. Plk1 localizes to specific subcellular locations through the targeting activity of the polo-box domain (PBD) that specifically interacts with S-pS/pT epitopes. However, the mechanism underlying this event remains largely elusive. Here we showed that Plk1 activity not only self-regulates the timing of its association with the PBIP1-Cenp-Q complex to stable localize to the kinetochores but also self-promotes its own delocalization from the kinetochores by hyperphosphorylating the complex. During interphase and early mitosis, Plk1 phosphorylated PBIP1 at T78 and interacted with the latter via its PBD, which then allowed the former to phosphorylate Cenp-Q in a manner that required the prior formation of the PBIP1-Cenp-Q complex. Either inhibition of Plk1 activity or disruption of the PBIP1-Cenp-Q complex was sufficient to abolish the Plk1-dependent Cenp-Q phosphorylation. Later in mitosis, Plk1 activity induced dissociation of the Plk1-PBIP1-Cenp-Q complex from the kinetochores by the p-T78-dependent hyperphosphorylation of the complex. Depletion of Plk1 activity increased the fraction of the PBIP1-Cenp-Q complex associating with chromatin and induced relocalization of the complex to the kinetochores. Taken together, our results presented here demonstrate that Plk1 autonomously regulates the timing of not only its localization to the centromeres/early mitotic kinetochores but also its dissociation from the late mitotic kinetochores in a fashion that does not require an activity from a third component.

DIFFERENTIAL CATABOLISM OF UBIQUITINATED PROTEINS IN PROLIFERATING AND QUIESCENT CELLS

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Quiescence (G0) is the reversible arrest of cell proliferation, during which a cell does not actively divide but nevertheless retains the ability to re-enter the cell cycle. Quiescence is a mechanism for cellular survival. For example, certain subsets of tumor cells exploit quiescence to avoid apoptosis normally induced by anticancer agents, and survive. We have found that primary quiescent fibroblasts are relatively resistant to proteasome inhibition-mediated cell death. This finding is of particular interest because proteasome inhibitors have been approved by the FDA for treatment of specific tumor types. Thus, understanding the mechanisms by which cells are resistant to proteasome inhibition is of basic and clinical importance. We hypothesized that the resistance of quiescent fibroblasts to proteasome inhibition could reflect the existence of other proteolytic pathways that permit quiescent cells to survive by compensating for the inhibition of proteasome activity. In primary fibroblasts we found that proteasome inhibition, both in proliferating and quiescent cells, leads to induction of autophagy, as measured by the conversion of the known autophagy marker LC3I to LC3-II. Down-regulation of the autophagy pathway gene using shRNA-targeting Beclin 1 decreases the resistance of contact-inhibited quiescent cells to proteasome inhibition. These findings suggest that autophagy may reflect a compensatory mechanism for the turnover of ubiquitinated proteins, and that there may be cross-talk between the two protein degradation pathways. Additionally, we monitored the levels of a reporter for ubiquitin proteasome system (UPS) activity in proliferating and quiescent fibroblasts in the presence and absence of proteasome activity. In proliferating cells, the reporter protein accumulated to high levels in all cells when proteasome activity was inhibited. By comparison, in quiescent cells, the reporter protein was degraded in some cells even when proteasome activity was inhibited. Furthermore, proteasome inhibition leads to an accumulation of the poly-ubiquitinated form of the reporter in proliferating cells as expected, whereas decreased levels of the poly-ubiquitinated form were observed in quiescent cells. This may indicate differential activity of the ubiquitination or deubiquitination pathways in proliferating and quiescent cells, or the induction of an alternative pathway that eliminates ubiquitinated proteins in the quiescent cells. We hypothesize that pathways used by quiescent cells to maintain viability may be co-opted by tumor cells to ensure survival during dormancy and to resist chemotherapy, resulting in tumor recurrence. Elucidation of these pathways may assist in developing effective therapeutic approaches.

HUMAN TIMELESS PROTEIN HAS ROLES IN TELOMERE MAINTEANCE

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Timeless is a component of the replication fork protection complex (FPC) that is involved in the activation of the replication checkpoint and stabilization of replication forks. Timeless moves with replication forks and cooperates with the ChlR1 DNA helicase to preserve genomic integrity in human cells. Interestingly, in yeast, Timeless homologs have been reported to play a critical role in replication of repetitive DNA including rDNA and trinucleotide repeats. This is likely due to frequent arrest of replication forks in repeat regions, necessitating the demand of FPC to stabilize stalled forks. In an analogous fashion, telomeres, which are composed of hexameric nucleotide repeats, are likely to stall replication forks. Here, we describe the effect of Timeless-depletion on telomere DNA replication to understand how replisome components facilitate replication of repeat regions. We show that telomeres are significantly shortened when Timeless is inhibited. Additionally, we show that Timeless-depletion results in collapse of replication forks at telomeres and formation of telomere dysfunction-induced foci (TIFs). When Timeless is downregulated, chromatin interaction of telomere-associated factors was altered while telomerase activity is still largely unaffected. Thus, Timeless seems to be involved in telomere lengthening in a manner independent of telomerase activity.

NUCLEAR MATRIX ASSOCIATION OF ORC AND UTP7P IS INTER-DEPENDENT, CELL CYCLE-REGULATED, AND REQUIRED FOR PRE-REPLICATIVE COMPLEX ASSEMBLY IN BUDDING YEAST

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Eukaryotic DNA replication is regulated by the stepwise assembly and disassembly of pre-replicative complexes (pre-RCs) at replication origins. Some pre-RC proteins may become attractive markers for cancer detection and targets for cancer therapy, as they are highly expressed in cancer cells but not in non-proliferating normal cells.

DNA synthesis occurs at replication factories or foci on the nuclear matrix. However, it is less certain whether or not DNA replication is also initiated at the nuclear matrix, although this is supported by some experimental evidence.

From a yeast genetic screen, we have identified Utp7p as a protein required for initiation and elongation of DNA replication. We found that *utp7-td* cells lose single-ARS containing plasmid at a high rate and multiple-ARS containing plasmid at a much lower rate. *utp7-td* cells are defective in S phase entry and progression when Utp7-td protein is degraded. Utp7p interacts with ORC and MCM in co-IP from yeast extracts and in yeast two-hybrid assays. *utp7-td* is synthetic lethal with replication mutants, and multicopy *UTP7* can partially suppress the lethality of *orc5-1* cells. Importantly, Utp7p is required for loading MCM proteins onto chromatin during the M-to-G1 transition, without affecting the levels of MCM proteins. Utp7p is also required for MCM chromatin association in both G1 and S phases.

We have also investigated the association of Utp7p and other replication-initiation proteins with chromatin and the nuclear matrix by performing cellular fractionation assays using a high salt buffer extraction method combined with nuclease digestion of chromatin from formaldehyde crosslinked cells. We found that histone H3 is present mostly in the chromatin fraction, and Mcm2p, Orc3p, Utp7p and ARS elements exist mainly in the nuclear matrix fraction in G1 cells (pre-RC state) but mainly in the chromatin fraction in G2/M cells (post-RC state). Importantly, the nuclear matrix attachment of ORC and Utp7p is inter-dependent and is required for pre-RC assembly during the M-to-G1 transition, shedding new light on the mechanism of pre-RC assembly on the nuclear matrix.

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CONTROL OF THE DREAM COMPLEX AND QUESCENCE BY A NOVEL TUMOR SUPPRESSOR PROTEIN KINASE, DYRK1A.

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The retinoblastoma (RB) family of proteins including pRB, p107 and p130, is essential for the ability of mammalian cells to enter G0/G1 or quiescent state. In fly and worm, RB-like proteins form a multisubunit complex with E2F, DP and five proteins related to the MuvB genes in C. elegans. In human cells, both p130 and p107 but not pRB are recruited into the related DREAM complex (DP, RB-like, E2F and MuvB-like proteins including LIN52, LIN54, LIN9, LIN37 and RBBP4). The DREAM binds to the promoters of more than 800 cell cycle-regulated genes and contributes to their repression in G0/G1. Upon entry into G0, p130 binds to the MuvB proteins and recruits E2F4 and DP1 to form the DREAM complex. During the G1/S phase transition, p130 dissociates from the MuvB proteins.
To understand the mechanisms governing the DREAM assembly and entry into quiescence, we performed a global proteomic analysis of the post-translational modifications of all components of the DREAM complex. This analysis revealed that serine 28 of LIN52 was always phosphorylated when p130 was bound to the MuvB proteins. The S28A-LIN52 point substitution mutant was not able to bind p130 and interfered with the DREAM assembly.
Proteomic analysis also revealed that LIN52 bound to DYRK1A, the kinase that could directly phosphorylate S28 of LIN52. DYRK1A is located within the minimum region of chromosome 21 that has been implicated in both the mental defects and the decreased cancer incidence associated with Down syndrome. Array CGH analysis of human cancers revealed that DYRK1A gene copy number is reduced in a variety of human tumors. We confirmed the reduced DYRK1A copy number by in situ hybridization in U2OS osteosarcoma cells. Re-introduction of DYRK1A into U2OS cells resulted in an increase in S28-LIN52 phosphorylation, DREAM assembly and suppression of proliferation, supporting the tumor suppressor function of DYRK1A. Moreover, we found that DYRK1A is required for human cells to enter G0/G1 arrested state and for the DREAM assembly. In addition, stable expression of the LIN52 S28A mutant enabled primary human fibroblasts to escape oncogenic RAS induced senescence and to continue proliferation. Our findings identify an essential role for DYRK1A in the entry into quiescence that has implications relevant to cancer, stem cell biology and development.
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PHOSPHORYLATION OF CLIP-170 BY BOTH PLK1 AND CK2 IS ESSENTIAL FOR THE FORMATION OF KINETOCHORE-MICROTUBULE ATTACHMENTS

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CLIP-170 is implicated in the formation of kinetochore-microtubule attachments through direct interaction with the dynein/dynactin complex. However, whether this important function of CLIP-170 is regulated by phosphorylation is unknown. Herein, we have identified Plk1 (Polo-like kinase 1) and CK2 (Casein kinase 2) as two kinases of CLIP-170 and mapped S195 and S1318 as their respective phosphorylation sites. We showed that a CK2 unphosphorylatable mutant lost its ability to bind to dynactin and to localize to kinetochores during prometaphase, indicating that the CK2 phosphorylation of CLIP-170 is essential for its dynactin-mediated kinetochore localization. Furthermore, we provide evidence that Plk1 phosphorylation of CLIP-170 at S195 positively regulates its association with CK2. Finally, we showed that wild type CLIP-170, but not the unphosphorylatable mutants, S175A and S1318A, was able to reverse CLIP-170 depletion-induced mitotic defects, confirming that Plk1- and CK2-associated phosphorylations of CLIP-170 are essential for the formation of kinetochore-microtubule attachments in mitosis.

PLK1-MEDIATED PHOSPHORYLATION OF GTSE-1 IS ESSENTIAL FOR P53-DOWNREGULATION DURING G2 CHECKPOINT RECOVERY

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In order to enter mitosis after G2 DNA damage-induced arrest, mammalian cells have to go through a process called checkpoint recovery, in which the G2 checkpoint network will be silenced. Previous report suggested that p53 is required to sustain the G2 arrest, but how p53 is eliminated during the recovery process is poorly understood. As an important cell-cycle regulator, Polo-like kinase-1 (Plk1) is essential for the G2 checkpoint recovery. During the recovery process, Plk1 were reported to play dual roles: it silences the DNA damage checkpoint through phosphorylation-induced degradation of Claspin, the adaptor protein between ATR and Chk1; it also directly contributes to abolishing the inhibition of cyclin B-Cdk1 via regulation of the phosphatase Cdc25B and Wee1 kinase. We found that Plk1-mediated phosphorylation of GTSE-1, a negative regulator of p53, is one mechanism to facilitate p53-degradation during the G2 checkpoint recovery. We showed that GTSE-1 is required for the G2 checkpoint recovery. Furthermore, phosphorylation of GTSE-1 at Serine 435 by Plk1 promotes its nuclear localization, and then it shuttles p53 out of the nucleus to induce p53 degradation. Taken together, our results demonstrated that Plk1 plays an essential role in p53 elimination during G2 checkpoint recovery.

STUDY OF CYCLIN A2 INTERACTIONS DURING THE CELL CYCLE IN LIVING CELLS

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Cyclin-dependent kinases (Cdks), which govern progression through the cell cycle, are mainly controlled by transient interactions with cyclin regulatory subunits and by reversible phosphorylation reactions. Among cyclins, cyclin A2 is particularly interesting since it can activate two different Cdks and hence participates in the regulation of S phase (associated to Cdk2) and mitosis (associated to Cdk1). In order to better understand the biological significance of cyclin A2, we work on getting a dynamic map of cyclin A2 interactions with its partners, at the subcellular level and throughout the cell cycle. To this aim, we use *in vivo* imaging and interaction techniques, namely FRET (Förster/Fluorescence Resonance Energy Transfer) measured by FLIM (Fluorescence Lifetime Imaging Microscopy). These techniques are non-invasive, quantitative, and produce high resolution data in time and space. The proteins of interest are expressed as fusions with fluorophores (EGFP or mCherry), in breast epithelial cells. Vectors allowing transient expression of the proteins of interest as fusions with fluorophores have been produced and tested (human cyclin A2-EGFP, Cdk1-mCherry, Cdk2-mCherry). We have checked that the fluorescent proteins are functional (subcellular localisation, colocalisation with known partners by confocal microscopy, binding to interactors by immunoprecipitation, kinase assays, ubiquitination assays) and that their expression does not perturb cell cycle progression. Using FRET/FLIM, we have detected interactions of cyclin A2-EGFP with Cdk2-mCherry in S phase and with ubiquitin-mCherry in mitosis, in MCF-7 cells. We were able to follow these interactions through time in the same cells. We thus got original data about the subcellular localisation of these interactions and their spreading through the cells during the cell cycle.

FUNCTIONAL ANALYSIS OF THE RETINOBLASTOMA PROTEIN N-TERMINAL DOMAIN

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The retinoblastoma tumour suppressor protein (pRb) is a key regulator of cell cycle progression and is functionally inactivated in most tumours. Moreover reactivation of pRb in cancer cell lines induces senescence which is associated with cell cycle exit and ‘flat cell’ morphology.
The well characterised central 'pocket' and carboxy C-terminal domains of pRb mediate regulation of E2F dependent transcription and block the G1/S cell cycle transition. Less is known about the function of the amino N-terminal region, although it is conserved in pRb paralogues and orthologues. Importantly, a substantial number of missense mutations in this region are associated with hereditary retinoblastoma indicating that loss of function in this region is oncogenic. Recently the structure of this domain was solved revealing four potential surfaces that could be involved in protein-protein interactions.
We aim to address the significance of the N-terminus through using functional analysis of structure-guided and cancer associated mutants. A panel of expression vectors with frame preserving pRbN mutations were prepared and tested in cell based assays for pRb function. We have identified three different types of defects within specific pRb variants: 1) mutants showing decreased stability, 2) mutants associated with reduced flat cell formation and 3) mutants associated with change in flat cell morphology. The first group of mutations affects the hydrophobic protein core and decreases proper folding of the whole pRb. The second group localises around two surfaces called: the “projection” and the cyclin wedge, whereas the third group touches the cyclin wedge and the hydrophobic conserved patch 2.
This data demonstrates for the first time a loss of function in cancer derived mutants and confirms the existence of discrete functional surfaces within this pRbN domain.

CDC5-DEPENDENT ASYMMETRIC LOCALIZATION OF THE MEN REGULATOR BFA1 FINE-TUNES TIMELY MITOTIC EXIT

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In budding yeast, the major regulator of the mitotic exit network (MEN) is Tem1, a GTPase, which is inhibited by the GTPase-activating complex (GAP), Bfa1/Bub2. Asymmetric Bfa1 localization to the bud-directed spindle pole body (SPB) during anaphase controls mitotic exit, but the molecular mechanism and function of this localization are not well understood, particularly in unperturbed cells. Here, we identified novel Cdc5 target residues within the Bfa1 C-terminus, 452S, 453S, 454S, and 559S. A Bfa1 mutant in which all of these residues have been changed simultaneously (called Bfa14A) persisted on both SPBs at anaphase and was hypo-phosphorylated, despite retaining its GAP activity against Tem1. These observations demonstrate a tight link between localization and phosphorylation, and no direct connection between asymmetric localization and GAP activity of Bfa1. Consistent with this, in kinase-defective cdc5-2 cells, Bfa1 was unphosphorylated and localized to both SPBs. The BFA14A cells progressed through anaphase normally, but displayed a delayed mitotic exit in unperturbed cell cycles. Altogether, we suggest that Cdc5 modulates the asymmetric Bfa1 distribution to the bud-directed SPB independently of Bfa1 GAP activity at anaphase, and that Bfa1 asymmetry fine-tunes the timing of MEN activation.

THE EARLY EMBRYONIC CDK1-APC OSCILLATOR IS PULSE-DRIVEN: CYCLIN SYNTHESIS IS INHIBITED PRIOR TO MITOSIS

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Protein synthesis and proteolysis are critical to cell growth and division. Recent studies have revealed important connections between translation and cell cycle control systems. Cyclin—a protein whose synthesis is essential for mitotic entry—has long been considered and modeled to be synthesized continuously to drive the CDK1-APC oscillator. However, past studies of the stimulus-response relationship between cyclin and CDK1 showed that cyclin accumulation plateaus prior to mitosis. It was unclear if this result occurred due to a balance between cyclin synthesis and initial low APC activity, or if cyclin synthesis is blocked. We recently learned that cyclin levels plateau in proteasome-inhibited Xenopus cycling egg extract. This supports the idea that inhibition of cyclin synthesis alone is responsible for the lack of cyclin accumulation prior to mitosis. This pause correlates with increasing CDK1 activity, and we hypothesized there exists some kind of negative feedback that blocks cyclin synthesis. We then discovered that supplementing a proteasome-inhibited cycling egg extract with non-degradable cyclin accelerated mitotic entry and blocked cyclin synthesis prematurely; this reduced the production of endogenous cyclin. Next, we determined that inhibiting CDK1 with roscovitine or constitutively active Wee1 delayed M-phase entry while permitting a prolonged period of cyclin synthesis. As a result, both treatments caused the extract to produce more cyclin. Finally, we found that adding cycloheximide to extracts at the point of this translational inhibition did not delay mitosis. Blocking translation at even earlier time points—in order to induce a premature and reduced plateau level of accumulated cyclin—delayed but permitted M-phase onset. Together, these results demonstrated that early embryonic mitoses do not require continuous cyclin synthesis. In fact, the oscillator itself confers an implicit negative feedback on cyclin production once a sufficient amount of stimulus has been produced. Modeling of this CDK1-APC system—driven by pulses rather than constant cyclin synthesis—yielded dynamics even more representative of experimentally measured CDK1 oscillations. We are currently investigating how the embryonic oscillator senses when sufficient stimulus has been produced, and are working to identify the factors in addition to CDK1 that are involved in this negative-feedback loop.

A NOVEL WD-REPEAT PROTEIN IS ESSENTIAL FOR LOADING ORC TO CHROMATIN

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Origin Recognition Complex (ORC) plays critical roles in the initiation of DNA replication and cell cycle progression. In metazoans, ORC associates with origin DNA during G1 and with heterochromatin in post-replicated cells. However, what regulates the binding of ORC to chromatin is not understood. We have identified a novel, highly conserved, leucine-rich repeats and WD40 repeat domain-containing protein 1 (LRWD1) in human cells that interacts with multiple subunits of ORC. LRWD1 co-localizes with ORC and shows similar cell cycle dynamics. By using a cell line containing an artificially generated chromatin region, we have demonstrated that LRWD1 efficiently recruits ORC to chromatin. Furthermore, depletion of LRWD1 in human embryonic stem cells results in loss of ORC binding to chromatin and a subsequent arrest in G1 phase. Our findings demonstrate that LRWD1 is an essential protein that is required for loading ORC to chromatin and that in turn is critical to initiate preRC assembly in G1 and chromatin organization in post-G1 cells.

STEPWISE ASSEMBLY OF A DOUBLE-HEXAMERIC MCM2-7 COMPLEX DURING LICENSING OF EUKARYOTIC DNA REPLICATION

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Initiation of DNA replication depends on loading of a MCM2-7 helicase complex at a replication origin prior to kinase dependent activation of the helicase and formation of the replication fork. We have reconstituted the first step of budding yeast replication initiation – formation of the pre-Replication Complex (pre-RC) using purified proteins. During pre-RC formation ORC, Cdc6 and Cdt1 cooperate to load the MCM2-7 helicase onto DNA. The reconstituted reaction depends on all pre-RC proteins, DNA and Cdc6 ATP-hydrolysis for MCM loading. During pre-RC formation the MCM2-7 structure changes and loaded MCM2-7 assemble into a double-hexamer. MCM2-7 loading depends on Cdc6 and Orc1 ATP hydrolysis, but it is unclear how ATP-hydrolysis and MCM double-hexamer formation are coordinated. Using a biochemical approach we found that a Cdc6 ATP-hydrolysis mutant promotes an ORC-MCM2-7-single hexamer complex and that an Orc1 ATP-hydrolysis mutant allows assembly of a MCM2-7 double-hexamer. We have analysed the MCM2-7 double hexamer interface using a mutagenesis approach and found that MCM-interface mutations block MCM2-7 loading onto DNA in vitro. When MCM interface mutants were over-expressed in vivo they resulted in a dominant lethal phenotype. These data suggest that ORC-Cdc6 initially associate with an MCM2-7 single hexamer, but after Cdc6 ATP hydrolysis the double-hexamer must be loaded onto DNA as a unit.

CELLULAR STRESS INDUCES RAPID UNLOADING OF THE MCM REPLICATIVE HELICASE THROUGH P38 AND JNK MAP KINASES.

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The complete and precise duplication of a eukaryotic genome presents a unique challenge during the cell cycle. During G1 phase thousands of replication origins are loaded with an inactive form of the replicative DNA helicase, the MCM complex. This replication licensing step is accomplished through the recruitment of MCM by the Cdt1 protein to origins where the Origin Recognition Complex (ORC) and Cdc6 protein then load MCM complexes onto DNA. ORC, Cdc6, and Cdt1 are then either destroyed or inhibited from the onset of S phase until mitosis to prevent relicensing and rereplication, whereas MCM complexes are unloaded as replication completes.

Many perturbations to intracellular or extracellular environments have profound effects on cell cycle progression. Since MCM loading is coordinated with the cell cycle, we propose that any acute environmental change capable of perturbing cell cycle progression has the potential to directly influence MCM loading. One example of such a perturbation is DNA damage which triggers the degradation of both Cdt1 and Cdc6. We have investigated MCM loading in response to cellular stress which blocks the cell cycle but does not involve direct DNA damage. Unlike DNA damage, cellular stress does not cause the degradation of Cdt1, Cdc6, or any other licencing proteins. We find however, that treatment of cells in G1 with either the inflammatory cytokine, TNFα, or an osmotic shock not only blocks *de novo* MCM loading, but also induces the rapid unloading of previously-loaded MCM complexes. This apparent licensing reversal is blocked by inhibitors of the MAP kinases central to the cellular stress signaling pathway, p38 and JNK. Activation of MAP kinases has no effect on the chromatin association of Orc2, Cdc6, or Cdt1.

MCM reloading during S phase and G2 is normally blocked by the combined activities of CDKs and the Cdt1 inhibitor, geminin. During a G2/M arrest, direct inhibition of CDK activity results in both geminin destruction and MCM reloading. Activation of MAP kinases by acute cellular stress does not prevent this geminin destruction, but stress kinase signaling blocks inappropriate MCM reloading even when geminin levels are reduced. These findings may have additional implications for the regulation of MCM loading in normal cell cycles.

QUANTITATIVE ANALYSIS OF GENOME-WIDE REPLICATION KINETICS IN BUDDING YEAST PREDICTS THAT REPLICATION TIMING IS REGULATED BY MCM LOADING

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Eukaryotic genomes replicate in define patterns with some parts of the genome replicating early in S phase and other parts replicating later. Replication timing correlates with transcription, chromatin modification, sub-nuclear localization and genome evolution, suggesting an intimate association between replication timing and other important aspects of chromosome metabolism. However, the mechanism of replication timing is currently unknown.

Genome-wide replication kinetics have been mapped in several organisms. The rich datasets that result contain more information than has been extracted by current methods of analysis. We have developed an analytical model that exploits previously unutilized information on the temporal aspects of replication. The model incorporates probabilistic initiation of origins, variable fork-progression rates, and passive replication. We used the model to fit a set of recently published time-course microarray data from *Saccharomyces cerevisiae*. We extracted the distribution of firing times for each origin and found that the later an origin fires on average, the greater the variation in firing times. This trend leads naturally to a model where earlier-firing origins have more initiator complexes loaded and a more-accessible chromatin environment. The model demonstrates how initiation can be stochastic and yet occur at defined times during S phase, without an explicit timing program. Furthermore, we propose that the initiators in this model correspond to loaded MCM complexes and that the timing of origin firing is regulated in by the number of MCM complexes loaded at an origin. Thus, for the first time, our model suggests a detailed, testable, biochemically plausible mechanism for the regulation of replication timing in eukaryotes. Preliminary experimental validation of our model confirms that early firing origins have more MCM loaded. We are currently extending this validation by ChIP-seq genome-wide quantitation of MCM occupancy.

CHECKPOINT DEPENDENT INHIBITION OF DNA REPLICATION INITIATION VIA SLD3 AND DBF4 PHOSPHORYLATION

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The initiation of eukaryotic DNA replication is regulated by three protein kinase classes: cyclin dependent kinases (CDK), Dbf4-dependent kinase (DDK) and the DNA damage checkpoint kinases. CDK and DDK trigger initiation by activating the pre-loaded Mcm2-7 replicative helicase and promoting replisome assembly. CDK phosphorylation of two key initiation factors, Sld2 and Sld3, promotes essential interactions with Dpb11 whilst DDK acts by directly phosphorylating Mcm2-7 subunits. CDK plays a second essential role in replication by preventing the re-loading of Mcm2-7 during S, G2 and M phases, thus preventing origin re-firing and re-replication. During G1-phase, both CDK and DDK are down-regulated, which is required to prevent premature replication initiation. Origin firing is also inhibited during S phase when DNA damage or replication fork stalling activates the checkpoint kinases. Here we show that, similar to the situation in G1 phase, the checkpoint kinase Rad53 inhibits origin firing in budding yeast by interfering with both CDK- and DDK-dependent pathways. Rad53 acts on DDK directly by phosphorylating Dbf4, whereas the CDK pathway is blocked by Rad53 phosphorylation of the downstream substrate, Sld3. Rad53 phosphorylation of Sld3 blocks its interactions with Dpb11 and Cdc45, an essential replisome component. This allows CDKs to remain active during S phase in the presence of DNA damage, which is crucial to prevent reloading of Mcm2-7 onto origins that have already fired. We show that global origin firing in the presence of active Rad53 only occurs when the phosphorylation of both Sld3 and Dbf4 is prevented. Our results explain how checkpoints regulate origin firing and show that the slowing of S phase by the ‘intra-S checkpoint’ is primarily due to the inhibition of origin firing.

GEMC1 IS A TOPBP1 BINDING PROTEIN REQUIRED FOR CHROMOSOMAL DNA REPLICATION

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Many factors required for chromosomal DNA replication have been identified in unicellular eukaryotes. However, DNA replication in complex multicellular organisms is poorly understood. Here, we report the identification of GEMC1, a novel vertebrate protein required for chromosomal DNA replication. GEMC1 is highly conserved in vertebrates and is preferentially expressed in proliferating cells. Using Xenopus egg extract we show that Xenopus GEMC1 (xGEMC1) binds to checkpoint and replication factor TopBP1, which promotes xGEMC1 binding to chromatin during pre-replication complex (pre-RC) formation. We demonstrate that xGEMC1 directly interacts with replication factors such as Cdc45 and Cdk2-CyclinE by which it is heavily phosphorylated. Phosphorylated xGEMC1 stimulates initiation of DNA replication whereas depletion of xGEMC1 prevents DNA replication onset due to impairment of Cdc45 loading onto chromatin. Likewise, inhibition of GEMC1 expression by morpholino and siRNA oligos prevents DNA replication in embryonic and somatic vertebrate cells. These data suggest that GEMC1 promotes initiation of chromosomal DNA replication in higher eukaryotes by mediating TopBP1 and Cdk2 dependent recruitment of Cdc45 onto replication origins.

THE REGULATION OF S PHASE BY CYCLIN-DEPENDENT KINASES (CDKS)

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The G1/S phase transition is controlled by the Retinoblastoma protein (Rb) and cyclin-dependent kinases (Cdks). Cdks are known to phosphorylate Rb on several residues, which controls the interaction of Rb with E2F transcription factors. During the G1 phase, phosphorylation of Rb increases to the point where it dissociates from E2F and therefore E2F mediated transcription is activated, resulting in S phase entry. None of the published mouse knockout models displayed a sever defect in DNA replication. This included Cdk2, Cdk4, Cdk6, cyclin D's, cyclin E's, and cyclin A's, all of which ought to have an important role in S phase. Only cyclin $E1^{-/-}E2^{-/-}$ double knockout MEFs could not enter S phase after serum starvation&release but these DKO MEFs were fine under continuous growth conditions. The most pronounced S phase entry delay was observed in $Cdk2^{-/-}Cdk4^{-/-}$ double mutants where Rb remains hypophosphorylated and as a consequence Cdk1 transcription is shut off. Previously, we had demonstrated that Cdk1 by interacting with cyclin E compensates for the loss of Cdk2 in S phase. In summary, these results suggested a possible role of Cdk1 in DNA replication. We have now generated conditional knockout mice for Cdk1 and are studying S phase in the absence of Cdk1. We will report our results of these investigations.

SWITCHING CDK2 ON AND OFF WITH SMALL MOLECULES TO REVEAL ESSENTIAL FUNCTIONS IN HUMAN CELL DIVISION

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In human cells, CDK/cyclin complexes form in precise temporal order with a high degree of specificity. Cdk2 binds cyclin E nearly exclusively and out-competes Cdk1 in binding cyclin A—thus delaying activation of Cdk1 until mid-S phase—despite being ~10-fold less abundant than Cdk1. Inhibition of CDK-activating kinase (CAK) in vivo revealed kinetic differences in activation of Cdk1 and Cdk2 that can account for Cdk2's priority. A computational model suggests that higher affinity of cyclin A for Cdk2, and phosphorylation of the Cdk2 T-loop in the absence of cyclin by CAK, collaborate to ensure that cyclin A binds Cdk2 before Cdk1. This implied essential requirements for Cdk2 activity during G1 and early S phase. To test this idea, we replaced Cdk2 with a version sensitized to bulky purine analogs, $Cdk2^{as}$, in human cells. Compared to wild-type Cdk2, $Cdk2^{as}$ had diminished binding to cyclin A (but not E) and T-loop phosphorylation in vivo; there were corresponding increases in abundance of Cdk1/cyclin A complexes in $Cdk2^{as/as}$ cells. ATP analogs rescued both cyclin-binding and phosphorylation defects in vivo, with efficacy that correlated with their inhibitory potency towards $Cdk2^{as}$ in vitro. We recapitulated the inverted cyclin A-binding preference (Cdk1>Cdk2), and its correction, in extracts of $Cdk2^{as/as}$ cells; different analogs restored binding of added cyclin A to endogenous $Cdk2^{as}$—at the expense of Cdk1—with or without stimulating T-loop phosphorylation, consistent with the reinforcing mechanisms predicted by the model. A weak inhibitor, 6-benzylaminopurine (IC_{50} ~1 μM), promoted $Cdk2^{as}$ activation in vivo and did not slow division of $Cdk2^{as/as}$ cells. An analog that restored $Cdk2^{as}$-cyclin A pairing and potently inhibited the kinase, 3-MB-PP1 (IC_{50} ~5 nM), decreased phosphorylation of Cdk2 substrates in vivo, impeded G1/S progression and blocked long-term cell proliferation. Therefore, when normal levels and pairing specificities of CDKs and cyclins are maintained, Cdk2 activity is essential for the mitotic cell cycle.

REAL-TIME OBSERVATION OF REPLICATION INITIATION AND ELONGATION ON SINGLE DNA MOLECULES

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Eukaryotic genomes are duplicated from multiple origins during each cell cycle. To study the timing and location of origin firing events, two powerful approaches, chromatin-immunoprecipitation (ChIP) and DNA combing, have been widely used. However, these methods have drawbacks. ChIP measures the average behavior of replication events in many cells, such that rare events are lost and imperfect synchronicity obscures transient behaviors. Molecular combing eliminates averaging by studying replication profiles of single molecules, but provides only static data obtained at different time points, from which a dynamic picture must be inferred. To obtain a complete kinetic picture, one needs a dynamic means of observing origin firing and fork progression.

We have developed a novel, fluorescence-based technique that allows us to directly visualize replication initiation and elongation on single DNA molecules in real time. Briefly, a well-stretched lambda phage DNA (48.5kb) is tethered at both ends to a coverslip within a microfluidic flow cell. Addition of *Xenopus laevis* egg extract promotes multiple initiations on each immobilized DNA molecule, leading to complete duplication. Importantly, the observed replication is dependent on Cdt1 and Cdk2, indicating it involves a physiological mechanism. By supplementing egg extracts with fluorescently-labeled Fen1, which binds PCNA during Okazaki fragment processing, we are able to mark sites of ongoing DNA replication. Thus, we can follow in real time the firing of individual origins and movement of individual forks. We validate this approach by comparing our kinetic data to static snapshots of nascent DNA at different times after initiation. Our preliminary data suggest that the inter-origin distance measured dynamically is lower than when using a static imaging approach. Thus, the number of initiation events is likely higher than suggested by previous approaches.

UBC4 AND NOT4 REGULATE STEADY-STATE LEVELS OF DNA POLYMERASE-A TO PROMOTE EFFICIENT AND ACCURATE DNA REPLICATION.

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The accurate duplication of chromosomal DNA is required to maintain genomic integrity. However, from an evolutionary point of view, a low mutation rate during DNA replication is desirable. One way to strike the right balance between accuracy and limited mutagenesis is to employ a DNA polymerase that lacks proofreading activity, but contributes to DNA replication in a very restricted manner. DNA polymerase-α (pol-α) fits this purpose exactly, but little is known about its regulation at the replication fork. Minichromosome maintenance protein (Mcm) 10 regulates the stability of the catalytic subunit of pol-α in budding yeast and human cells. Cdc17, the catalytic subunit of pol-α in yeast, is rapidly degraded following depletion of Mcm10. Here, we show that Ubc4 and Not4 are required for Cdc17 degradation. Disruption of Cdc17 degradation resulted in sensitivity to hydroxyurea, suggesting that this pathway is important for DNA replication. Furthermore, overexpression of Cdc17 in ubc4 and not4 mutants caused slow growth and synthetic dosage lethality, respectively. Our data suggest that Cdc17 levels are very tightly regulated through the opposing forces of Ubc4 and Not4 (degradation) and Mcm10 (stabilization). We conclude that regular turnover of Cdc17 via Ubc4 and Not4 is required for proper cell proliferation.

DAMAGE-INDUCED PHOSPHORYLATION OF SLD3 IS IMPORTANT TO BLOCK LATE ORIGIN FIRING

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Upon checkpoint activation, the kinase Rad53 (Chk2 in mammals) blocks the firing of late replicating origins. The mechanism underlying this function of Rad53 remains unknown. Here, we show that the replication initiation protein Sld3 is phosphorylated by Rad53, and that this phosphorylation, along with phosphorylation of the Cdc7 kinase regulatory subunit Dbf4, blocks late origin firing. Upon exposure to DNA damaging agents, cells expressing SLD3 and DBF4 phosphorylation-deficient mutants (*SLD3-m25* and *dbf4-m25*, respectively) proceed through S-phase faster than wild-type cells, and inappropriately fire late origins of replication. Moreover, *SLD3-m25 dbf4-m25* cells grow poorly in the presence of the replication inhibitor hydroxyurea. We found that *SLD3-m25 dbf4-m25* cells are delayed in recovering from transient blocks to replication, accumulate multiple Rad52 foci in S-phase and subsequently arrest at the DNA damage checkpoint. These data suggest that the intra-S-phase checkpoint functions to block late origin firing in adverse conditions to prevent genomic instability and maximize cell survival.

THE FANCM/FAAP24 COMPLEX IS SELECTIVELY REQUIRED FOR THE DNA INTERSTRAND CROSSLINK-INDUCED CHECKPOINT RESPONSE

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DNA inter-strand crosslink (ICL) occurs in the opposite strand of DNA that severely blocks replication fork progression. The products of lipid peroxidation and other forms of oxidative DNA damage are potential sources for endogenous ICLs. On the other hand, exogenous ICL agents, such as Cisplatin and Mitomycin C, are commonly used for anticancer drugs. Therefore, understanding cellular response to DNA ICL is critical for more effective utilization of these compounds and for the identification of novel therapeutic targets.

ICL repair involves many distinct DNA repair pathways, including nucleotide excision repair, translesion synthesis and homologous recombination. Importantly, proteins implicated in the multigenic disease Fanconi anemia have a role in ICL repair. Fanconi anemia (FA) is a rare human genetic disease that causes severe bone marrow failure and an increased susceptibility to cancer. Thirteen different FA genes (FANC-A, -B, -C, -D1, -D2, -E, -F, -G, -I, -J, -L, -M, and –N) have been identified and mutation of any one of the FA genes results in distinct chromosomal abnormalities and hypersensitivity to ICL agents.

Despite the identification of thirteen of the FA genes, the mechanism by which proteins encoded by these genes protect a cell from ICLs remains to be elucidated. Among the open questions are how do FA proteins respond to DNA ICLs and interact with DNA, and what is the functional significance of these interactions.

FANCM (FA complementation group M) and its binding partner, FAAP24 (FA-associated protein of 24 kDa) anchor the multisubunit FA nuclear core complex to chromatin. Here we show that the FANCM/FAAP24 heterodimer is selectively required for the recruitment of replication protein A (RPA) to ICLs-stalled DNA replication forks in vivo and in vitro. FANCM/FAAP24-dependent RPA foci formation is required for chromatin association of ATR and checkpoint activation. Interestingly, the DNA translocase activity of FANCM is dispensible for RPA recruitment, but DNA binding activity of FAAP24 is critical for this function. Taken together, our study demonstrate that FANCM/FAAP24 forms a unique structure with RPA and promote RPA binding at ICL-stalled replication forks, and this is required for ATR-mediated checkpoint response.

DNA DAMAGE SIGNALING RECRUITS THE RTT107-SLX4 SCAFFOLDS VIA DPB11 TO MEDIATE REPLICATION STRESS RESPONSE

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The DNA damage checkpoint kinase $Mec1^{ATR}$ is critical for maintaining the integrity of replication forks. While it has been proposed to promote fork repair, the mechanisms by which Mec1 regulates DNA repair factors remain unclear. Here we found that Mec1 mediates a key interaction between the fork protein Dpb11 and the DNA repair scaffolds Slx4-Rtt107 to regulate replication stress response. Dissection of the molecular basis of the interaction reveals that Slx4 and Rtt107 jointly bind Dpb11 and that Slx4 phosphorylation is required. Mutation of Mec1 phosphorylation sites in Slx4 disrupts its interaction with Dpb11 and compromises the cellular response to replisomes blocked by DNA alkylation. Proteomic analysis reveals that multiple fork repair factors associate with Rtt107 or Slx4 supporting that Mec1-dependent assembly of the Rtt107-Slx4-Dpb11 complex functions to coordinate fork repair. Our results unveil a new role for Mec1 and establish a mechanistic link between DNA damage signaling and fork repair.

INTEGRATION OF THE DNA DAMAGE CHECKPOINT WITH A CELL-FREE CDK1 ACTIVATION SYSTEM

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Entry into mitosis must occur abruptly, irreversibly, and at the correct time. Although the molecular players in this process are well known, their quantitative relationships had been lacking for mammalian cells. Recently, we established an in vitro system from human (HeLa) cell extract that recapitulates activation of the “engine” of mitosis, CDK1, in response to the mitotic cyclins. We found that CDK1 is activated in a multistage response to cyclin B that is orchestrated by the WEE1 and CDC25 feedback loops. As purified cyclin B is added to the extract, a low level of CDK1 activity is produced, and a complex is formed between cyclin B-CDK1, and WEE1. This low CDK1 activity phosphorylates and appears to activate WEE1 to prevent further increases in CDK1 activity despite addition of more cyclin B. Eventually, however, at a cyclin B concentration (~600 nM) equivalent to what drives mitosis in intact cells, cyclin B can overcome WEE1 buffering, dissociate WEE1 from cyclin B-CDK1 complex, and activate CDC25 phosphatases to promote full CDK1 activation. Cyclin A augments the activation of CDK1 by weakening the WEE1 negative feedback loop. This system has helped elucidate several unappreciated facets of mitotic regulation. However, in cells the integrity of chromosomes is under constant assault. As a result the mitotic circuit is accompanied by a DNA damage response that blocks cell division in the presence of genomic insults. To understand the integration at a mechanistic level beyond what has been feasible in cells, we combined our mitotic entry system with DNA damage signaling. Addition of broken DNA to the extract leads to ATM and CHK2 phosphorylation with in vivo-like kinetics, and the subsequent reduction of both cyclin A- and B-dependent kinase activity. Importantly, the CDK1 inhibition is equivalent to that seen when CDC25 is inhibited, suggesting CDC25 as the damage response target. This effect can be overcome by blocking the checkpoint with caffeine to inhibit ATM or by obviating the need for CDC25 with an inhibitor of the WEE1 kinase. Currently we are assessing the relationship between the level of checkpoint (CHK2) activation and the inhibition of CDK1 activity. From these data we are creating a quantitative model that describes how a small amount of DNA damage can be amplified to a signal that can suppress CDK1 from fully activating and driving cell division.

USP10 REGULATES P53 LOCALIZATION AND STABILITY BY DEUBIQUITINATING P53

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p53 stability and localization is essential for its tumor suppressor function. Ubiquitination by the E3 ubiquitin ligase Mdm2 is the major regulatory mechanism of p53, which induces p53 nuclear export and degradation. However, it is unclear whether ubiquitinated cytoplasmic p53 can be recycled. We found that USP10, a cytoplasmic ubiquitin-specific protease, deubiquitinates p53, reversing Mdm2-induced p53 nuclear export and degradation. Following DNA damage, USP10 is stabilized and a fraction of USP10 translocates to the nucleus to activate p53. The translocation and stabilization of USP10 is regulated by ATM -mediated phosphorylation of USP10 at Thr42 and Ser337. USP10 suppresses tumor cell growth in cells with wild-type p53, with USP10 expression downregulated in a high percentage of clear cell carcinomas, known to have few p53 mutations. Most recently, we also found that USP10 is negatively regulated by G3BP1 (RasGAP binding protein 1), which is frequently overexpressed in several cancers. We show that G3BP1 promote cancer cell growth by inhibiting p53 through USP10. Overall, our findings reveal USP10 to be a novel regulator of p53, providing an alternative mechanism of p53 inhibition in cancers with wild-type p53.

SENSITIVITY OF P53- AND P21-DEFICIENT EPITHELIAL CELLS TO THE COMBINATION OF DNA DAMAGE AND CHK1 INHIBITION

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Genome integrity is protected by checkpoint proteins such as Chk1 and p53 that halt cell cycle progression in cells experiencing genotoxic- or replicative-stress. Half of all human cancers harbor a deletion or inactivating mutation of *TP53* rendering them resistant to DNA damaging agents and anti-metabolites. Chk1 serves a protective function in p53 deficient tumors as the combination of DNA damage and Chk1 inhibition induces selective killing of p53-deficient tumors. To date, experiments monitoring the selective killing of p53-deficient cells by combining DNA damage with Chk1 inhibition have utilized transformed cells cultured *in vitro* or grown as xenografts *in vivo*. Cultured cells accumulate many mutations as a result of continuous passaging in vitro. Thus, although *TP53* can be sequenced in each cell line and the integrity of the p53 pathway assessed, it is impossible to know all of the genetic changes that have taken place and how these alterations contribute to the experimental outcome. Therefore, in this study mice that were wild-type or null for either *TP53* or its downstream effector *p21Waf1/Cip1* were treated with the DNA damaging agent irinotecan followed by the Chk1 inhibitor UCN-01 and small intestinal epithelial cells were assessed for cell cycle arrest, DNA damage, checkpoint abrogation and apoptosis. The combination of irinotecan and UCN-01 induced selective checkpoint bypass and apoptosis in intestinal epithelial cells that were null for *TP53* demonstrating that p53 status is a key determinant of how cells with DNA damage respond to Chk1 inhibition. intestinal epithelial cells null for *p21Waf1/Cip1* exhibited enhanced DNA damage, checkpoint bypass and apoptosis relative to *TP53* null cells after exposure to the combination treatment. These results demonstrate that basal levels of p21 provide a protective effect against DNA damage and indicates that tumor with low levels of p21 may be particularly sensitive to therapies that combine a DNA damaging agent or anti-metabolite with a Chk1 inhibitor.

CHK1-GCN5 AND PP1Γ-HDAC3 COLLABORATELY REGULATE E2F-DEPENDENT GENE EXPRESSION UPON DNA DAMAGE

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Eukaryotic cells are equipped with coordinated systems to contend with DNA damage, such as cell cycle arrest mechanisms, DNA repair pathways and the apoptotic response. Together, these maintain genomic integrity. These systems are partly regulated by transcriptional activation and repression. DNA damage alters the global pattern of gene expression to orchestrate a variety of cellular events. Following UV irradiation, about 4 % of transcripts show more than 3 fold changes. Transcriptional activation is relatively well characterized in this context with sequence specific transcriptional activators, such as p53 and NF-κB. However, transcriptional repression is largely unknown although 90 % of transcripts are downregulated. We recently found that DNA damage-induced suppression of cell cycle regulatory genes correlates with a reduction in histone H3-T11 phosphorylation (H3-pT11) on their promoters that is partly mediated by dissociation of Chk1 from chromatin. The molecular mechanism(s) by which H3-T11 dephosphorylation triggers transcriptional repression remains unclear. Here, we identify protein phosphatase 1γ (PP1γ)-HDAC3 complexes that provide combined phosphatase-HDAC activity that cooperatively deacetylates/dephosphorylates at H3-K9/T11. PP1γ activity is regulated by Cdk1-dependent PP1γ-T311 phosphorylation and NIPP1 interaction. During unperturbed S phase, a Chk1-GCN5-E2F1 complex resides at the E2F binding sites of cyclin B1 and cdk1 promoters. After DNA damage, activated PP1γ dephosphorylates pRb, which results either in recruitment of the PP1γ-HDAC3-pRB complex to E2F1, or replacement of Chk1-GCN5-E2F1 with PP1γ-HDAC3-p107-E2F4. Both events ultimately lead to transcriptional repression of cyclin B1 and cdk1. Our results suggest that H3-K9/T11 at E2F binding sites functions as a “phospho-acetyl” code regulated by Chk1-GCN5 and PP1γ-HDAC3 complexes, whose usage is dependent on the level of Cdk activity.

ELIMINATION OF PIF1- AND EXO1-DEPENDENT NUCLEASE ACTIVITIES PERMITS TELOMERE MAINTENANCE WITHOUT CAPPING BY CDC13

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Telomeric DNA resembles a DNA Double Strand Break (DSB), making telomeres vulnerable to recognition and processing by the DNA Damage Response (DDR). single-stranded DNA (ssDNA) binding proteins of the telomere cap, such as Cdc13 in budding yeast and POT1 in human cells, help prevent recognition of the telomeric DNA by the DDR. When Cdc13 is inactivated telomeres become 'uncapped' and extensive resection of the telomeric DNA occurs by nuclease activities to reveal many kilobases of ssDNA. Telomeric ssDNA then stimulates a potent checkpoint response and arrests the cell at metaphase, ultimately leading to cell death.

Exo1 is the only nuclease so far identified that resects uncapped telomeres. We report that the helicase Pif1 as an essential component of a nuclease activity that functions in parallel to Exo1 to resect uncapped telomeres. The Pif1- and Exo1-dependent nucleases have different properties; ssDNA generated by Pif1 is sufficient, but not necessary, to cause checkpoint activation in the majority of cells, while ssDNA generated by Exo1 is sufficient to cause checkpoint activation in all cells. Inactivation of both the Pif1- and Exo1-dependent nuclease activities eliminates resection of the telomeric DNA at uncapped telomeres, preventing Rad53-dependent checkpoint activation and metaphase arrest in response to telomere uncapping.

Remarkably, elimination of Pif1 and Exo1 overcomes the requirement for telomere capping by Cdc13, as CDC13 can be deleted. Interestingly, telomeres are maintained and increase in length over time in cells lacking Cdc13, suggesting that Cdc13-independent mechanisms of telomerase recruitment occur.

STRUCTURAL INSIGHTS INTO TELOMERASE ELONGATION COMPLEX FORMATION

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A common hallmark of human cancers is the overexpression of telomerase, a ribonulceoprotein complex responsible for maintaining the length and integrity of chromosome ends. Telomere length deregulation and telomerase activation is an early and perhaps necessary step in cancer cell evolution. In fact ~85% of human tumors show high levels of telomerase activity when it is dormant in most somatic cells. Efforts to elucidate in detail the complex mechanism of telomere replication by telomerase as well as attempts to discover cancer and aging therapies that target this enzyme have been hindered to a certain extend by the absence of structural information.

The recent high-resolution structure of the catalytic subunit of telomerase from T. castaneum reveals a number of novel and unexpected results that greatly enhance our understanding of telomerase action on telomeres. The protein consists of four highly conserved domains, organized into a ring-like structure that shares common features with retroviral reverse transcriptases, viral RNA polymerases and to a certain extend the bacteriophage B-family DNA polymerases. Domain organization places motifs implicated in substrate binding and catalysis in the interior of the ring, which can accommodate seven-to-eight bases of double stranded nucleic acid, which corresponds to the length of the RNA templating region of telomerase from this organism. We recently determined the high resolution structure of TERT in complex with its RNA-template and telomeric DNA, data that provides for the first time direct evidence of a partial telomerase elongation complex.

RECOVERY FROM A DNA DAMAGE-INDUCED ARREST

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Activation of the DNA damage checkpoint causes a cell cycle arrest via inhibition of cyclin-dependent kinases (cdks). In order to successfully recover from the arrest, a cell should somehow be maintained in the proper cell cycle state. This problem is particularly eminent when a cell arrests in G2, as cdk-activity is important to establish a G2 state. We have identified the phosphatase Wip1 (PPM1D) as a factor that maintains a cell competent for cell cycle re-entry during an ongoing DNA damage response in G2. We show that Wip1 function is required throughout the arrest, and that Wip1 acts by antagonizing p53-dependent repression of crucial mitotic inducers. Our data show that the primary function of Wip1 is to retain expression of the proper cell cycle regulators during the arrest, rather than to silence the checkpoint to promote recovery. Our findings uncover Wip1 as a first in class recovery “competence” gene, and suggest that the principal function of Wip1 in cellular transformation is to retain proliferative capacity in the face of oncogene-induced stress. In addition we found that the Forkhead transcription factor FoxM1, an important regulator of gene expression in the G2-phase of the cell cycle, is also required for cell cycle re-entry following DNA damage. We show that FoxM1 is transcriptionally active during a DNA damage-induced G2 arrest and this is essential for checkpoint recovery. Paradoxically, Cdk activity, although reduced after checkpoint activation, is required to maintain FoxM1-dependent transcription during the arrest and is essential for sustained expression of pro-mitotic targets, such as Cyclin A, Cyclin B and Plk1. Indeed we find that cells need to retain sufficient levels of Cdk activity during the DNA damage response to maintain cellular competence to recover from a DNA damaging insult. These data demonstrate that cellular competence to recover from a DNA damage-induced arrest needs to be actively sustained during the ongoing arrest. In addition, our studies have uncovered the first factors that control recovery competence and open the way for studies aimed to understand how cells determine their fate following a DNA damaging insult.

ALZHEIMER'S AMYLOID-BETA ABROGATES THE FORMATION OF THE MITOTIC SPINDLE AND DIRECTLY INHIBITS MITOTIC MOTOR KINESINS

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Alzheimer's disease is the predominant cause of age-associated dementia and one of the leading causes of death. Because this slow-progressing pathology affects some 10% of individuals over age 65 and almost half of those over 85, it imposes a tremendous emotional and financial burden on our society. It is widely accepted that a small 40-42 amino acid long Alzheimer's Amyloid-beta (Aβ) peptide plays a key role in the generation and progression of this neuropathology. We established that aneuploidy is one of the hallmarks of Alzheimer's disease and demonstrated that it might be induced by an Aβ-dependent mechanism. Our recent studies showed that Aβ impairs formation and stability of the mitotic spindle. Indeed, as was shown in the Xenopus egg extracts, addition of Aβ resulted in spindles that do not reach full length, are bent, atrophic, collapsed and display a disarray of microtubule fibers within the spindle. We attribute these phenotypes to malfunction of mitotic microtubule-based motor kinesins, particularly Eg5, KIF4A, and MCAK. Our further analysis confirmed that above-mentioned motor kinesins are directly bound to and inhibited by Aβ. Our results indicate that Aβ may disrupt binding of motor kinesins to microtubules, whereby abrogating the motor functions. We propose that Aβ-mediated inhibition of the kinesin motors is directly implicated in the generation of aneuploidy typical of Alzheimer's disease.

THE DEUBIQUITINATING ENZYME BAP1 REGULATES CELL PROLIFERATION VIA INTERACTION WITH HCF-1

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The deubiquitinating enzyme BAP1 possesses growth inhibitory activity and functions as a tumor suppressor. In this study, we report that BAP1 also plays positive roles in cell proliferation. BAP1 depletion by RNAi inhibits cell proliferation as does overexpression of a dominant negative mutant of BAP1. Mass spectrometry analyses of copurified proteins revealed that BAP1 is associated with a cell cycle regulator, HCF-1, and transcription factors FoxK1 and FoxK2, suggesting that BAP1 may be involved in regulation of gene expression. We show that HCF-1, BAP1, and FoxK1/K2 form a ternary complex, in which BAP1 bridges HCF-1 and FoxK1/K2. HCF-1 is a heterodimer of HCF-1N and HCF-1C, which are produced by proteolytic processing of a large precursor protein. The Kelch domain in HCF-1N binds to transcription factors such as E2F family members by recognizing a conserved peptide sequence, (D/E)HXY, known as the HCF-1-binding motif (HBM). By functioning as a scaffold to recruit histone-modifying enzymes to promoters, HCF-1 regulates gene expression through selective modulation of chromatin structure. We show that HCF-1N is modified with K48-linked polyubiquitin chains on its Kelch domain. The HBM of BAP1 is required for interaction with HCF-1N and mediates deubiquitination of HCF-1N by BAP1. The importance of the BAP1-HCF-1 interaction is underscored by the fact that growth suppression by the dominant negative BAP1 mutant is entirely dependent on the HBM. These results suggest that BAP1 regulates cell proliferation by deubiquitination of CF-1.

AURORA B REGULATES FORMIN MDIA3 MICROTUBULE STABILIZATION ACTIVITY AT KINETOCHORES

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A mammalian homologue of Drosophila diaphanous and a member of the formin-homology (FH) family of proteins, mDia3, has been reported to localize at kinetochores and siRNA-induced defects in metaphase chromosome alignment. However, how direct these effects are remains unclear. We are able to reproduce the observation that knock down of formin mDia3 using siRNA in mammalian cultured cells led to chromosome misalignment. The alignment could be rescued by a siRNA-resistant form of wild-type mDia3, as well as a siRNA-resistant mDia3 mutant that is defective in actin nucleation, but not in stabilizing microtubules. We subsequently mDia3 is phosphorylated by Aurora B kinase in vitro with purified recombinant components and in cultured cells. Cells expressing the non-phosphorylatable mDia3 mutant cannot position chromosomes at the metaphase plate. Strikingly, cells expressing the phosphomimetic mDia3 mutant are able to achieve metaphase chromosome alignment even in the presence of an Aurora B kinase inhibitor. However, the kinetochore fibers are not stable against cold treatment in cells with the phosphomimetic mDia3 mutant. Further, Aurora B phosphorylation reduced mDia3 microtubule stabilization activity in vitro using purified recombinant proteins. Thus, mDia3 is a physiological substrate of Aurora B in achieving stable kinetochore microtubule attachment in which Aurora B kinase directly regulates mDia3 microtubule stabilization activity.

REPLICATION FACTOR C COMPLEXES FUNCTION IN SISTER CHROMATID COHESION

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Recent insights have expanded our understanding of sister chromatid cohesion pathways. Of particular interest is the growth in number of anti-establishment factors. Current models suggest that anti-establishment factors preclude precocious pairing reactions until Ctf7/Eco1-dependent acetylation of Smc3. Of particular interest is deletion of anti-establishment factors greatly suppresses growth defects of *ctf7/eco1* mutant cells. New studies have identified two classes of anti-establishment factors: those that are cohesin-associated (Rad61/WAPL and Pds5) and those that are DNA replication fork-associated (Elg1-RFC).
Here, we report that anti-establishment activities of *RAD61* and *ELG1* are indistinguishable when assayed both genetically and functionally. These findings suggest that both perform within a singular anti-establishment mechanism that is sensitive to Smc3 acetylation. We also characterize further pro-establishment activities such as Ctf18-RFC - currently thought to promote Ctf7/Eco1 acetylation of Smc3. In contrast to this model, our results reveals that *smc3* acetylation mimics fail to bypass the roles of *CTF18* in cohesion establishment. In combination, these findings reveal that cohesion regulatory activities are much more complicated than currently discussed in the literature.

CENTROSOME DISJUNCTION: A CONCERTED ACTION BETWEEN NEK2A AND COMPONENTS OF THE HIPPO PATHWAY

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Separation of the duplicated centrosomes upon mitotic entry is essential for spindle formation and to avoid aneuploidy, the latter being one of the hallmarks of cancer cells. A critical kinase that is involved in centrosome separation during mitotic entry is the NIMA-related Nek2A kinase that displaces linker proteins between two centrosomes upon phosphorylation. Current models suggest that Nek2A activity reaches a critical threshold in prophase, which then dissolves this linkage. The data presented in this study revise this model, describing a novel function for Hippo pathway components at the centrosome. Hippo pathway is well known for its function in tissue/organ growth and apoptosis. Here, we report that two Hippo pathway components, the mammalian sterile 20-like kinase 2 (Mst2) and the scaffold protein Salvador (hSav1), regulate the NIMA-related kinase Nek2A in its ability to initiate centrosome disjunction at mitotic entry. Nek2A has a SARAH-like coiled-coil region at its extreme C-terminus that mediates direct interaction with the SARAH domains of hSav1 and Mst2. siRNA depletion experiments reveal that Mst2 and hSav1 are important for the centrosomal localization of Nek2A. Moreover, hSav1/Mst2-dependent recruitment of Nek2A to centrosomes regulates Nek2A-dependent phosphorylation of C-Nap1 and rootletin, centrosomal linker proteins whose phosphorylation triggers centrosome separation. Mst2 directly phosphorylates Nek2A and Nek2A mutants that are no longer phosphorylated by Mst2 fail to induce centrosome splitting. We propose that hSav1 and Mst2 regulate localized activity of Nek2A at centrosomes thus resolving centrosome cohesion in early mitosis.

SPINDLE ASSEMBLY CHECKPOINT REGULATED BY NON-EQUILIBRIUM COLLECTIVE SPINDLE-CHROMOSOME INTERACTION—RELATIONSHIP TO SINGLE DNA MOLECULE FORCE-EXTENSION FORMULA

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The spindle checkpoint, which blocks segregation until all sister chromatid (SC) pairs have been stably connected to the two spindle poles, is perhaps the biggest mystery of the cell cycle. The main reason seems to be that the spatial correlations imposed by microtubules between kinetochores and the nonlinear dependence on the increasing number of attached kinetochores, have been disregarded in earlier studies. From these missing parts a non-equilibrium collective spindle-chromosome interaction is obtained for budding yeast (Saccharomyces cereviciae) (J. Phys. Cond. Matter 21 (2009) 502101). In this interaction tension decreases nonlinearly with the number of attached kinetochores and becomes equally distributed between all bioriented SC pairs, blocking segregation until all sister chromatids pairs are bi-oriented without need of a "wait-anaphase" signal. The model thus explains how the committment to anaphase could be synchronized. It also predicts kinetochore oscillations at a frequency which agrees well with observation. Finally, a relationship between this model interaction and the force-extension formula of a single DNA molecule is obtained.

FACT PREVENTS THE ACCUMULATION OF FREE HISTONES EVICTED FROM TRANSCRIBED CHROMATIN AND A SUBSEQUENT CELL CYCLE DELAY IN G1

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Background
The FACT complex participates in chromatin assembly and disassembly during transcription elongation. Yeast mutants affected in the SPT16 gene, which encodes one of the FACT subunits, exhibit defects in the G1-S transition.

Methodology/Principal Findings
Here we show that the dysfunction of chromatin reassembly factors like FACT or Spt6 down-regulates the expression of the gene encoding the cyclin that modulates G1 length (CLN3) during this phase of the cell cycle, by specifically triggering the repression of its promoter. The G1 delay suffered by spt16 mutants is not mediated by the DNA-damage checkpoint, although mutation of RAD53 enhances the cell-cycle defects of spt16-197. We show that the dysfunction of FACT produces an accumulation of free histones evicted from transcribed chromatin. This accumulation is enhanced in a rad53 background and it leads to a delay in G1. We also show that the overexpression of histones in wild-type cells down-regulates CLN3 and causes a delay in G1.

BAYESIAN CELL-CYCLE PARAMETER INFERENCE: A NOVEL APPROACH TO TIME SERIES DATA COMPARISON

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Time series experiments on synchronized cell populations reveal dynamic behaviors underlying cell-cycle progression. Cell cycle-regulated genes, for example, often oscillate, peaking in expression at a particular cell-cycle phase. Comparison of measurements from two different time series experiments is made all the more difficult without knowledge of the time in which cells traverse the cell cycle and the time required for recovery from synchronization. Previous approaches (Orlando *et al.*, *Nature* 2008) have relied on morphological features (eg. presence of buds in budding yeast, *S. cerevisiae*) in the cell populations to estimate these two parameters. Also, these approaches infer only one set of parameters per experiment, omitting the possibility of different period lengths. To address these two issues we developed a Bayesian statistical method for cell-cycle parameter inference. We applied the approach to time series gene expression data for a wild type strain and two different mutant strains of budding yeast. For one of the mutant strains, a strain capable of budding but lacking S phase and mitotic cyclin activity, we were able to consistently recover parameters comparable to those identified by previous methods. Upon comparison with wild type data, we found that nearly 1100 genes retained periodic patterns of expression in this strain. We were also able to recover parameters for the second mutant strain, *cdc28-4*. This strain lacked budding capability and had non-functional cyclin-dependent kinase activity at restrictive temperature. Comparison with wild type data revealed an extended cycle time for the *cdc28-4* mutants and, surprisingly, almost 800 genes that remained periodically expressed in spite of the absence of a functional cyclin-dependent kinase. While we applied this approach to budding yeast experiments, the method is adaptable to time series measurements from other experiments and organisms.

ESSENTIAL ROLE FOR THE DEAD-BOX HELICASE DDX5 IN S-PHASE PROGRESSION AND CANCER CELL PROLIFERATION.

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DNA replication is fundamental for cell division where it is required for the duplication of genetic information that will be equally distributed into daughter cells during mitosis. Understanding factors required for DNA replication will enrich our knowledge of this important process and potentially identify vulnerabilities that can be exploited therapeutically in disease states typified by uncontrolled cell division such as cancer. To identify proteins with essential roles in DNA replication we developed an assay that measures the stability of maintenance of an episomal plasmid in human cells that have been transduced with short hairpin RNAi (shRNA) targeting the expression of 54 genes of poorly understood function. These genes are either up-regulated during G1/S or have been observed to interact with human ORC. RNA interference of known replication factors significantly impairs plasmid stability in cells supporting that the plasmid can be used as a reporter of DNA replication activity. We identified 6 genes from the screen that had no appreciated role in DNA replication but are required for plasmid stability. AND-1 is one of the genes identified and has recently been reported to have an essential activity in DNA replication. DDX5, a member of the family of DEAD-box RNA helicases, is another gene identified and multiple shRNA's targeting this gene significantly impair both cell proliferation and plasmid stability. Consistent with an important role in DNA replication DDX5 knockdown results in down-regulated expression of replication factors, reduced association of replication factors with chromatin during S-phase, and impaired S-phase entry/progression. Moreover we have found that the DDX5 locus is frequently amplified in breast cancer where cell lines containing amplification of the locus also overexpress DDX5 protein compared to cell lines that do not have amplification of the locus. Intriguingly, knockdown of DDX5 in an amplification containing breast cancer cell line impairs proliferation whereas knockdown in a non-amplification breast cancer cell line does not suggesting that DDX5 contributes toward breast cancer development. These results demonstrate a new activity for DDX5 in regulating DNA replication and support an oncogenic role for DDX5 in breast tumorigenesis.

GEMININ CONTROLS THE PATTERN OF STEM CELL DIVISION IN THE BONE MARROW

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Stem cell division patterns are unusual in that they generate two types of daughter cells: daughters that remain stem cells (self-renewal) and daughters that will differentiate into somatic cells. The balance between self-renewal and somatic cell production must be carefully balanced in order to produce an adequate number of somatic cells without depleting the stem cell population. A defect in this balance could cause tissue degeneration. Despite their obvious importance, the mechanisms that control the choice between self-renewal and somatic cell production are poorly understood.

The unstable regulatory protein Geminin is thought to maintain cells in an undifferentiated state while they proliferate. Geminin is a bi-functional protein. It limits the extent of DNA replication to one round per cell cycle by binding and inhibiting the essential replication factor Cdt1. Loss of Geminin leads to replication abnormalities that activate the DNA replication checkpoint and the Fanconi Anemia (FA) pathway. Geminin also influences patterns of cell differentiation by interacting with Homeobox (Hox) transcription factors and chromatin remodeling proteins.

To examine how Geminin affects the proliferation and differentiation of hematopoietic stem cells, we created a mouse strain in which Geminin is deleted from the proliferating cells of the bone marrow. Geminin deletion has profound effects on all three hematopoietic lineages. The production of mature erythrocytes and leukocytes is drastically reduced and the animals become anemic and neutropenic. In contrast, the population of megakaryocytes is dramatically expanded and the animals develop thrombocytosis. Interestingly, the extent of DNA replication is largely normal. We conclude that Geminin deletion causes a maturation arrest in some lineages and directs cells down some differentiation pathways at the expense of others. We are now testing how Geminin loss affects cell cycle checkpoint pathways, whether Geminin regulates hematopoietic transcription factors, and whether Geminin deficient cells give rise to leukemias or lymphomas.

THE RELATIONSHIP BETWEEN THE GSK3B PROTEIN KINASE, MCK1 AND THE HIGHLY CONSERVED CDC25 PHOSPHATASE, MIH1.

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The Mck1 protein kinase has proposed functions in a variety of pathways from chromosome segregation to calcium stress response. By performing a synthetic dosage lethality screen in an mck1 mutant, we identified Mih1 as a putative target of the Mck1 kinase. Mih1 is a tyrosine phosphatase that removes the Cdc28/Cdk1 inhibitory tyrosine phosphorylation imposed by the Swe1 kinase to transition cells through mitosis. Mih1 is hyper phosphorylated early in the cell cycle and is dephosphorylated as cells enter mitosis. We find changes in the phosphorylation state of Mih1 during the cell cycle in an mck1 mutant strain. Yet, we do not detect dramatic defects in the mck1 mutant's mitotic cell cycle transition. Instead, our data suggests that the regulation of Mih1 by Mck1 may be important for a target other than mitotic Cdc28/Cdk1. Our current work is aimed at identifying additional targets of Mih1 and characterizing the interaction between Mck1 and Mih1.

REGULATION OF GENOME MAINTENANCE BY NOPO/TRIP, AN E3 UBIQUITIN LIGASE REQUIRED FOR CELL-CYCLE PROGRESSION DURING EARLY EMBRYOGENESIS IN *DROSOPHILA*

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In a screen for cell-cycle regulators, we identified a *Drosophila* maternal effect-lethal mutant that we named "*no poles*" (*nopo*). Embryos from *nopo* females undergo mitotic arrest with barrel-shaped, acentrosomal spindles during the rapid S-M cycles of syncytial embryogenesis. The *no poles* (*nopo*) phenotype is similar to that observed when Checkpoint kinase 2 (Chk2) is activated in the early *Drosophila* embryo. We showed that the spindle/centrosomal and developmental defects of *nopo* mutants are strongly suppressed by *Chk2* mutation. Furthermore, we found that *Chk2 nopo* mutants exhibit a significantly shorter interphase 11 as compared to wild type, suggesting that *nopo* mutants may enter mitosis with incompletely replicated DNA. We hypothesize that NOPO regulates timing of S-M transitions in syncytial embryos to ensure that S-phase is of sufficient length to allow completion of DNA replication prior to mitotic entry; however, the precise mechanism of NOPO function is unknown.

nopo encodes an E3 ubiquitin ligase. We showed that NOPO interacts with the E2 conjugating enzyme, Bendless (BEN), via yeast two-hybrid assay. We also found that *ben*-derived embryos arrest during early embryogenesis and exhibit a *nopo*-like phenotype. We hypothesize that BEN and NOPO form an E2-E3 complex required during syncytial embryogenesis for genomic integrity, cell-cycle progression, and continuation of development. NOPO's mammalian homolog, TRAF-interacting protein (TRIP), was identified for its role in TNF signaling, although it has been proposed to have additional functions.

To gain insight into the biological roles of NOPO/TRIP, we performed a yeast two-hybrid screen for human TRIP interactors. We identified a family of DNA polymerases that facilitate the replicative bypass of damaged DNA (translesion synthesis) as TRIP interactors. We have also shown that NOPO interacts with *Drosophila* homologs of these DNA polymerases via yeast two-hybrid assays. We are currently testing these proteins as potential substrates of NOPO/TRIP E3 ligases. Future efforts will focus on placing NOPO/TRIP within a molecular framework so as to elucidate the mechanism by which it promotes genomic stability.

THE POLO-LIKE KINASE CDC5 AND THE CLB2/CDK COMPLEX COOPERATE TO RELEASE CDC14 IN EARLY ANAPHASE

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In budding yeast the protein phosphatase Cdc14 controls exit from mitosis. The activity of Cdc14 is regulated by a competitive inhibitor, Cfi1/Net1, which binds to and sequesters the phosphatase in the nucleolus from G1 up to metaphase. During anaphase, Cdc14 is released from its inhibitor and spreads throughout the nucleus and the cytoplasm, where it reaches its targets and triggers mitotic exit. The Cdc Fourteen Early Anaphase Release (FEAR) network promotes the release of Cdc14 from its inhibitor during early anaphase whereas the Mitotic Exit Network (MEN) promotes and maintains the release of Cdc14 during late stages of anaphase and telophase. Both the Polo-like kinase Cdc5 and the Clb2/CDK complex have been involved in promoting the release of Cdc14 in early anaphase as components of the FEAR network but their function and relationship in the network have remained unclear. We found that Cdc5 and Clb2/CDK cooperate to promote the release of Cdc14 from the nucleolus. Interestingly, we observed that in response to *CDC5* overexpression and concomitant to Cdc14 release, Clb2 becomes phosphorylated. We are currently investigating the consequences of this(ese) phosphorylation event(s) on Clb2 and its(their) relevance for the release of Cdc14 in early anaphase.

FRIED MUTANTS EXHIBIT DEFECTS IN OOGENESIS AND LARVAL AND PUPAL DEVELOPMENT

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The Drosophila egg chamber provides the opportunity to study regulation of mitosis, meiosis, and the endocycle in a single cluster of 16 germ cells. In wild type oogenesis, a single germ cell undergoes four incomplete, synchronous divisions to generate a cyst of 15 nurse cells and one oocyte. As the egg chamber grows, the oocyte is transcriptionally quiescent and arrests in Meiotic Prophase I. The oocyte chromosomes compact into a spherical karyosome. Meanwhile, the nurse cells are transcriptionally active and undergo many endocycles. For the first four endocycles, the nurse cell chromosomes are polytene and they exhibit a characteristic clumped morphology. Starting at the fifth endocycle, the chromosomal organization changes, leading to a more dispersed morphology.

Three alleles of the previously unidentified Drosophila gene, *fried*, were uncovered in a clonal germline screen for oogenesis mutants on chromosome 3R. *fried* egg chambers contain 15 nurse cells and one oocyte, indicating that the early mitotic divisions and cell fate decisions occur normally. *fried* egg chambers grow poorly and fail to develop beyond stage 8 (mid-oogenesis). The nurse cells in *fried* egg chambers accumulate less DNA than wild type chambers of a similar age. *fried* nurse cell chromosomes retain a polytene-like morphology until throughout egg chamber development. The *fried* mutant oocyte's karyosome is misshapen, and it appears fragmented. *fried* mutants are therefore likely to be defective in the cell cycle and/or DNA repair and packaging.

Examination of *fried* homozygotes reveals that the gene is required earlier in development. *fried* larvae are small, and often fail to pupariate. *fried* pupae do not eclose. We have mapped fried to a 40 kb region of 3R using the Bloomington Deficiency kit. The *fried* region includes 8 candidate genes, including four genes with previously studied loss-of-function alleles. We will present our mapping and complementation data and phenotypic analysis.

A NOVEL ROLE FOR THE DROSOPHILA MELANOGASTER´S POLO KINASE ON THE NATURE OF CHROMOSOME-SPINDLE INTERACTIONS.

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Polo-kinase has been shown to be an essential regulator of many events that take place during preparation, entry, progression and exit from mitosis. Previous studies have shown that during these stages of the cell cycle Polo localizes to centrosomes, spindle microtubules, kinetochores and the midbody. However while much is known about its role in centrosomes, microtubules and the midbody little is know about the role of Polo at kinetochores.
To study this process we have carried out a careful analysis of microtubule-kinetochore interaction in the absence of Polo in Drosophila S2 cell and observed a new important phenotype that had been overlooked in previous studies. We observed that in POLO-depleted cells the majority of chromosomes fail to show normal amphitelic attachment and are mostly attached syntelically to spindle microtubules.
The synthelic attachments are not corrected and Aurora B, which shows partial delocalization over chromosome arms and spindle microtubules, has compromised kinase activity. Yet in the absence of Aurora B, chromosomes are not as prone to syntelic attachments as when POLO is depleted from cells which clearly indicates that other aspects of POLO function are also required for attaining correct chromosome attachments.
Indeed, the synthelic attachment observed in the absence of Polo is associated with changes on centromere structure. First, interkinetochore distance which represents centromere length is significantly decreased in POLO-depleted chromosomes. In addition, we observed that Mei-S332, a centromeric protein whose removal from mitotic chromosomes is dependent on POLO, changes its localization in the absence of Polo occupying a different spatial domain within the centromere. Accordingly, double-depletion of POLO and Mei-S332 partially rescues the syntelic phenotype.
Our results indicate that Polo is essential for the establishment of correct chromosome-spindle attachments not only through a regulatory role of the correction mechanism but also by conducting proper chromosome architecture.

E2F4 BINDING WITHOUT E2F SITE: THE CHR ELEMENT IS SUFFICIENT FOR BINDING OF E2F4, P107, P130 AND LIN PROTEINS TO CELL CYCLE PROMOTERS REPRESSED BY CDE/CHR SITES.

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Expression of genes like cyclin A, cyclin B, Cdc2 (cdk1) and Cdc25C on the transcriptional level is regulated by CDE and CHR promoter sites through repression in G0. The CDE is related to E2F sites. However, CDEs are distinct from E2F elements as they are always found in conjunction with a CHR four nucleotides downstream. Recently, the mammalian DREAM/LINC protein complex was shown by ChIP analyses to bind to several CDE/CHR-regulated promoters. Particularly more detailed experiments on the Cdc2 promoter showed binding of E2F4 to the E2F/CDE site requiring an intact CHR four nucleotides downstream. Furthermore, Lin54 appears to contact the CHR contributing to binding of DREAM/LINC to this CDE/CHR promoter. Until now the assumption was that E2F4 binding to E2F-like CDE sites is central to cell cycle-dependent transcriptional regulation and this association is only supported by the neighboring CHR stabilizing the interaction. However, some promoters are just controlled by a CHR with the sequence 5'-TTTGAA-3' lacking a functional CDE or E2F site in the promoters. We have looked at protein binding to the mouse and human cyclin B2 promoters in nuclear extracts from G0 cells and observe that the DREAM/LINC complex binds to both promoters. Binding of E2F4, p107, p130, Lin9, Lin37 and Lin54 is decreased to background when the CHR is mutated. Importantly, cell cycle-dependent regulation of the human cyclin B2 promoter solely depends on the CHR since it does not contain a functional E2F or CDE site. Thus, E2F4 in a complex with other DREAM/LINC proteins can bind to the promoter through the CHR. We also observe binding of the complex in vivo in resting cells, but binding of E2F4, p107, p130 and Lin proteins is clearly reduced when cells enter S-phase. NF-Y-binding CCAAT-boxes are required for most of the transcriptional activity of mouse and human cyclin B2 promoters throughout the cell cycle. Our data suggest a model by which E2F4/DREAM components repress transcription in G0 and are primarily bound through the CHR. Binding of this complex is lost when the promoter is activated. Activation is dependent on NF-Y proteins binding to CCAAT-boxes.

A PROTEIN PHOSPHATASE 2A-DEPENDENT PATHWAY TO QUIESCENCE OPERATING IN G2

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Quiescence (G0) is a cellular fate that allows cycling cells to reversibly cease cell division in culture and *in vivo*. The final decision to enter quiescence has long been suspected of occurring in the early G1 phase of the cell cycle, where cells can monitor their environment for conditions favoring either continuous growth or cell cycle arrest. The signaling events transmitting this information all converge on the pocket protein family, i.e. retinoblastoma protein (pRb), p107 and p130, all of which can interact with chromatin to suppress the G1-S transition and enable quiescent growth arrest.
Here we report results suggesting that a heretofore unanticipated pathway that is centered in the G2 phase of the cell cycle and depends on the ubiquitous cellular phosphatase, PP2A, regulates quiescence. Inhibition of PP2A using okadaic acid during a limited time period in G2 released pocket proteins from chromatin and impaired quiescence in growth factor-deprived cultured cells as shown by continuous uptake of BrdU. The specific PP2A subcomplex necessary for quiescence contains the B56γ subunit, previously implicated in the transformation of mammalian cells when functionally inhibited [1]. Interestingly, inhibition of PP2A-B56γ using the oncoprotein SV40 small t, okadaic acid, or shRNA-mediated B56γ depletion caused hyperphosphorylation of several components of the so-called DREAM complex, comprising the pocket protein p130, E2F4 as well as members of the mammalian MuvB complex that has been shown previously to be crucial for quiescence establishment [2].
These data suggest a novel function for PP2A in G2 that is essential for cells to enter quiescence in the next G1. It likely depends, at least in part, on a functional interaction – direct or indirect – between PP2A and certain chromatin-bound pocket protein-containing complex(es). These findings challenge the notion that G1 is the one cell cycle phase during which the decision to enter quiescence occurs and could help to enhance one's understanding of the mechanisms that trigger this key cellular state. Importantly, normal access to G0 is potentially involved in regulating such crucial processes as stem cell self-renewal and suppression of cancer development.

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ROLE OF MITOTIC SPINDLE GEOMETRY IN CANCER CELL CHROMOSOME INSTABILITY

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Most cancer cells exhibit high rates of mitotic chromosome mis-segregation, which results in chromosome instability (CIN). CIN not only maintains aneuploidy (i.e., incorrect chromosome numbers), but correlates with malignancy. We recently found that one mechanism responsible for chromosome mis-segregation in CIN cells is the transient assembly of multipolar spindles. Within these multipolar spindles, chromosomes are very likely to establish erroneous attachments with the mitotic spindle, and this eventually leads to chromosome mis-segregation at later stages of mitosis. This study suggests that mitotic spindle geometry influences chromosome mis-attachments and mis-segregation. Thus we hypothesized that another mechanism by which chromosomes can establish erroneous attachments with spindle microtubules in CIN cells is by starting mitosis with incompletely separated spindle poles. To test our hypothesis, we first performed live-cell experiments using a cell line with GFP-labeled spindle poles, and found that cells with incomplete spindle poles separation upon mitotic entry, exhibited chromosome segregation defects indicative of defective chromosome/spindle attachment. We then experimentally delayed spindle pole separation to identify the mechanism by which chromosome mis-attachments are formed. Finally, to determine whether this mechanism is involved in cancer cell CIN, we analyzed a panel of cancer cell lines and found that 30-70% of them display unseparated spindle poles in early mitosis. In conclusion, our results suggest that spindle geometry plays a key role in CIN, and proteins involved in mitotic spindle morphology are potential targets for cancer therapy

DIFFERENTIAL REQUIREMENTS FOR CDK4 AND CDK6 CATALYTICAL AND STOICHIOMETRIC FUNCTIONS

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The cyclin D-cdk4/6 complex has two distinct functions. Its catalytic role involves its ability to act as serine/threonine kinase, responsible for phosphorylation of substrates required for cell cycle transitions, while its sequestration function involves its ability to act as a reservoir for p27Kip1. This association sequesters p27 from cyclin E-cdk2 complexes, allowing them to remain active. We exploited the ability of TGF-β to induce p15Ink4b, to ascertain whether the catalytic and sequestration roles of cdk4 and cdk6 were required equally during proliferation. p15 binding destroys the cyclin D/cdk4 complex, negating both the catalytic and sequestration functions and results in G1 arrest. Expression of wild-type, catalytically inactive or p15-resistant cdk4 or cdk6 mutants rendered Mv1Lu cells resistant to TGF-β-mediated growth arrest. Cells expressing a catalytically-inactive, p15-resistant double cdk6 mutant were also resistant to TGF-β-mediated arrest, proliferating in the absence of all cdk4/6 kinase activity. Expression of this cdk6 mutant acted as a sink for p27, allowing cdk2 to remain active, and suggested that the sequestration function was sufficient in asynchronously growing cells. Interestingly, the homologous cdk4 mutant was unable to overcome TGF-β-mediated arrest. When contact arrested cells were released from quiescence in the presence of exogenous cdk4, the G0-G1 transition was accelerated in a kinase-dependent manner. In contrast, overexpression of both catalytic and non-catalytic cdk6 alleles accelerated G0 exit, suggesting that cdk6 may play a different role in the G0-G1 transition. Gel filtration analysis of cdk4 and cdk6 pools in proliferating and arrested cells revealed the presence of a previously unidentified cyclin D-cdk6 dimer, which may contribute to the differential activity of these kinases to promote proliferation of cells both in cycle and upon reentry from the quiescent state. Cdk4 appears unable to form a similar dimeric complex with cyclin D, suggesting that the structural interactions of these two kinases are distinct.

COMBINED INACTIVATION OF THE PRB AND HIPPO PATHWAYS INDUCES DEDIFFERENTIATION IN THE *DROSOPHILA* RETINA.

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Functional inactivation of the Retinoblastoma (pRB) pathway is an early and obligatory event in tumorigenesis. This is widely attributed to the need of tumor cells to bypass the ability of pRB to promote cell cycle exit. While support of this is well documented, pRB is involved in other cellular processes that are less physiologically understood. Here we demonstrate that independently of cell cycle exit control, pRB, in cooperation with the Hippo tumor suppressor pathway, functions to maintain the terminally differentiated state. We found that mutations in the Hippo signaling pathway, *wts* or *hpo*, trigger widespread dedifferentiation of *rbf* mutant cells in the *Drosophila* eye. Initially *rbf wts* or *rbf hpo* double mutant photoreceptors are morphologically indistinguishable from their wild type counterparts as they properly differentiate, express mature neuronal markers, and develop axonal projections. However, double mutant cells stochastically lose their neuronal identity as they undergo dedifferentiation and become uncommitted eye specific cells. Surprisingly, this dedifferentiation is fully independent of cell cycle exit defects and occurs even when inappropriate proliferation is blocked by a *de2f1* mutation. The implications of these findings suggest that one of the post-mitotic roles of the pRB pathway is to maintain a state of terminal differentiation and to prevent cells from reverting to a progenitor-like state. Thus, in addition to its role in promoting cell cycle exit, our results implicate another tumor suppressor property of the pRB pathway.

PHOSPHORYLATION CONTROLS LOCALIZATION OF THE DROSOPHILA SHUGOSHIN MEI-S332 TO THE CENTROMERE

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The Drosophila MEI-S332 protein localizes to centromeres in mitosis and meiosis when sister chromatids are attached. It is essential for the maintenance of centromere cohesion in meiosis until the sister chromatids segregate at anaphase II, and it also contributes to centromere cohesion in mitosis. We previously showed that in cell culture centromere localization of MEI-S332 is controlled by phosphorylation, with phosphorylation by Aurora B kinase being necessary for proper localization and phosphorylation by POLO kinase being required for MEI-S332 dissociation from the centromere at anaphase. To investigate the function of phosphorylation *in vivo* in mitosis and meiosis we have generated transgenic lines expressing mutant forms of MEI-S332 resistant to Aurora B phosphorylation or with mutations that mimic phosphorylation. We also have produced transgenic lines in which MEI-S332 is mutated to reduce binding to POLO or contains a phosphomimetic mutation in the POLO binding domain. We will present the effects of these mutations on MEI-S332 centromere localization and chromosome segregation.

REGULATION OF DNA REPLICATION AND FORK PROGRESSION.

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Defects in DNA replication can lead to increased gene copy number, known to be present in many cancer cells, but regulatory mechanisms that link DNA replication activity to the cell cycle in metazoan cells remain poorly understood. *Drosophila melanogaster* ovarian follicle cells are a superb model system to study metazoan DNA replication, as replication origins repeatedly fire at precise points in differentiation, resulting in gene amplification. We have uncovered EMS-induced mutations that result in BrdU incorporation patterns consistent with increased DNA replication activity in amplifying follicle cells. One such mutant, *quadruple double*, displays enhanced replication fork separation, premature cessation of origin firing, and ectopic sites of amplification. Mapping of these additional sites of amplification by comparative genomic hybridization (CGH) indicates that specific regions of the genome are susceptible to origin activation. Key regulators of the G1/S transition control gene amplification in *Drosophila* ovarian follicle cells. Loss of Rbf1 activity (the *Drosophila* retinoblastoma family homologue) results in additional rounds of endoreplication, which normally precede gene amplification. *Rbf* mutants also display BrdU incorporation patterns consistent with ectopic sites of gene amplification, and preliminary results to identify those sites of amplification will be presented. Additionally, CyclinE activity is necessary for proper gene amplification, and we have uncovered a mutant allele of *cyclinE*, *cyclinE*^{lf36}, that results in increased replication fork progression. Our data suggest a direct role for CyclinE in modulating replication fork progression. Taken together, gene amplification in *Drosophila* ovarian follicle cells provides a tractable genetic, cytological, and biochemical system to uncover the regulatory mechanisms responsible for coordinating metazoan DNA replication with the cell cycle.

CYCLIN A / CDK2 IS REQUIRED FOR EXIT FROM A G2 CHECKPOINT.

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Cyclin A / cdk2 is the primary cyclin/cdk regulating G2 phase progression and the timing of progression into mitosis. Cyclin A / cdk2 activity peaks in G2 phase and its depletion by siRNA or drug inhibition causes a G2 phase delay. In this study we have examined the mechanisms by which cyclin A / cdk2 regulates the timing of entry into mitosis. Checkpoint kinase 1 is well known for its role in DNA damage responses, but published data has also suggested a role for Chk1 in normal G2 / M phase progression. In cyclin A / cdk2 depleted G2 phase cells, there was an elevated level of activated Chk1, and inhibiting or depleting Chk1 substantially reversed the G2 delay seen with cyclin A depletion. The activation of Chk1 was dependent on ATR. We have investigated the mechanisms by which cyclin A / cdk2 regulates Chk1. Cyclin A / cdk2 can bind to Chk1 in vivo and has been reported to phosphorylate Chk1 on two novel inhibitory sites, Ser286 and Ser301 in vitro [1]. We found that cyclin A / cdk2 phosphorylates these sites in vivo, and mutation of these sites can delay entry into mitosis. In addition, we have examined the role of a known cdk2 substrate and mitotic regulator Cdh1, and its downstream targets Plk1 and Claspin. Our data revealed that surprisingly Plk1 levels, regulated by Cdh1, were relatively unaffected by cyclin A / cdk2 depletion. However, depletion of Cdh1 did substantially reverse the G2 phase delay imposed by cyclin A / cdk2 depletion, suggesting that Cdh1 is involved in the cyclin A / cdk2 dependent G2 phase delay. We have evidence of increased Claspin levels suggesting that this may be the point of Cdh1 involvement and that cyclin A / cdk2 may regulate both the activation as well as inactivation of Chk1. In addition to its role in regulating normal G2 / M progression, we will present evidence that cyclin A / cdk2 has a critical role in progression out of a G2 phase checkpoint arrest.

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MST1 PROMOTES STABLE KINETOCHORE-MICROTUBULE ATTACHMENT BY REGULATING THE KINASE ACTIVITY OF AURORA B.

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The establishment and maintenance of proper attachment of kinetochores to microtubules are required to prevent chromosome missegregation and consequent chromosomal instability and tumorigenesis. Although MST1 (mammalian sterile 20–like kinase 1) has been implicated in many aspects of cell cycle regulation and tumor suppression, its precise mechanism of action has remained largely unknown. We now show that MST1 promotes accurate kinetochore-microtubule attachment by modulating the kinase activity of Aurora B. HeLa cells depleted of MST1 failed to develop stable end-on kinetochore-microtubule attachment, giving rise to unaligned mitotic chromosomes. The misaligned chromosomes activated the Mad2- and BubR1-dependent spindle checkpoint response, resulting in a delay in anaphase onset. The kinase activity of Aurora B, which promotes destabilization of kinetochore-microtubule attachment, was increased in cells depleted of MST1 or NDR1, a downstream kinase of MST1. Moreover, MST1 associated with and directly phosphorylated Aurora B. Depletion of Aurora B restored the stability of kinetochore-microtubule attachment in cells depleted of MST1 or NDR1. The MST-NDR1 complex is thus required for precise regulation of Aurora B activity and thereby ensures mitotic chromosome congression and accurate chromosome segregation.

AN HDM2 ISOFORM, HDM2C, PROMOTES CELL PROLIFERATION WITHOUT DECREASING P53

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Hdm2 oncoprotein is the key regulator of p53. In cells with Hdm2 over-expression due to a single nucleotide polymorphism (SNP309 T to G) in the *hdm2* P2 promoter, p53 activity is often compromised. Over forty *hdm2* splice variants exist, however, little is known about their functions. In both G/G and T/T cells, we found various splice variants such as P2*hdm2C* which is missing exons 5 through 9 that contain important domains for p53 regulation and cell growth inhibition. Using exon-junction (4-10) specific primers for RT-PCR, we found that P2*hdm2C* was over-expressed in cancer cells with high Hdm2 levels. We developed an antibody to Hdm2C using the amino acid sequence of part of exons 4 and 10. Using this antibody we showed that cells over-expressing Hdm2 had more Hdm2C as compared to low Hdm2 expressing cells. In cells with wild type p53 and low Hdm2 protein levels, the activation of p53 resulted in increased Hdm2C protein; suggesting a role for Hdm2C in normal feedback regulation of p53. In order to compare the influence of Hdm2 and Hdm2C on p53 function, we used isogenic cell lines derived from H1299 cells with inducible p53 and exogenous full length Hdm2 (FL Hdm2) or Hdm2C. We found that in the presence of induced p53, Hdm2C reduced the amount of p21 transcript while FL Hdm2 did not. In addition, Hdm2C expressing cells proliferated faster as compared to parental and FL Hdm2-expressing cells. Interestingly, FL Hdm2-expressing cells grew the slowest, thus correlating our work with that of others who have shown that FL Hdm2 has growth inhibitory effects. FL Hdm2 caused no decrease in p53 DNA binding activity as seen via chromatin immumoprecipitation and gel shift assays. Our results suggest that Hdm2C may be a reason for compromised p53 activity in cells that over-express Hdm2. Experiments are in progress to determine the influence of Hdm2C on p53 transcription, p53-chromatin interactions and co-factor recruitment.

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REGULATION OF THE CELL CYCLE BY A STABLE NUCLEAR-LOCALIZED RETINOBLASTOMA TUMOR SUPPRESSOR COMPLEX

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The retinoblastoma (RB) tumor suppressor is a cell cycle regulator that is thought to repress cell cycle progression when bound to E2F-DP transcription factor heterodimers. Cell cycle regulated phosphorylation of RB by cyclin dependent kinases (CDKs) causes release from E2F-DP, thereby allowing cell cycle activation. However, there is now accumulating data suggesting that RB-E2F-DP ternary complexes may have roles in regulating gene expression in addition to their well established function as a cell cycle repressor.

In the unicellular alga *Chlamydomonas reinhardtii*, RB (encoded by the *MAT3* gene) controls cell division and regulates size homeostasis. As is the case in metazoans, MAT3/RB represses the activity of E2F-DP that are encoded by single copy genes. We have examined the cell cycle dynamics of MAT3/RB, E2F1 and DP1 and found that MAT3/RB is a phosphoprotein; that E2F1-DP1 can bind to a consensus E2F site; and that all three proteins interact in vivo to form a complex that can be quantitatively immunopurified. We examined the abundance of MAT3/RB and E2F1-DP1 in highly synchronous cultures of *Chlamydomonas* and found that they are synthesized and remain stably associated throughout the cell cycle with no detectable fraction of free E2F1-DP1. Consistent with their stable association, MAT3/RB and DP1 are constitutively nuclear and MAT3/RB does not require E2F1-DP1 heterodimers for nuclear localization. Together, our data show that cell cycle regulation by an RB-related protein can occur without dissociation from E2F-DP subunits, and that other changes associated with the complex may be sufficient to convert RB-E2F-DP from a cell cycle repressor to an activator.

INDUCTION OF SPINDLE MULTIPOLARITY VIA CENTROSOME AMPLIFICATION IS A CHEMOTHERAPEUTIC STRATEGY

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Precise regulation of centrosome duplication and inheritance is pivotal to the execution of error-free cell division. Centrosome amplification occurs frequently in most cancer types, and is a cause of chromosomal instability in cancer cells. It also underlies aberrant spindle organization which usually results in a chronic mitotic arrest, a phenotype elicited by most tubulin-binding drugs. The exact nature and mechanism of the mitotic arrest, and factors dictating abnormal spindle morphogenesis however, remain elusive. We show that a novel microtubule-modulating agent, 9-bromonoscapine, induces formation of multipolar spindles containing 'real' centrioles at all the poles, indicating drug-induced centrosome amplification and persistent centrosome declustering. Cell-cycle phase-specific experiments showed that the cytotoxicity window of the drug encompasses the late S-G2 period. Drug treatment excluding the S-phase not only resulted in lower sub-G1 population, but also significantly attenuated centrosome amplification and spindle multipolarity phenotypes, suggesting that drug-induced centrosome amplification is a chemotherapeutic strategy. Subsequent to a robust mitotic arrest, EM011-treated cells displayed diverse cellular fates suggesting a high-degree of intraline-variation. EM011 caused a range of irremediable outcomes including prolonged mitotic arrest, mitotic catastrophe and slippage, unrestrained asymmetric cytoplasmic and nuclear division culminating in apoptosis. Some 'apoptosis-evasive' cells underwent aberrant cytokinesis to generate rampant aneuploidy that perhaps contributed to drug-induced cell death. Our results therefore underscore that spindle multipolarity induction via centrosome amplification is potentially a powerful chemotherapeutic strategy.

IMPAIRED PLK1 LOCALIZATION AND MITOTIC ARREST IN CELLS EXPRESSING NONSELF-PRIMEABLE PBIP1-CDK MUTANT

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Mammalian polo-like kinase 1 (Plk1) has been studied extensively as a critical element in regulating various mitotic events during M-phase progression. Plk1 function is spatially regulated through the targeting activity of the polo-box domain (PBD) that specifically recognizes S-pS/pT phosphoepitopes. The current prevailing model is that the PBD binds to a phospho-epitope generated by Cdk1 or other Pro-directed kinases. In addition to this well-appreciated nonself-priming and binding mechanism, evidence accumulated in recent years suggests the existence of an alternative self-priming and binding mechanism whose PBD-binding site is generated by Plk1 itself. However, whether the latter unusual biochemical mechanism is a physiologically significant process or a passively evolved event as a consequence of the unavailability of nonself-priming kinase at a particular time and subcellular location remains unknown. Here we demonstrated that mutation of a Plk1-dependent self-priming and binding site of PBIP1 to a Cdk-dependent nonself-priming site (PBIP1-cdk) resulted in an early recruitment of Plk1 to the interphase centromeres, but failed to achieve a drastic accumulation of Plk1 at the mitotic kinetochores as observed in the cells expressing wild-type PBIP1. As a consequence, both PBIP1-cdk and its binding protein, Cenp-Q, exhibited a prolonged association with chromatin fraction, thus likely causing chromosome missegregation and mitotic arrest. Taken together, the data provided here suggest that the self-priming and binding of PBIP1 by Plk1 is a unique biochemical event designed to maximize the efficiency of Plk1 recruitment to and delocalization from the kinetochores and to promote proper M-phase progression.

AURORA A DEPLETION DURING ADULTHOOD INHIBITS CELL PROLIFERATION AND REGENERATION BY INDUCING MITOTIC ARREST AND DNA DAMAGE

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Aurora kinase A is a critical mitotic regulator that has been associated with tumor development and progression. Aurora A has become a relevant objective of study in cancer cell biology since it participates in the formation and maturation of the spindle, the main target of microtubule poisons, and it confers resistance to Taxol. Although the cellular effects of Aurora A inhibition have been deeply studied in culture cells and embryos, the consequences of Aurora A depletion have not been explored in adult mammals. Here, we generated and characterized a mouse model in which Aurora A depletion can be achieved in a conditional manner during adulthood. We show that Aurora A ablation is associated with a general lack of proliferation in vivo which induces loss of weight and bone density and a significant alteration of proliferative tissues. Furthermore, Aurora A depleted tissues are characterized by a significant increase in mitotic, apoptotic and DNA damage markers. We also show how the lack of proliferation found in Aurora A depleted tissues has an important impact in the regenerative processes that take place in mouse. Together, our results demonstrated an essential role of Aurora A in vivo for proliferative and regenerative processes and are of special significance for understanding possible side effects of Aurora inhibitors currently in development as anticancer agents.

CIRCADIAN CLOCK REGULATION OF THE CYCLIN-DEPENDENT KINASE INHIBITOR P21 AND ITS ROLE DURING ZEBRAFISH DEVELOPMENT

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The zebrafish circadian system is highly decentralized with the majority of tissues and cells of the body containing independent clocks, which are directly light-responsive. These circadian oscillators regulate the timing of fundamental aspects of cell biology, including the cell cycle. A functional circadian clock is present early in zebrafish development and appears to gate the timing of S phase in developing embryos. However, to date, very little is known about the molecular interactions between the circadian clock and the cell cycle during zebrafish development. To address this issue, we focused on the cyclin-dependent kinase inhibitor p21, a key regulator of the G1/S transition and a known inhibitor of DNA replication. Here, we demonstrate by quantitative PCR that p21 is expressed with a robust circadian rhythm in embryos raised on a light-dark cycle. Importantly, these rhythms peak at a time when DNA synthesis is low. Rhythmic expression of p21 starts early in development at one day post fertilization, persists in constant darkness, and is suppressed by constant light, a condition known to inhibit circadian clock function. These results indicate that p21 is regulated by the circadian clock and provide the first molecular link between the clock and the cell cycle during zebrafish development. By whole-mount *in situ* hybridization, we have localized p21 mRNA primarily to the developing digestive system, where its expression is strongly rhythmic, consistent with our qPCR data. We are currently investigating whether the circadian clock plays a role in the timing of S phase in the developing gut. Moreover, we are exploring the possibility that the circadian clock, through its regulation of p21, might directly influence the timing of gut development and differentiation.

LRR-1 IS A NOVEL REGULATOR OF THE ATL-1/CHK-1 CHECKPOINT PATHWAY DURING C. ELEGANS DEVELOPMENT

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Acquisition of lineage-specific cell cycle duration is fundamental during development. In early C. elegans embryo, the ATL-1/CHK-1 (ataxia telangectasia mutated and rad3 related/Chk1) checkpoint pathway controls the timing of cell division in the P lineage, or future germline, of the animal. However, the mechanisms regulating this pathway are still poorly understood. Here, we present a novel ATL-1 checkpoint pathway regulator, called LRR-1 (Leucine-Rich Repeat 1). We show that LRR-1 is a nuclear substrate-recruitment factor of a cullin 2-RING E3-ligase that promotes cell cycle progression by inactivating the ATL-1/CHK-1 checkpoint pathway in C. elegans. Loss of lrr-1 function causes a G2 cell-cycle arrest in the mitotic region of the germ line resulting in sterility owing to depletion of germ cells. Inactivation of ATR/Chk1 signaling components ATR/ATL-1, TopBP1/MUS-101, claspin/F25H5.5 and Chk1/CHK-1, fully suppresses these phenotypes and restores lrr-1 mutant fertility. Likewise in early embryo loss of lrr-1 function induces Chk1 phosphorylation and a severe cell cycle delay in the pace of P-lineage division causing embryonic lethality. Our findings demonstrate that the CRL2-LRR-1 complex silences the ATL-1/CHK-1 pathway in C. elegans and contributes to the timing of cell division in early embryos

A BIOCHEMICAL APPROACH TO STUDY CDC7 KINASE REGULATION IN THE CELL CYCLE

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In eukaryotic cells, DNA synthesis is initiated at numerous origins of replication, which are licensed by the sequential recruitment of initiation factors including the Mcm2-7 complex. During S-phase of the cell division cycle, initiation of licensed origins of replication requires the action of two S-phase promoting kinases, the cyclin-dependent kinase (CDK) and the Cdc7 kinase. Cdc7 is an essential serine/threonine kinase that is activated by association with its regulatory subunits, Dbf4 or Drf1. Cdc7 is able to phosphorylate several subunits of the chromatin-bound Mcm2-7 complex. Phosphorylation is thought to be required for the activation of Mcm2-7 helicase activity, leading to DNA unwinding and chromatin association of Cdc45 and the DNA polymerase. Cdc7 is also believed to play a role in the response to DNA damage.

Several small chemical molecules have recently been reported to be selective inhibitors of Cdc7/Dbf4 function and to exhibit anti-tumour activity. Pharmacological inhibition of the Cdc7 kinase represents a novel approach to cancer treatment by targeting origin initiation, an earlier stage in the process of DNA replication compared to anti-cancer therapeutics that affect DNA elongation. We present preliminary results from the characterisation of an ATP-competitive inhibitor, PHA-767491, in the cell-free system derived from *Xenopus* eggs. We show that PHA-767491 inhibits replication in a dose-dependent manner, which correlates with the inhibition of hyper-phosphorylation of chromatin-bound Mcm4. Mcm4 hyper-phosphorylation has been shown to be Cdc7-dependent. This hyper-phosphorylation is not affected when extracts were treated with $p27^{Kip1}$ to inhibit CDK activity.

Using cell-free extracts derived from *Xenopus* eggs, we are able to characterise the effects of PHA-767491 on DNA replication at a molecular level. This will allow the inhibitor to be used as a powerful tool for biochemical analyses of Cdc7 regulation during the cell cycle.

STABILIZATION OF M-PHASE KINASES IN G1 BY POSITIVE FEEDBACK-INSENSITIVE CDK1 INDUCES EARLY SPINDLE ASSEMBLY AND INCOHERENT MITOTIC CYCLES

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In frogs, flies, and other multicellular eukaryotes, the somatic cell cycle has an intrinsically slower rate of progression relative to its early embryonic counterpart. Prior work described a requirement for positive feedback in the CDK1-Wee1-Cdc25 module to sustain rapid CDK1-APC oscillations in early embryos. Using a non-phosphorylatable, positive-feedback-insensitive CDK1 (CDK1AF), this feedback loop was then shown to prevent rapid oscillatory behaviors in G1-phase somatic (HeLa) cells. Recent studies have uncovered surprising insights into why this behavior can be generated and the consequences that arise as a result. Live-cell imaging revealed that premature spindle assembly and disassembly corresponds with cellular oscillations of cyclin B1 in these cells, but becomes incoherent with chromatin condensation state. Incompletely condensed chromatin is maintained in a mass that is initially congressed within relatively normal bipolar spindles, but then undergoes decondensation and ejection to the spindle periphery. Cells stained for the nuclear pore complex showed that the large masses of decondensed chromatin appeared to possess a nuclear envelope but no nuclear pores. The chromatin in these pseudo-nuclear structures—as well as chromosome fragments remaining congressed within the spindle—stained positive for phospho-histone H3, indicating that the temporal organization of these mitotic processes had been lost. Staining for Aurora A, Aurora B, and Polo kinase revealed that these incoherent spindle and chromatin dynamics were found to occur in conjunction with the presence of all of these improperly accumulated, but properly localized mitotic kinases. With the occurrence of such premature but semi-functional mitotic structures in interphase cells, we inquired whether these abnormal spindle structures were inducing the metaphase-arrest checkpoint. Surprisingly, cyclin B1-YFP oscillated more rapidly in CDK1AF-expressing daughter cells treated with hesperadin, and in cells also knocked down for Mad2. Positive feedback in CDK1 activation is therefore critical for the absolute destruction of mitotic substrates upon cell division. Without it, improperly stabilized components permit the premature initiation of cycling, though with a loss of coherence between all of the necessarily concurrent mitotic processes.

TEMPORAL INDEPENDENCE IN MITOTIC EVENTS DUE TO FEEDBACK REGULATION

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Temporal precision in mitosis is important in maintaining proper cell cycle progression. However, the duration of cell cycles between mitoses can be highly variable, depending on a variety of environmental and intracellular factors. Here we ask whether and how the events of mitosis are temporally insulated from the variable events that precede mitosis. Cdk1, the kinase which serves as the main trigger of mitosis, is regulated by multiple feedback loops. Our hypothesis is that the double-negative feedback loops (regulated by Wee1 and Myt1) and positive feedback loops (controlled by Cdc25 isoforms) involved in the rapid activation of Cdk1 serve to insulate the timing between mitotic events. To test the idea, we made use of Xenopus egg extracts incubated with different doses of non-degradable cyclin to make mitosis occur either quickly (high cyclin) or slowly (low cyclin), and we monitored the timing of the phosphorylation of early mitotic substrates (Wee1 and/or Cdc25) or a late mitotic substrate (the Cdc27 subunit of the APC). We found that although the absolute timing at which the early mitotic phosphorylations occur can be made to vary over a huge range, the time interval between the early phosphorylations and the late phosphorylations is more robust. Compromising the positive feedback by immunodepleting Wee1 compromises the robustness of this interval; the high-cyclin-treated extracts tended to have shorter intervals, and the low-cyclin-treated extracts tended to have longer intervals. This suggests that the abrupt, all-or-none activation of Cdk1 made possible by the bistable Cdk1/Cdc25/Wee1 system insulates the timing of mitotic events from the timing of the events that lead up to mitosis.

THE MITOTIC EXIT TRANSCRIPTIONAL PROGRAM: EFFECTS AT LOCKED MITOTIC CYCLIN LEVELS.

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Many genes involved in cell cycle processes have altered expression during the cell cycle to impose the order and timing of events. During mitotic exit in budding yeast, two transcription factors, Swi5 and Ace2, transcribe many of the genes required for physically dividing the mother and daughter cells and beginning the next round of cell division. As cells exit mitosis, the cyclin dependent kinase (CDK) that has driven many of the cell cycle processes is inhibited and degraded, allowing many of the kinase substrates to return to their unphosphorylated state. Previously our lab has shown that locking mitotic cyclin levels, by inducing transcription of an undegradable form of the protein, causes dose-dependent delays in different cell cycle events. Interestingly, in these cells where mitotic cyclin levels were sustained, deletion of the transcription factor Swi5 increases the mitotic cyclin inhibition specifically of budding and cytokinesis. The contribution of Ace2 is still being investigated.

Importantly, when phosphorylated by mitotic cyclin/CDK, Swi5 is excluded from the nucleus. Nevertheless in cells with sustained mitotic cyclin, Swi5 still enters. In fact in some cells, Swi5 enters the nucleus several times before advancing in the cell cycle. Given previous studies from our lab showing that the release of Cdc14 phosphatase also oscillates under these conditions, the reentry of Swi5 may support a model that a kinase/phosphatase balance allows cell cycle progression in these cells. All this suggests that Swi5 transcribes genes important for promoting cytokinesis and budding despite high mitotic cyclin levels. Subsequent analysis will be necessary to see how specific targets of the mitotic exit transcriptional program contribute to the mitotic cyclin regulation of cytokinesis and budding.

REGULATION OF GENE EXPRESSION DURING THE CELL CYCLE AND IN RESPONSE TO DNA DAMAGE IN *SCHIZOSACCHAROMYCES POMBE*.

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The yeast *Schizosaccharomyces pombe* is used as a model organism to study the regulation of gene expression, both during the cell cycle and in response to DNA damage.

The transcription factor complex MBF, which is analogous to metazoan E2F, regulates the cell cycle-dependent expression of a group of genes at the transition from G1 to S phase. Many of these genes encode components necessary for DNA replication. In order to ensure genomic integrity, eukaryotic cells respond to exogenous and endogenous DNA damage via checkpoint signalling pathways that are mediated by the conserved kinases ATM and ATR. It has recently become apparent that regulation of cell cycle-dependent gene expression is a vital feature of the checkpoint response in *S. pombe* cells. Stalled replication in *S. pombe* activates the Rad3 kinase (equivalent to ATR), which then upregulates MBF-dependent genes.

However, at present there is still much to learn about the mechanisms underlying gene expression during the cell cycle and in checkpoint responses. We have identified a new regulator of MBF-dependent gene expression, and we are currently investigating the role of this protein both in cell cycle-dependent and checkpoint-mediated gene expression.

MODELING THE START TRANSITION IN BUDDING YEAST – HOW SBF AND MBF TURN ON AND OFF.

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Cln3 plays a significant role in initiating the START transition in budding yeast by activating two transcription factors SBF (a heterodimer of Swi4/Swi6) and MBF (a heterodimer of Mbp1/Swi6). SBF promotes the production of G1 cyclins (Cln1, Cln2) responsible for bud initiation, while MBF promotes the production of S phase cyclins (Clb5, Clb6) needed for DNA synthesis. SBF is turned off by mitotic cyclins (Clb1, Clb2); whereas MBF is turned off by Nrm1. A detailed mechanism of how these events are controlled is still unclear.

Early on, it was thought that Cln3 phosphorylates Whi5, an inhibitor of SBF, and causes Whi5 to be exported from the nucleus, thereby removing its inhibition of SBF. However recent evidence (Wagner *et al.*, PLoS ONE 4: e4300, 2009) showed that this might not be the sole mechanism. They found that Whi5-12A (non-phosphorylable by CDK) has the same size as wild type cells, indicating that Cln3 may activate SBF through an alternative mechanism, probably through phosphorylating Swi6. However, the authors found that Swi6-SA4 (non-phosphorylable by CDK) also has the same size as wild type cells. Only the double mutant 'Whi5A Swi6-SA4' shows a delay in START transition with a size 1.4 times that of the wild type. Hence, SBF activation seems to require phosphorylation of either Whi5 or Swi6.

There are additional problems with the Whi5 nuclear export mechanism. Evidence from Wagner's paper (2009) and Queralt *et al.*, (Mol. Cell. Biol. 23:3132, 2003) suggests that Swi4 and Swi6 are needed for the nuclear export of phosphorylated Whi5. Furthermore it is known that Whi5 export occurs when SBF is activated in late G1, whereas Swi6 export occurs only when SBF is turned off in mid S-phase. To accommodate these findings, we propose that free Swi4/Swi6 dimers (unbound to promoters and not phosphorylated) may shuttle in and out of the nucleus to promote the export of phosphorylated Whi5 when SBF is active. When SBF is turned off, Swi4/Swi6 become phosphorylated, and Swi6-P translocates to the cytoplasm.

We have built a mathematical model of the START transition (SBF and MBF regulation) based on these hypothetical mechanisms. We use the model to explain the sizes of various START mutants and the localization of Whi5, Swi6 and Swi4 at different phases of the cell cycle.

A NOVEL ROLE FOR THE RSC CHROMATIN-REMODELING COMPLEX IN THE ADAPTATION TO THE SPINDLE ASSEMBLY CHECKPOINT BY CONTROLLING ACTIVATION OF THE CDC14 PHOSPHATASE

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The survival of cells requires accurate chromosome segregation and transmission. The spindle assembly checkpoint (SAC) delays anaphase and mitotic exit until all chromosomes are bipolarly attached to the mitotic spindle. The duration of SAC-induced arrest is not indefinite and after a variable time, cells can progress into cell cycle even if errors have not been corrected with a process called “adaptation”. This process is largely responsible for the failure of chemotherapeutic compounds targeting the mitotic spindle to block tumour progression. Through a genetic screen designed to find adaptation-defective mutants in budding yeast, we implicated the RSC chromatin remodeling complex, and in particular its form bound to the Rsc2 accessory subunit, in this process. We found that RSC-Rsc2 inactivation prolongs the SAC activation's time and suppresses the ability of SAC mutants to exit mitosis upon spindle disruption, protecting cyclinB from degradation. Since we found that in yeast adaptation to the SAC requires the factors that are normally involved in mitotic exit, such as the Cdc14 phosphatase, we investigated a possible role of RSC-Rsc2 in Cdc14 regulation. Cdc14 is kept inactive in the nucleolus throughout most of the cell cycle, and released from the nucleolus by two pathways: the FEAR (fourteen early anaphase release) that promotes a partial Cdc14 release in early anaphase, and the MEN (mitotic exit network) that drives its complete release in telophase. We found that RSC2 deletion abolishes Cdc14 release in SAC-defective mutants treated with nocodazole and is either lethal or decreases the maximal permissive temperature of MEN-defective mutants, similarly to FEAR mutations. These data suggest that RSC-Rsc2 controls Cdc14 release acting together with the FEAR or through a parallel pathway. Strikingly, Rsc2 physically interacts with the polo kinase Cdc5, which has been extensively implicated in both the FEAR and MEN pathways. Thus, RSC-Rsc2 might promote adaptation to the SAC by stimulating Cdc14 release through regulation of the polo kinase.

A COMMON TELOMERIC GENE SILENCING ASSAY IS AFFECTED BY NUCLEOTIDE METABOLISM AND THE DNA DAMAGE RESPONSE

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In budding yeast, a reversible form of heterochromatin at telomeres, the telomere position effect (TPE), was discovered when auxotrophic markers were placed near chromosome ends. Selection for or against these markers demonstrated roles for several proteins in telomeric heterochromatin formation; these include the SIR protein complex, chromatin assembly factors (CAF-I, Asf1), DNA replication and DNA damage checkpoint proteins. One widely used assay to identify mutants with improper heterochromatin employs the *URA3* gene at the telomere of chromosome VIIL; 5-Fluoroorotic Acid (5-FOA) containing medium requires cells to completely silence *URA3* expression in order to survive.
A component of the core DNA replication machinery that is central to chromatin formation is PCNA (*POL30*), tethering DNA polymerases to leading and lagging strands during DNA replication. To understand the role of PCNA in telomeric silencing, we analyzed the phenotype of the silencing defective *pol30-8* (RD61,63AA) mutant using various TPE reporter strains. We found that while this mutant exhibits a heterochromatin defect, it is very subtle in comparison to a *sir3Δ* mutant. Through a genetic screen we identified new candidates involved in repression of *URA3*-VIIL in *pol30-8* mutant cells, amongst them thymidylate synthase (*CDC21*). *CDC21* also suppresses the telomeric silencing defect of a strain deleted for *DOT1*, encoding the only histone H3K79 methyltransferase, but it does not suppress the 5-FOA sensitivity of a strain carrying endogenous *URA3*. Gene expression arrays for the *pol30-8* mutant showed an upregulation of the ribonucleotide reductase (RNR) genes *RNR2*, *RNR3* and *RNR4*. In agreement, we found less of the transcriptional co-repressor Ssn6 bound to the *RNR2* promoter in *pol30-8* cells by chromatin immunoprecipitation. Inhibition of RNR activity as well as mutations in the *RAD53* DNA damage response pathway rescued the sensitivity of *pol30-8 URA3*-VIIL cells to 5-FOA. These data raise the interesting possibility that in the context of low *URA3* expression such as in a *URA3*-VIIL strain increased RNR activity is responsible for a higher conversion rate of 5-FOA into its toxic metabolites which accounts for the majority of the 5-FOA sensitivity seen in *pol30-8 URA3*-VIIL mutants. The implications for the role of DNA damage checkpoint proteins in telomere repression will be discussed.

DAMAGED DNA BINDING PROTEIN-2 PLAYS CRITICAL ROLES IN APOPTOSIS AND PREMATURE SENESCENCE FOLLOWING DNA DAMAGE

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Damaged DNA binding protein-2 (DDB2) is encoded by the nucleotide excision repair gene XP-E. In human cells, expression of DDB2 is induced by p53 following DNA damage. We discovered two new functions of DDB2: (1) Ubiquitination and proteolysis of p21 and (2) transcriptional repression of the antioxidant genes. The findings are somewhat similar to the E3-ligase adapter Skp2, which functions in ubiquitination as well as in regulation of transcription. More importantly, these functions of DDB2 are critical in deciding cell fate following DNA damage.
We observed that the DDB2-deficient cells fail to undergo apoptosis following DNA damage. The lack of apoptosis resulted from high-level accumulation of p21. We observed evidence that DDB2 induces proteolysis of p21 through the ubiquitin-proteasome pathway, which is critical for cells to undergo apoptosis after DNA damage. Deletion of p21 restores apoptosis in the DDB2-deficient cells. Because of the high-level accumulation of p21 in the DDB2-deficient cells, we expected these cells to undergo premature senescence after DNA damage with greater efficiency. Surprisingly, we observed that the DDB2-deficient cells fail to undergo premature senescence induced by DNA damage. Moreover, the cells are refractory to premature senescence induced by culture shock, exogenous oxidative stress and oncogenic stress. We found that these cells did not accumulate reactive oxygen species (ROS) following DNA damage. The lack of ROS accumulation in DDB2-deficiency results from high-level expression of the antioxidant genes, in vitro and in vivo. DDB2 represses antioxidant genes by recruiting Cul4A and Suv39h, and by increasing histone-H3K9 tri-methylation. Moreover, expression of DDB2 also is induced by ROS. Therefore, upon oxidative stress, DDB2 functions in a positive feed back loop, by repressing the antioxidant genes, to cause persistent accumulation of ROS and induce premature senescence.

KINASE AND PHOSPHATASE COMPETE FOR RETINOBLASTOMA PROTEIN BINDING TO REGULATE ITS ACTIVITY

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A balance of opposing kinase and phosphatase activities regulates the function of many cell cycle proteins, however the mechanisms through which an appropriate balance is achieved remain relatively uncharacterized. Here we describe how competitive binding of Cyclin dependent kinases and protein phosphatase 1 (PP1) to the retinoblastoma tumor suppressor protein controls Rb phosphorylation state and activity in suppressing cell growth. A crystal structure identifies an enzyme-docking site in the Rb C-terminal domain that is required for efficient PP1 activity towards Rb. The phosphatase-docking site overlaps with the known Cyclin dependent kinase-docking site, and we demonstrate that each enzyme can directly inhibit the other by sequestering Rb substrate. PP1 competition with Cdk-Cyclins for Rb binding is sufficient to maintain hypophosphorylated Rb and block cell cycle advancement. These results provide the first detailed molecular insights into Rb activation and establish a novel mechanism for regulation in which kinase and phosphatase compete for substrate docking.

THE ABUNDANCE OF SIR4 REGULATES THE CELL CYCLE-DEPENDENT ESTABLISHMENT OF NEW SITES OF HETEROCHROMATIN IN BUDDING YEAST

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Heterochromatin in the budding yeast Saccharomyces cerevisiae is composed of polymers of the SIR (Silent Information Regulator) complex bound to nucleosomal DNA. Assembly of heterochromatin requires all three proteins of the SIR complex: the histone deacetylase, Sir2 and the histone binding proteins Sir3 and Sir4. Changes in the locations and boundaries of heterochromatin are critical during development of multicellular organisms, and de novo establishment of heterochromatin in yeast is the best understood example of how these dynamic changes occur in the context of cell cycle progression.

De novo assembly of heterochromatin in budding yeast requires between one and five cell divisions, and at least one cycle of DNA replication. Previous studies have shown that this assembly is not regulated by active DNA replication but by another event that occurs in the S-phase of the cell cycle. Recent work has suggested that the cell cycle inhibition of chromatin modifying enzymes that block heterochromatin formation may be the mechanism for how cells limit when they can assemble new sites of heterochromatin. Halving the levels of Sir4 in cells causes a dramatic loss of silencing, and these data have led us to test an alternative hypothesis: the abundance of Sir4 protein regulates the assembly of new regions of heterochromatin, and its regulated destruction may prevent this assembly during the G1 phase of the cell cycle.

We are currently examining *de novo* assembly of heterochromatin using an assay for establishment of a site of heterochromatin in individual cells. Introduction of additional copies of Sir4 on low copy plasmids leads to a marked increase in the number of cells that are able to establish heterochromatin after only one cellular division. Addition of Sir4 at high copy number leads to ablation of this effect, while overexpression of Sir4 prevents establishment of heterochromatin.

Sir4 abundance in G1 may be regulated by the anaphase-promoting complex (APC), an E3 ubiquitin ligase. Mutation of potential APC recognition sites (destruction boxes) in Sir4 lead to an increase in silencing and we are currently testing if these mutants will allow more rapid de novo assembly of heterochromatin. The APC is shut off at the start of S-phase, and its regulation of Sir4 abundance may provide an additional explanation for why cell cycle establishment of heterochromatin occurs only after the initiation of S-phase.

A REGULATORY NETWORK CONTROLLING CELL DIVISION DURING *C. ELEGANS* DEVELOPMENT

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During the development of the model organism, *C. elegans*, the cell cycle is tightly controlled such that the location and timing of each division is essentially invariant. We use the development of the vulva as a model to reveal the general mechanisms used to achieve such a level of spatiotemporal control over the cell cycle. In particular, we examine the vulva precursor cells (VPCs) that are directed to undergo cell-cycle quiescence soon after their generation.

We performed genome-wide RNAi and forward genetic screens which collectively identified over one hundred genes that are necessary for the cell-cycle quiescence of the VPCs. Each of the identified genes is an important component of the developmental network regulating the VPC cell cycle, since the loss of activity of any single gene disrupts the temporary cell-cycle arrest and results in extra VPC divisions. Thus, we have identified the core components of a developmental pathway that promotes cell-cycle quiescence. Our ultimate aim is to generate a comprehensive description of the regulatory interactions that determine the selection between division and quiescence. The organization of this hierarchy is being revealed using a combination of genetic and biochemical approaches.

To establish a foundation from which to extend our analyses, we focus initially on the regulation of *cdc-14*. The *C. elegans cdc-14* locus encodes the singular Cdc14-like phosphatase. Unlike its fungal counterparts, *cdc-14* is not required for mitosis and, in fact, it is not intrinsically necessary for cell-cycle progression. Rather, *cdc-14* displays a role that is conserved with mammalian Cdc14B in the negative regulation of G1 progression. Our analyses have determined that a collaboration between alternative splice form usage and Crm1-mediated nucleocytoplasmic shuttling of CDC-14 protein restricts *cdc-14* activity to guide cell-cycle quiescence during development. Currently, experiments are ongoing to integrate the remaining, newly identified genes into a rational interpretation of this cell cycle regulatory network.

COOPERATIVE ACTION OF MAP4- AND KINETOCHORE-BASED MECHANISMS ARE ESSENTIAL FOR SPINDLE BIPOLARITY

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An important challenge in cell biology is to understand how the combination of molecular motors and microtubule-associated proteins (MAPs) contribute to bipolar spindle assembly and cooperate to govern its steady-state architecture. Although much is known about molecular motors, such as Kinesin-5 or Kinesin-14, little information is available about the role of non-motor MAPs, like PRC1 or MAP4. Here we show, using siRNA-mediated protein depletion, that MAP4, the major structural MAP in non-neuronal cells, is essential for building a mitotic spindle with normal morphology. In MAP4-depleted cells, around half of spindles lose their normal elliptical shape, appear bent or diamond-shaped and undergo a series of rotational movements in the z-axis. We also show that the timing of chromosome alignment is delayed, with 1-2 chromosomes failing to reach the metaphase plate, resulting in a mitotic arrest. Why does loss of MAP4 result in such phenotypes? We reveal that depletion of MAP4 leads to a decrease in the rate of kinetochore-MT (KT-MT) plus-end turnover, thus explaining the failure in chromosome congression. To confirm that MAP4 affects MT dynamics, we directly measured the dynamic properties of individual MTs in living cells using the plus-end marker EB3-tdTomato. So far these experiments have revealed an increase in the growth rate of astral-MTs, thereby confirming that MAP4 regulates MT dynamics and explaining the spindle orientation defects. To test whether MAP4 also affects non-KT MTs in the spindle, we removed KT-MTs by depleting the KT protein Nuf2R. In Nuf2R/MAP4 co-depletion, we observed an additive effect in which spindles completely lost their normal shape and began rotating in both z- and x-y axes. Surprisingly, new spindle poles also formed de novo within the existing mitotic spindle. We propose that MAP4 sets the base-rate of MT dynamics throughout the mitotic spindle, probably as a result of physical interactions with the MT lattice, and cooperates with kinetochore-mediated mechanisms to maintain spindle architecture and orientation.

CHARACTERIZING THE ROLE OF MYOSIN-10 IN REGULATING MITOTIC SPINDLE STRUCTURE AND FUNCTION

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The unconventional myosin, myosin-10, contributes to the structural integrity and/or function of various cellular structures including filopodia and meiotic and mitotic spindles. Like all myosins, myosin-10 is comprised of an amino-terminal head domain that binds actin and hydrolyzes ATP and a carboxy-terminal tail domain. The tail of myosin-10 harbors multiple functional domains, which facilitate interactions with various macromolecules including microtubules, phospholipids and integral membrane proteins. Previous imaging studies from our lab in the fully intact epithelium of the *Xenopus laevis* embryo revealed that myosin-10 localizes to the mitotic spindle and depletion of myosin-10 protein results in several mitotic spindle-related phenotypes including spindle elongation, failed anchoring, mitotic delay and pole fragmentation. However, the mechanisms by which myosin-10 contributes to proper spindle structure and function are poorly understood. For instance, while myosin-10 distributes throughout the entire mitotic spindle it appears to concentrate near the spindle poles. How this restricted localization is achieved and whether or not it is important for mitotic functions of myosin-10 is not known. In the current research we have characterized the distribution of GFP-tagged myosin-10 as well as several GFP-tagged myosin-10 fragments in the embryo epithelium via 4-D microscopy (4-D = x, y, z and time). Our data demonstrate that different fragments of myosin-10 exhibit unique distributions with respect to the mitotic spindle and cell cycle stage, indicating that various interaction domains within the myosin-10 protein regulate different aspects of its function. Accordingly, in order to identify novel myosin-10 interacting proteins that may contribute to its mitotic spindle-related localization and/or functions, a yeast 2-hybrid screen was performed with a fragment of the myosin-10 tail. Interestingly, the mitotic kinase Wee1 scored the most positive hits in this screen, and here we show independent verification of a direct interaction between myosin-10 and Wee1 proteins. Furthermore, we provide evidence for a genetic interaction between myosin-10 and Wee1 with respect to some of the spindle phenotypes. These data lead us to hypothesize that myosin-10, with its ability to interact with Wee1, a mitotic regulatory kinase, and cytoskeletal components such as filamentous actin and microtubules, functions as an important link between cell cycle regulatory systems and physical regulation of the mitotic spindle.

CUX1 CAUSES CHROMOSOMAL INSTABILITY BY ACTIVATING A GENE EXPRESSION SIGNATURE PREDICTIVE OF OUTCOME IN BREAST CANCER

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Cell populations able to generate a large repertoire of genetic variants have increased potential to generate tumor cells that can survive through the multiple selection steps involved in tumor progression. A proposed mechanism for the generation of aneuploid cancer cells involves the formation of tetraploid cells that gradually lose chromosomes. A major obstacle in this scenario, however, is that induction of tetraploidy in normal cells causes cell cycle arrest or cell death.

We show that the p110 CUX1 homeodomain transcription factor contributes to the establishment of a transcriptional program that allows tetraploid cells to sustain a robust mitotic checkpoint thereby favoring multi-centrosomes clustering and bipolar division. CUX1 enables the survival of tetraploid cells in tissue culture, but frequent defective chromosome attachments leads to the emergence of chromosomally unstable aneuploid cells that evolve to become tumorigenic. A similar passage through a tetraploid state is also apparent in a transgenic mouse model. The fact that over 80% of mammary tumor cells in MMTV-CUX1 transgenic mice display a sub-tetraploid chromosome content suggests that CUX1 induces tumor development by enabling tetraploid cells to undergo bipolar mitosis and generate aneuploid genetic variants.

Transcriptional targets of CUX1 involved in replication and bipolar mitosis defines a gene expression signature that, across 12 breast cancer gene expression datasets, is associated with poor clinical outcome. Not only is high expression of these genes found more frequently in breast tumor subtypes that exhibit a poor prognosis, like the basal-like and HER2+, but it also identifies poor outcome among estrogen receptor positive/node negative patients, a sub-group considered to be at lower risk.

The CUX1 signature therefore represents a novel criterion to stratify patients and provides insight into the molecular determinants of poor clinical outcome.

INSIGHTS INTO THE ROLE OF AURORA B AND MPS1 IN THE SPINDLE ASSEMBLY CHECKPOINT

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The Spindle Assembly Checkpoint (SAC) is a safety device that monitors microtubule-kinetochore attachment and delays anaphase onset until all sister chromatids have achieved bipolar attachment to the mitotic spindle. Protein phosphorylation by mitotic kinases is crucial for the SAC. Among the core components of the SAC, several proteins, including Aurora B, Bub1, BubR1, and Mps1, have a kinase domain. Here, we report the characterization of two checkpoint kinases, Aurora B and Mps1, using cell-permeable small molecules selectively targeting their kinase activities. Firstly, we describe the characterization of a new Mps1 inhibitor named Reversine as an inhibitor of the SAC. Importantly, Reversine treatment led to undetectable levels of Zwilch and Mad1 at unattached kinetochores, which is consistent with the powerful SAC override observed in the presence of this inhibitor. Intriguingly, we found that after a recovery from a STLC block (that leads to a monopolar spindle whit several synthelic KT-MT attachments), Reversine-treated cells show a large number of mis-aligned chromosomes, indicating that besides its role in the SAC, Mps1 also plays a role in the correction of improper kinetochore-microtubule attachments. Aurora B was also implicated in error correction whereas its role in the SAC is controversial. Using the Aurora B inhibitor Hesperadin, we demonstrate that Aurora B, like Mps1, is strictly required for the SAC. While partial inhibition of Aurora B is still compatible with the checkpoint response to unattached kinetochores, complete ablation of its kinase activity leads to severe impairment of localization of SAC proteins at unattached kinetochores. Our findings suggest that a core component of the SAC, including the Aurora B and Mps1 kinases, responds to unattached as well as tensionless kinetochores. Thus, error correction is intimately intertwined with the sac, establishing a new paradigm in the sac.

CDK1-CYCLINB1 SPATIAL POSITIVE FEEDBACK REGULATION AT THE ONSET OF MITOSIS

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Mitosis is unquestionably one of the most spectacular events in cell biology, as it brings unparalleled morphological changes in the cell. At the heart of most of these changes are the mitotic cyclin dependent kinase, Cdk1, and its regulatory subunit cyclin B1. The Cdk1:CyclinB1 complex in embedded in two interlinked positive feedback regulatory loops generating a bistable switch, which allows the activity of the complex to change abruptly as a stimulus (like the concentration of cyclin) is continuously varied. In addition, the spatial distribution of the Cdk1Cyclin B1 complex is also dynamically regulated. During interphase, Cdk1:CyclinB1 shuttles between nucleus and cytoplasm with export rates much faster then import rates. At the onset of mitosis, however Cdk1:CyclinB1 translocate from the cytoplasm to the nucleus in a rapid, abrupt manner. The switch-like nature of this import led us to hypothesize that spatial regulation of the Cdk1:CyclinB1 complex may be coupled to the control of its activation. In other words, is the switch-like nature of the Cdk1:CyclinB1 nuclear import an in-build spatial component that confers robustness to Cdk1:CyclinB1 bistable switch? We approached this question by combining mathematical modeling with quantitative single cell imaging studies in HeLa cells and discovered that a spatial-positive feedback triggers the translocation of active Cdk1:CyclinB1 from the cytoplasm to the nucleus, where a supra-threshold increment of localized nuclear Cdk1:CyclinB1 results in more Cdk1:CyclinB1 import. Importantly, when feedback regulation is compromised, Cdk1:CyclinB1 nuclear import is delayed and becomes more graded. In addition, the normal interval of timing of mitotic events is disrupted rendering completion of mitosis a more variable, sluggish process. Together, our studies strongly suggest that coupling of spatial positive feedback regulation to activation control in the cascades that govern mitosis promotes robust unidirectionality, insulation and synchronicity of cell division.

MOLECULAR BASIS FOR LYSINE SPECIFICITY IN THE UBIQUITIN-CONJUGATING ENZYME CDC34

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Protein ubiquitination of cell cycle regulators plays a critical role in cell cycle progression. The SCF and APC multiprotein ubiquitin ligases (E3s), with their cognate ubiquitin-conjugating enzymes (E2s) are required for G1-S phase and mitotic cell cycle progression. Ubiquitin-conjugating enzymes and ubiquitin ligases catalyze the attachment of ubiquitin (Ub) to lysine residues in substrates and Ub during monoubiquitination and polyubiquitination. Lysine selection is important for generating diverse substrate-Ub structures, which provides versatility to this pathway in targeting proteins to different fates. The mechanisms of lysine selection remain poorly understood, with previous studies suggesting that ubiquitination site/s are selected by E2/E3-mediated positioning of lysine/s toward the E2/E3 active site. By studying polyubiquitination of the yeast Cdk inhibitor and regulator of G1-S phase progression, Sic1, by the E2, Cdc34, and the RING E3, Skp1/Cul1/F box protein (SCF), we now demonstrate that in addition to E2/E3-mediated positioning, proximal amino acids surrounding the lysine residues in Sic1 and Ub are critical for ubiquitination. This mechanism is linked to key residues composing the catalytic core of Cdc34 and independent of SCF. Changes to these core residues altered the lysine preference of Cdc34 and specified if this enzyme monoubiquitinated or polyubiquitinated Sic1. These new findings indicate that compatibility between amino acids surrounding acceptor lysine residues and key amino acids in the catalytic core of ubiquitin-conjugating enzymes is an important mechanism for lysine selection during ubiquitination.

KSP/EG5 FACILITATES ELONGATION OF CHROMATIN-DERIVED MICROTUBULES DURING MITOTIC SPINDLE FORMATION.

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During cell division, mitotic spindle formation is essential for proper segregation of chromosomes. Mitotic kinesin KSP (human Eg5) plays a critical role in centrosome separation before nuclear membrane breakdown, and is required for bipolar organization of spindle. KSP is localized along onto k-fiber microtubules of metaphase bipolar spindle, but the function of KSP on k-fibers remains to be elucidated.
To make a better understanding of KSP function on spindle formation after nuclear membrane breakdown, we utilized mitotic inhibitors on HeLa cells; a microtubule destabilizing agent and KSP inhibitors. In mitotic cells treated with nocodazole, KSP seemed to translocate from the peri-centrosome area to the chromosome area to form foci surrounded with kinetochores just after nuclear membrane breakdown. In the fraction of immuno-purified KSP from such nocodazole-treated cells, several factors related to chromosome-derived microtubule nucleation and elongation, including TPX2, Aurora A, RHAMM and γ-tubulin, were also identified by MALDI-TOF MS and werstern blotting assay. Corresponding to the biochemical result, co-localization of KSP with TPX2 and RHAMM was observed on the chromosome area in nocodazole-treated cells. Simultaneous treatment with nocodazole and an STLC-derived KSP inhibitor altered the KSP localization to whole cell area diffuse distribution to result in dissolution of the co-localization with TPX2 and RHAMM. Removal of nocodazole from such double-treated cells could promote microtubule elongation preferentially from kinetochores, but the microtubule fibers associated with TPX2 seemed to be thinner and shorter compared with those released from nocodazole in the cell pretreated with only nocodazole. These results suggest that, in normal mitotic progression, a portion of KSP localizes on kinetochores with TPX2 and RHAMM after nuclear membrane breakdown to play a role in proper elongation of chromatin-derived microtubules through its activity of microtubule bundling.

ANALYSIS OF RECOMBINANT APC/C BY ELECTRON MICROSCOPY AND MASS SPECTROMETRY DEFINES APC/C SUBUNIT STOICHIOMETRY AND THE POSITION OF INDIVIDUAL SUBUNITS WITHIN THE APC/C MOLECULAR ENVELOPE

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The anaphase promoting complex (APC/C) is a multisubunit E3 ubiquitin ligase governing the metaphase to anaphase transition and mitotic exit. Knowledge of its structure is essential for understanding its mode of action and regulation.
So far electron microscopy (EM) studies have provided structural insights into the overall shape and dimensions of the APC/C, and characterised functionally important states of the APC/C bound to co-activators (Cdc20 and Cdh1) and the mitotic checkpoint complex. Although antibody labeling experiments could localise the approximate positions of APC/C subunit epitopes, three dimensional mapping of individual APC/C subunits has not previously been reported.
To map the position and extent of APC/C subunits within the overall molecular envelop of the APC/C we developed a recombinant expression and purification method for *S. cerevisiae* APC/C. We demonstrated its correct assembly by showing its specific ubiquitylation activity dependent on Cdh1 and functional D-box and KEN-box motifs within substrates. Furthermore, we compared the structures of recombinant and endogenous APC/C by using single particle EM analysis. Additionally, we have analysed various subcomplexes by EM and mass spectrometry. These studies provide insights into APC/C stoichiometry as well as its three dimensional subunit architecture at around 20Å. The established subunit boundaries enable unambiguous docking of available X-ray structures and homology models.

THE CDK5 KINASE REGULATES THE STAT3 TRANSCRIPTION FACTOR TO PREVENT DNA DAMAGE UPON TOPOISOMERASE I INHIBITION

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The STAT3 transcription factors are cytoplasmic proteins that induce gene activation in response to growth factor stimulation. Following tyrosine phosphorylation, STAT3 proteins dimerize, translocate to the nucleus, and activate specific target genes involved in cell-cycle progression. Despite its importance in cancer cells, the molecular mechanisms by which this protein is regulated in response to DNA damage remain to be characterized.
In this study, we show that STAT3 is activated in response to topoisomerase I inhibition. Following treatment, STAT3 is phosphorylated on its C-terminal serine 727 residue but not on its tyrosine 705 site. We also show that topoisomerase I inhibition induced the upregulation of the cdk5 kinase, a protein initially described in neuronal stress responses. In coimmunoprecipi-tations, cdk5 was found to associate with STAT3 and pull down experiments indicated that it associates with the C-terminal activation domain of STAT3 upon DNA damage. Using RNA interference and chromatin immunoprecipitation assays, we found that cdk5 phosphorylates the transcription factor on its serine 727 residue and that this is necessary to downregulate the cyclin D1 and Myc genes. Importantly, the cdk5-STAT3 pathway reduced DNA damage in response to topoisomerase I inhibition through the upregulation of Eme1, an endonuclease involved in DNA repair.
We therefore propose that the cdk5-STAT3 oncogenic pathway plays an essential role in the expression of DNA repair genes and that these proteins could be used as predictive markers of tumors that will fail to respond to chemotherapy

HIF1A−DEPENDENT ARREST OF ACINAR MORPHOGENESIS INDUCED BY DYSREGULATED CYCLIN E EXPRESSION IN MAMMARY EPITHELIAL CELLS

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Cyclin E is often over-expressed in breast cancers and is an adverse prognostic indicator. Cyclin E expression is regulated by the ubiquitin proteasome system, whereby degradation of active cyclin E occurs via the SCF^{Fbw7} pathway. We have utilized organotypic, three-dimensional (3D) cultures of MCF10a human mammary epithelial cells (HMECs) on a reconstituted basement membrane, which yields acini-like spheroids that recapitulate several aspects of glandular architecture in vivo. Unexpectedly, we found that overexpression of hyper-stable cyclin E mutant (T->A substitution at position 380 (T380A)), which is resistant to Fbw7-mediated degradation, leads to reduced cell proliferation and impaired acinar morphogenesis of HMECs, but only when cultured in 3D conditions. The arrested morphogenesis was associated with induction of $p21^{Cip1}$, $p27^{Kip1}$, and markers of cellular senescence. We investigated the mechanism of cyclin E- mediated growth arrest and found that the expression of hypoxia inducible factor (HIF1α) was increased in arrested acini. Small hairpin RNA-mediated knockdown of HIF1α resulted in reversal of growth arrest and normalization of p21 and p27 levels and acinar morphogenesis. HIF1α is one subunit of the heterodimeric transcription factor HIF1, which is a major regulator of cellular responses to decreased O2 concentration. Under normoxic conditions, hydroxylation of key proline residues of HIF1α by PHD proteins results in VHL binding and degradation. We found that dysregulated cyclin E causes reduced expression of PHD proteins in HMECs grown in 3D, resulting in stabilization of HIF1α. Also, high cyclin E expression, in 3D cultures and in mammary cells in vivo, caused increased expression of HIF1α. Our data indicate that HIF1α opposes proliferation induced by high cyclin E in mammary epithelial cells and suggest that HIF1α may be a barrier against tumorigenesis associated with high cyclin E and Fbw7 mutations.

EFFECT OF MELATONIN ON CANNABINOID 1 RECEPTOR (CB1R) IN RAT MODEL OF CORTICAL DYSPLASIA

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The effect of cannabinoid on the cell cycle and cell fate is little known. Cortical dysplasia is a malformation resulted from altered location of neurons in cortical layers during brain development. Recent studies have shown that the cyclin-dependent kinase (cdk) inhibitors play important roles in cell cycle progression in normal cells. Alterations in the cdk inhibitors may be important in cortical dysplasia development. Cannabinoid receptors (CBR) and melatonin have cellular protective effects in development. Thus, in this study, we evaluated alterations of cannabinoid receptor-1 protein expression and possible associations between melatonin and cannabinoid-1 receptor (CB1R) expression alterations in experimental cortical dysplasia model. Wistar female rats mated after vaginal smear controls and experimental cortical dysplasia model was generated by using administration of DNA alkilating agent (carmustin) during pregnancy. Five different experimental groups were conducted, including; control, ethanol, melatonin, carmustin, carmustin plus melatonin administered rats. Newborn rat brain tissue sections were evaluated under light microscopy after staining with hematoxylin-eosin, luxol-fast blue and immunohistochemistry. Comparisons of CB1R staining levels between five experimental groups have revealed that CB1R expression levels in brain tissues were reduced in carmustin and carmustin plus melatonin groups compared to remaining groups. Also, CB1R staining level was reduced in carmustin group compared to carmustin plus melatonin group. In conclusion; our preliminary dates give clues about the association between CB1R expression and melatonin administration should reveal formation mechanisms of cortical dysplasia and to detect new treatment approaches on cortical dysplasia.

THE DBF4-CDC7 KINASE PROMOTES S PHASE BY ALLEVIATING AN INHIBITORY ACTIVITY IN MCM4

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Eukaryotic DNA replication uses kinase regulatory pathways to facilitate coordination with other processes during cell division cycles and response to environmental cues. At least two cell cycle-regulated protein kinases, the S phase-specific cyclin-dependent protein kinases (S-CDKs) and the Dbf4-Cdc7 kinase (DDK, the Dbf4-dependent protein kinase) are essential activators for initiation of DNA replication. While the essential mechanism of CDK activation of DNA replication in Saccharomyces cerevisiae has been established, exactly how DDK acts has been unclear.

We have shown that the N-terminal serine/threonine-rich domain (NSD) of Mcm4 plays both inhibitory and facilitating roles in DNA replication control and that the sole essential function of DDK is to relieve an inhibitory activity residing within the NSD. By combining an mcm4 mutant lacking the inhibitory activity with mutations that bypass the requirement for CDKs for initiation of DNA replication, we show that DNA synthesis can occur in G1 phase when CDKs and DDK are limited. However, DDK is still required for efficient S phase progression. In the absence of DDK, CDK phosphorylation at the distal part of the Mcm4 NSD becomes crucial. Moreover, DDK-null cells fail to activate the intra-S-phase checkpoint in the presence of hydroxyurea-induced DNA damage and are unable to survive this challenge. Our studies have established that the NSD of Mcm4 has evolved to integrate multiple protein kinase regulatory signals for progression through S phase.

CYCLIN-DEPENDENT KINASES CONTRIBUTE TO ROBUST OSCILLATIONS DRIVEN BY A TRANSCRIPTION FACTOR NETWORK OSCILLATOR

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During the cell cycle in *Saccharomyces cerevisiae*, approximately 20% of the genome is transcribed periodically. Regulation of this periodic gene expression program is likely important for the timing of cell-cycle events. Although it is widely accepted that cyclin/CDK activity controls periodic gene expression, we have recently shown that a majority of the periodic gene expression program is maintained in yeast cells lacking both S-phase and mitotic cyclins. We proposed that a transcription factor network with the ability to oscillate maintains periodic gene expression in these mutants. This conflict raises the question: What is the role of CDKs in cell-cycle oscillations? To address this question, we examined global gene expression over time under conditions in which yeast cells lack all cyclin/CDK activity. We find that a significant number of genes are still periodically transcribed in the absence of all CDK activity, suggesting that a transcription factor network could maintain these oscillations independent of all CDK activity. However, with decreasing levels of CDK activity, oscillations appear to be less synchronous within the population and the average period length is extended. CDKs are known to interact with the transcription factor network at several points, providing both positive and negative feedback. Our data suggest that this feedback from CDK activity is important for robust oscillations in wild-type cells and that removal of this feedback may lead to damped oscillations. Together our data support a model in which a transcription factor network forms the core cell-cycle oscillator; however, the behavior of this oscillator is modulated by CDK activities.

ZOLEDRONIC ACID TARGETS A NUCLEAR NFATc2 PROTEIN STABILIZATION PATHWAY TO SUPPRESS CANCER GROWTH

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Zoledronic acid, a widely used aminobisphosphonate, has elicited significant attention due to its potential antitumoral activity, however, its underlying mechanism of action remains unclear. Here, we analyze the anti-tumor effects of zoledronic acid using both, in vitro and in vivo approaches to define the existence of a novel nuclear mechanism for promoting cancer growth which involves stabilization of the pro-proliferative transcription factor NFATc2 by GSK-3β-mediated phosphorylation of the serine rich N-terminal SP2 motif. Treatment with zoledronic acid, however, inhibits GSK3β kinase activity, thus disrupts NFATc2 phosphorylation and stabilization in the nucleus, and finally allows the 26S proteasomal machinery to target NFATc2 for degradation. Mechanistically, HDM2, the human homologue of the E3-ligase MDM2, accumulates in the nucleus upon treatment binds to NFATc2 and transfers ubiquitin to lysines K-684 and K-897. Ubiquitination of K-684 and K-897 requires an unphosphorylated status of NFATc2 in the nucleus and is key for the subsequent recognition and degradation by the 26S proteasome. The net cellular outcome of GSK3β -NFATc2 pathway disruption and degradation of the transcription factor is a progredient halt of cancer cells at the G1 cell cycle phase. Collectively, these results significantly expand our basic mechanistic understanding of a key oncogenic pathway (GSK-3β-NFATc2-HDM-2) and suggest that double-targeting of this pathway accounts for the potent and sustained antitumoral effects of zoledronic acid.

PI3K ACTIVATES P27-DEPENDENT TUMOR CELL MIGRATION AND METASTASIS: MECHANISMS BEYOND RHOA

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p27 plays an important role in regulating the G1-S phase transition. p27 also appears to promote tumor cell motility and invasiveness. Here we provide additional evidence for p27 as a mediator of tumor metastasis. The phospho-inositol 3' kinase (PI3K) is frequently activated in human cancers. Downstream of PI3K, AGC kinases catalyze p27 phosphorylation at T157 and T198 to stabilize p27 and impair its nuclear import, causing its accumulation in the cytoplasm. Prior work by Besson and Roberts showed that p27 can bind RhoA to disrupt the cytoskeleton and increase cell motility. We showed that T198 phosphorylation promotes p27 binding to RhoA. PI3K effectors RSK, SGK, and mTOR all promote p27:RhoA binding and enhance directed cell migration as assessed by wound-healing and modified transwell assays. This motility phenotype is p27-dependent since lentiviral p27 knockdown reverted this hyper-motile phenotype to levels in parental cells. The contribution of deregulated p27 to tumor cell invasion and metastasis was assayed in xenografts using variants of the human breast cancer line, MDA-MB-231, selected for metastatic tropism to lung, MDA-MB-231-4175, and to bone, MDA-MB-231-1833. Both cell lines demonstrate hyperactivation of PI3K effectors, mTOR, SGK, RSK, and AKT, with concomitant elevation of cellular p27, and cytoplasmic mislocalization of p27. These cells demonstrate increased p27:RhoA binding and increased p27-dependent invasiveness. Following tail vein injection, the high degree of lung tumor formation in the MDA-MB-231-4175 line was strikingly reduced to near parental levels following shRNA-mediated knockdown of p27. Notably, p27 knockdown in MDA-MB-231-4175 did not alter cell cycle profiles and Ki67 staining in lung tumors from 4175 and 4175 shRNA p27 xenografted animals were similar, thus the reduction of lung tumor formation was not due to cell cycle effects of p27. These results indicate the importance of PI3K activation in the pro-oncogenic role of p27: signaling via PI3K and AGC kinase family members promotes p27 phosphorylation, cytoplasmic mislocalization, RhoA inhibition and other cytoskeletal effects that promote tumor cell motility and invasion. Deregulation of p27 localization and its consequences on the cytoskeleton and cell motility may be pivotal to PI3K driven tumor metastasis in vivo. Effects of p27 knock-down on miRNA and genes known to regulate EMT will be presented.

MOLECULAR CAUSES FOR BUBR1 DYSFUNCTION IN THE HUMAN CANCER PREDISPOSITION SYNDROME MOSAIC VARIEGATED ANEUPLOIDY

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Genetic mutations in the mitotic regulatory kinase BubR1 are associated with the cancer susceptible disorder mosaic variegated aneuploidy (MVA). In patients with biallelic mutations, a missense mutation pairs with a truncating mutation. Here we show that cell lines derived from MVA patients with biallelic mutations have an impaired mitotic checkpoint, chromosome alignment defects, and low overall BubR1 abundance. Ectopic expression of BubR1 restored mitotic checkpoint activity, proving that BubR1 dysfunction causes chromosome segregation errors in the patients. Combined analysis of patient cells and functional protein replacement demonstrates that all MVA mutations fall in two distinct classes: those that impose specific defects in checkpoint activity or microtubule attachment and those that lower BubR1 protein abundance. Low protein abundance is the direct result of the absence of transcripts from truncating mutants combined with high protein turnover of missense mutants. In this group of missense mutants, the amino acid change consistently occurs in or near the BubR1 kinase domain. Our findings provide a molecular explanation for chromosomal instability in patients with biallelic genetic mutations in BubR1.

TOR COMPLEX 1 REGULATES IPL1/AURORA B FUNCTION VIA THE GLC7/PP1 PHOSPHATASE.

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Ipl1/Aurora B is the catalytic subunit of a complex that is required for chromosome segregation and nuclear division. Prior to anaphase, Ipl1/Aurora B localizes to kinetochores, where it is required to establish proper kinetochore-microtubule associations and regulate the spindle assembly checkpoint. At anaphase, the complex migrates to the spindle, where it stabilizes the spindle and regulates cytokinesis. The protein phosphatase Glc7/PP1 opposes Ipl1/Aurora B for some of these activities but the regulatory/targeting subunit(s) required for this activity are not well known. Several proteins have been identified that by genetic criteria could act as nuclear regulatory subunits for Glc7, but biochemical evidence suggests that they act as inhibitors. To more thoroughly characterize the Glc7/PP1 phosphatase activity that opposes Ipl1, we have isolated suppressors of *ipl1-2* inviability at the non-permissive temperature. In addition to mutations in genes previously known to suppress the temperature sensitivity of ipl1-2 (*GLC7, SDS22* and *YPI1*), we recovered a null mutant in *TCO89*, which encodes a subunit of the TOR Complex 1 (TORC1), the conserved rapamycin-sensitive kinase activity that regulates cell growth in response to nutritional status. Suppression of the growth defect of *ipl1-2* is accompanied by increased fidelity of chromosome segregation. The temperature sensitivity of *ipl1-2* can also be suppressed by null mutations in *TOR1*, or by administration of pharmacological TORC1 inhibitors, indicating that reduced TORC1 activity is responsible for the suppression. Nuclear Glc7 levels are reduced in a *tco89* mutant, suggesting that TORC1 activity is required for the nuclear accumulation of Glc7. In support of this hypothesis, several mutant *GLC7* alleles that suppress the temperature sensitivity of *ipl1-2* exhibit negative synthetic genetic interactions with TORC1 mutants. Together, our results suggest that TORC1 positively regulates the Glc7 activity that opposes Ipl1 and provide a mechanism to tie nutritional status with cell cycle regulation.

USING A NOVEL SMALL MOLECULE INHIBITOR AZ3146 TO PROBE THE ROLE OF MPS1 KINASE ACTIVITY DURING MITOSIS.

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The Mps1 protein kinase is a key component of the spindle assembly checkpoint. Here, we describe a novel Mps1 inhibitor, AZ3146 and use it to probe the role of Mps1's catalytic activity during mitosis.
When spindle assembly is inhibited, AZ3146 prevents both establishment and maintenance of the checkpoint. During an otherwise unperturbed mitosis, AZ3146 accelerates anaphase onset and frequently induces chromosome missegregation. Consistent with our previous observations (Tighe, A. *et al. J Cell Biol.* 181:893-901. 2008), we show that kinetochore localization of Mad2 is critically dependent on Mps1 kinase activity, while Mad1 localization is less so. We also demonstrate that the anti-Mad2 antibody used for these studies preferentially recognizes the open conformation of Mad2 (O-Mad2). Consequently this antibody cannot detect C-Mad2 bound to Mad1 at the kinetochore. Subsequently we show that when Mps1 activity is inhibited, the Mad1-C-Mad2 complex is still bound to kinetochores; thus Mps1 activity plays a key role in recruiting O-Mad2 to the Mad1-C-Mad2 complex.
Mps1 has also been shown to promote chromosome alignment via activation of Aurora B (Jelluma, N. *et al. Cell*. 132:233-246. 2008). While AZ3146 can indeed compromise chromosome alignment we see no obvious effect on Aurora B activity. By contrast, AZ3146 has a profound effect on kinetochore recruitment of the kinesin-related motor, Cenp-E. Moreover, we show that inhibition of Mps1 activity increases its own abundance at kinetochores and that Mps1 can dimerise in cells.
We propose a model whereby Mps1 trans-phosphorylation results in its release from kinetochores, thus facilitating recruitment of O-Mad2 and Cenp-E, thereby simultaneously promoting checkpoint signaling and chromosome congression.
*AT and LH contributed equally to this work.

REGULATION OF THE G1-SPECIFIC TRANSCRIPTION BY THE DNA REPLICATION CHECKPOINT IN *SACCHAROMYCES CEREVISIAE*

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The G1-S-phase transition is critical to cell cycle progression. In the budding yeast *Saccharomyces cerevisiae*, this transition is driven by a wave of gene expression, which is under the control of the cell cycle-regulated transcription factors SBF and MBF. SBF mainly targets genes involved in timing, morphogenesis and spindle pole body duplication, whereas MBF regulates many genes involved in DNA replication and repair. During S-phase the DNA replication checkpoint detects DNA replication stress and generates a global response, which includes the transcriptional induction of genes involved in DNA replication and repair. We have previously shown that transcriptional induction in response to DNA replication stress in the fission yeast *Schizosaccharomyces pombe* is exerted through the inhibition of SpNrm1, a transcriptional corepressor of MBF, by the checkpoint kinase Cds1. Here, we show that there is also a transcriptional induction of genes involved in DNA replication and repair in response to DNA replication stress in *S. cerevisiae*. That response depends upon the DNA replication checkpoint pathway acting via ScNrm1, like in fission yeast. To establish the set of genes induced in response to DNA replication stress, we have examined genome-wide transcription dynamics in synchronized populations of budding yeast treated with hydroxyurea (HU), methyl methane sulfonate (MMS) or camptothecin (CPT). We find that a large fraction of the genes induced in response to HU and MMS are MBF targets, while the majority of SBF target genes remain unaffected. The analysis also reveals that CPT activates only a small subset of G1/S genes along with a unique subset of compound specific genes. We conclude that the transcriptional induction of G1/S genes is a conserved response to DNA replication stress in eukaryotes.

MEDIATORS OF PRB FUNCTION

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The retinoblastoma susceptibility gene, RB1, is one of the best-known tumor suppressor genes. The functional inactivation of its product, pRB, is thought to occur in most human tumors. Despite many years of investigation, the precise molecular interactions that mediate the tumor suppressing activity of pRB remain unclear. pRB has been proposed to interact with a very large number of cellular proteins, and the difficulty in interpreting the pRB literature is that there are too many potential mediators of pRB function. Currently it is unclear whether "pRB function" is mediated through just a few of its interacting proteins, or whether "pRB function" is the compound effect of its interaction with many different targets. The current evidence suggests that different pRB-associated proteins are likely to be important in different cellular contexts.
We have assembled a collection of lentiviral expressed shRNAs that target the expression of 227 proteins that have been either reported to interact with pRB, or that associate with pRB in vitro. Using assays that assess specific readouts of pRB activity (cell cycle arrest, senescence, differentiation, transcriptional repression) we are screening this collection to identify pRB-binding proteins that are necessary for each activity. In parallel, we have screened through a set of shRNAs that target 411 human kinases to identify signaling pathways that are needed for the pRB-induced changes. The goal of these studies is to identify the critical mediators pRB function, and to link them with specific signaling pathways.

DUAL DETECTION OF CHROMOSOMES AND MICROTUBULES BY THE CHROMOSOMAL PASSENGER COMPLEX TRIGGERS SPINDLE ASSEMBLY

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Chromosomes trigger spindle formation through activating Aurora B, the kinase subunit of the chromosomal passenger complex (CPC). Spatial regulation of the signaling after this initial chromosomal activation step remains unclear. Here we reveal that the local phosphorylation reactions mediated by Aurora B are insufficient for spindle assembly: chromosomally activated Aurora B must be targeted to microtubules. While the CPC is enriched on chromosomes in metaphase, we establish that a fraction of the CPC is targeted to the metaphase spindle. Using *Xenopus* egg extracts, we demonstrate that active Aurora B must be linked to the microtubule-targeting module of the CPC to drive spindle assembly. Moreover, although the CPC-microtubule interaction can activate Aurora B, which further promotes microtubule assembly, this positive feedback is not initiated without chromosomes. We propose that the dual detection of chromosomes and microtubules by the CPC induces spindle assembly around and only around chromosomes.

MOLECULAR ARCHITECTURE OF THE DNA REPLICATION ORIGIN ACTIVATION CHECKPOINT

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Perturbation of the DNA replication initiation pathway arrests human somatic cells in G1, pointing towards a cell cycle checkpoint for replication-competent origins. We used RNAi against Cdc7 kinase to block replication initiation and dissect the molecular nature of this checkpoint in primary fibroblasts. Our results show that the checkpoint response is dependent on three axes coordinated through the Forkhead transcription factor FoxO3a. In G1 arrested cells, FoxO3a activates the ARF┤ Mdm2┤ p53→p21 pathway and mediates upregulation of p15. P53 in turn activates expression of the Wnt/ β-catenin signaling antagonist Dickkopf 3 (Dkk3), leading to Myc and cyclin D1 downregulation. Gene expression microarrays show the resulting loss of CDK activity inactivates the Rb-E2F pathway, overriding the regular G1/S transcriptional program. Primary fibroblasts concomitantly depleted of Cdc7/FoxO3a, Cdc7/p15, Cdc7/p53 or Cdc7/Dkk3 are all able to bypass the cell cycle blockade and proceed into an abortive S phase followed by apoptosis. Our findings explain how somatic cells delay S phase entry until a threshold of replication-competent origins is reached to ensure complete genome duplication. The lack of redundancy between the checkpoint axes and reliance on several tumour suppressor proteins commonly inactivated in human tumours provides a mechanistic basis for the cancer cell specific killing observed with cdc7 small molecule inhibitors.

CHARACTERIZATION OF THE G1/S DNA DAMAGE CHECKPOINT IN MOUSE EMBRYONIC STEM CELLS

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The efficiency by which embryonic stem cells respond to DNA damage and regulate the onset of apoptosis is crucial as these cells have the potential to differentiate in adult lineages and therefore represent outermost ideal candidates for therapeutic applications. The cell cycle of embryonic stem cells (mES) differs from that of somatic cells as it is characterized by a short duration, and a very short G1 phase. It has been proposed that p53 function may be compromised in these cells, although contrasting data on the role of p53 in mediating cell cycle arrest at the G1/S boundary after DNA damage have been reported.

We have thoroughly analyzed the DNA damage response of mouse embryonic stem cells after exposure to divers DNA damaging agents, in either asynchronous or synchronous cell populations, as well as using different cell culture conditions. Overall we have observed that mES cells are generally more sensitive to DNA damage than somatic cells. Nevertheless our results show that DNA damage induces rapid ATM/ATR-dependent p53 phosphorylation. Cell fractionation experiments demonstrate that phosphorylated p53 accumulates on chromatin after induction of DNA damage and analysis of gene expression by real time PCR demonstrates rapid induction of p53 targets. Moreover we observed degradation of the CDC25A phosphatase, induction of both Chk1 and H2AX phosphorylation as well as a clear cell cycle delay after exposure of mES cells to MMS. Altogether, these findings indicate that in mES cells p53 functions in a similar manner to somatic cells in the context of DNA damage, to induce cell cycle arrest.

IDENTIFYING NEW DEVELOPMENTAL CELL CYCLE REGULATORS IN DROSOPHILA EMBRYOGENESIS.

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The coordination of cell growth and proliferation with differentiation events is crucial during development. In addition to signals to arrest cell division or exit the cell cycle, modified cell cycles are exploited for particular developmental strategies such as rapid embryogenesis or the production of large cells with specialized functions. Despite the tight correlation between developmental and cell cycle events, the *in vivo* developmental signals that modify cell division are poorly understood in many species. We are using the Drosophila embryonic cycles, which undergo several modifications that are tightly correlated with key developmental transitions, to address this question. Following 13 rapid S-M nuclear divisions, the cellularized embryo goes through three postblastoderm divisions on a S-G_2-M cycle. Due to large maternal stockpiles and because cell division is not required for larval growth, the majority of the previously identified mitotic mutants in Drosophila arrest at late larval or early pupal stages. Mitotic mutants arresting at the postblastoderm stages (mid embryogenesis) are rare, but our laboratory isolated a collection of lethal mitotic mutants that arrest at the postblastoderm divisions. These mutants cause mitotic arrest or the presence of polyploid nuclei. We are currently mapping and identifying gene products affected by three of these mutants. In addition, we are defining the developmental role and regulation of these genes using a combination of cytological and live cell imaging analysis. These studies will provide insights into unique regulatory features of the S-G_2-M postblastoderm divisions.

PROTEOLYSIS OF RAD17 BY CDH1/APC REGULATES CHECKPOINT TERMINATION AND RECOVERY FROM GENOTOXIC STRESS

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Recent studies have demonstrated a critical role for the ubiquitin-proteasome system (UPS) in regulating the signaling network for DNA damage responses and DNA repair. To search for new UPS targets in the DNA damage signaling pathway, we have carried out a non-biased assay to identify fast-turnover proteins induced by various types of genotoxic stress. This endeavor led to the identification of Rad17 as a protein exhibiting a distinctive pattern of up regulation followed by subsequent degradation after exposure to UV radiation in human primary cells. Our characterization showed that UV-induced Rad17 oscillation is mediated by Cdh1/APC, a ubiquitin-protein ligase. Studies utilizing a degradation-resistant Rad17 mutant demonstrated that Rad17 stabilization prevents the termination of checkpoint signaling, which in turn attenuates the cellular re-entry into cell cycle progression. In addition, pathological analyses unveiled the aberrant accumulation of Rad17 in a variety of human tumor specimens. The findings provide an insight into how the proteolysis of Rad17 by Cdh1/APC regulates the termination of checkpoint signaling, as well as a possible connection between regulation of Rad17 proteolysis and carcinogenesis.

INVESTIGATION OF THE CELL-CYCLE-DEPENDENT EXPRESSION OF A LYSINE DEACETYLASE REVEALS THE REGULATION THROUGH FORKHEAD TRANSCRIPTION FACTORS

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Different transcription factors dictate the cell-cycle-dependent expression of various genes. Forkhead family transcription factors are conserved from yeast to human, and are important for expression of genes involved in cell growth, proliferation and differentiation. Two partially redundant forkhead genes, FKH1 and FKH2, are found in S. cerevisiae. and are important for a number of genes whose expression display a cell-cycle-related oscillation and peak at G2/M transition. Such expression patterns are essential for cell cycle control (especially G2/M transition) and activation of proper downstream effectors.

Hos3, which encodes for a Type II lysine deacetylase, has two Fkh1p and two Fkh2p binding sites within its promoter, and both these factors are known to be recruited to the HOS3 promoter. Upon release from G1 arrest, the Hos3p level decreases quickly but peaks later at G2/M transition. In forkhead transcription factor mutants, the cell-cycle-dependent expression pattern of Hos3p is altered. The effect of forkhead transcription factors on the expression of Hos3p suggests a cell-cycle-related function of this lysine deacetylase, which needs further characterization.

LOSS OF CDC14B LEADS TO PREMATURE AGING AND REDUCED FERTILITY IN MICE

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Cdc14 dual-specificity phosphatase plays key roles in yeast cell cycle control. There are two mammalian Cdc14 homologues, Cdc14A and Cdc14B. Cdc14A is thought to function in centrosome cycling, whereas Cdc14B's function remains elusive. To probe the physiological function of Cdc14B, we generated mice deficient in this phosphatase. We found that Cdc14B is not essential for mitosis and the mutant mice can survive to adulthood. However, Cdc14B-deficency resulted in reduced fertility and deficits in learning and memory. Furthermore, Cdc14B mutant mice showed elevated rates of early onset cataracts, a sign of premature aging. At cellular level, loss of Cdc14B also caused premature senescence with concomitant upregualtion of p21Cip1 and more accumulated endogenous DNA damage. These data indicate a role of Cdc14B in the prevention of premature aging at both cellular and organismic levels.

Keywords: Cdc14B, Knock-Out, Cellular Senescence, DNA Damage.

REGULATION OF CORTEX, AN APC/C ACTIVATOR ESSENTIAL FOR THE FEMALE *DROSOPHILA* MEIOTIC CELL CYCLE

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Regulated proteolysis of proteins is essential to progress cell division cycles. The Anaphase Promoting Complex/Cyclosome plays a role in both mitosis and meiosis by ubiquitinating and targeting specific substrate proteins for degradation by the 26S proteasome. Although known targets such as Securin, Cyclin A, Cyclin B, and Cyclin B3 must be degraded to permit exit from both mitosis and meiosis, there may be proteins uniquely targeted for degradation in meiosis. To explore this we are investigating a meiosis specific form of the APC/C. *Drosophila* express two APC/C activators during mitosis: *fzy/cdc20* and *fzr/cdh1*. During female meiosis, however, *fzy/cdc20* is expressed in addition to a novel, meiosis-specific activator *cortex*. Both *cortex* and *fzy/cdc20* are necessary for the completion of meiosis. The non-redundancy of *fzy/cdc20* and *cortex* suggests either they have unique substrates or are subject to unique regulation.

We are taking two approaches to identify potential substrates and regulators of Cortex. First, we screened for deletions in the *Drosophila* genome that dominantly suppress the meiotic arrest resulting from reduced levels of Cortex protein. These intervals may contain negative regulators of Cortex and/or novel meiotic substrates of the APC/C. Five suppressing deficiencies have been found. Second, proteins that bind to a functional Myc-Cort fusion protein expressed in the *Drosophila* ovary were identified by mass spectrometry. As expected, subunits of the APC/C were recovered. Additionally, proteins involved in processes such as microtubule binding/motors, chromosome segregation and mitosis/meiosis were immunoprecipitated with Myc-Cort. These proteins are candidate substrates and regulators of Cortex.

RIPCHIP AND RIPSEQ IDENTIFIES THE REPLICATION-DEPENDENT HISTONE MRNAS AS SLBP TARGETS

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Expression of the replication-dependent histone mRNAs are restricted to S phase of the cell cycle. Their regulation is primarily post-transcriptional and controlled in part by the histone Stem-loop Binding Protein (SLBP). We have used RNA-binding protein IPs (RIP) followed by microarray analysis (RIPchip) and ultra-highthroughput (UHTP) sequencing (RIPseq) to identify targets of the SLBP. Prior analysis of SLBP by RIPchip indicated replication-dependent histone mRNAs were the only verifiable targets. We have repeated the analysis of SLBP by RIPchip and RIPseq to confirm and extended these results. Nine SLBP IPs were performed from polyribosomes and in each case, a negative control was performed in parallel. RNA isolated from the bound and unbound fractions was analyzed on whole-genome DNA microarrays. Aliquots of the same samples were pooled and analyzed by Solexa UHTP sequencing. Analysis of SLBP targets on DNA microarrays and selection of probes by Significance Analysis of Microarrays (SAM; FDR <0.001%) identified 82 enriched probes. 66 probes mapped to 58 replication-dependent histone mRNAs, the remainder to non-histone mRNAs. Analysis by Solexa UHTP sequencing gave >15M sequencing reads of 25 nt from the SLBP-RNA complex, the Ab:peptide negative control and a no-antibody negative control. Overlapping reads were collapsed and mapped to known RefSeq genes. For each gene, we calculated the reads per kilobase of exon model per million mapped reads (RPKM), which is a representation of transcript abundance corrected for bias introduced by variable transcript size. We evaluated the reproducibility of this data by comparing the histone mRNA enrichment observed by RIPseq with that observed by RIPchip. Of the most abundant gene transcripts identified by RIPseq, there is significant enrichment for replication-dependent histone mRNAs. Of the top 82 genes by RIPseq, 53 are histone mRNAs representing 50 different genes. In total, 66 histone transcripts were identified by RIPseq and RIPchip, including 62 replication-dependent histone mRNAs, two pseudogene mRNAs (H2BFS, HIST2H3PS2), and the replacement variants H2AFX and H2AFJ. 42 genes were identified by both methods, 16 only by RIPchip, and 8 only by RIPseq. The non-histone genes enriched by RIPchip were not enriched by RIPseq and vice versa. Our data demonstrate that RIPseq provides similar sets of targets as RIPchip, but has the advantage that one does not need to pre-specify the RNA sequences on the array, allowing for discovery of new targets and binding sites. We are currently extending these methods to include protein-RNA cross-linking followed by nuclease digestion (CLIP) and UHTP sequencing to precisely identify binding sites for RNA-binding proteins involved in histone mRNA regulation.

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SPINDLE CHECKPOINT-INDEPENDENT RECRUITMENT OF CYCLIN B1-CDK1-CKS TO THE APC/C CONTROLS MITOTIC EXIT AND MITOTIC SLIPPAGE

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The APC/C ubiquitin ligase is activated at prometaphase by mitotic phosphorylation and increased binding of its activator Cdc20. This initiates cyclin A destruction, while cyclin B1 is stabilized by the mitotic spindle checkpoint.

Upon spindle checkpoint release, it was proposed that the RXXL destruction motif (D-box) directs recruitment of cyclin B1 to either core APC/C or Cdc20. We studied APC/C substrate-capturing in cells and report that endogenous cyclin B1-Cdk1 is recruited to the checkpoint-inhibited, phosphorylated APC/C in prometaphase, independently of Cdc20 or the cyclin B1 D-box.

Like cyclin A, cyclin B1 binds the phosphorylated APC/C in a way dependent on the Cdk-subunits of the Cks family and APC3. Prior binding to APC/C-Cdc20 mediates efficient degradation of cyclin B1 in metaphase to control mitotic exit and cytokinesis. We also show that this Cks-mediated recruitment of cyclin B1-Cdk1 to the APC/C contributes to mitotic slippage of cells that are arrested with an active spindle checkpoint.

We conclude that, in cells, factors that bind APC/C substrates can direct the processivity of their ubiquitination. Further we propose that the spindle checkpoint does not prevent cyclin B1 recruitment to APC/C-Cdc20, but rather may inhibit the ability of APC/C-Cdc20 to recognize the D-box of bound cyclin B1.

IDENTIFICATION OF FUNCTIONAL REGIONS OF SORORIN

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Faithful segregation of sister chromatids during cell division is essential for the accurate transmission of genetic material from one generation to the next. This process is mediated by sister chromatid cohesion (SCC), which ensures sister chromatids remain coupled until anaphase. SCC is mediated by the cohesin complex, a multi-subunit protein complex which is thought to form a ring that physically encircles the sister chromatids.
In addition to the cohesin complex, many other proteins are required for SCC, including sororin, a positive regulator of SCC that interacts with the cohesin complex in vitro. Sororin is required for the increased stability of the cohesin complex on chromatin following DNA replication and the maintenance of sister chromatid cohesion during G2, yet seems to be dispensable for cohesin complex loading. The mechanism by which sororin ensures cohesion is currently unknown.
In this study, we have undertaken a structure/function analysis of the sororin protein. The primary sequence of sororin does not suggest the presence of any known structural or functional motifs, though sequence alignment of sororin proteins across several species indicates the presence of particularly well-conserved regions. We hypothesize these regions are likely to be important for function. Here we show that mutations within the conserved c-terminus of sororin fail to rescue a sororin knockdown phenotype. We show that this region of the protein is not important for chromatin association. Together, these data suggest that sororin is recruited to the chromatin by a mechanism independent of its ability to regulate cohesion. Further studies are being conducted to identify regions of sororin that mediate interaction with the cohesin complex.

INTERACTION NETWORK OF DNA REPLICATION-INITIATION PROTEINS AND DIMERIZATION OF ORC AND NOC3P IN PRE-REPLICATIVE COMPLEX ASSEMBLY IN BUDDING YEAST

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Initiation of eukaryotic DNA replication involves the sequential association of replication-initiation proteins with replication origins, such as ORC, Noc3p, Cdc6p, Cdt1p, MCM, Cdc45p, Mcm10p, Sld2p, Dbp11p, Sld3p and GINS. Using the yeast two-hybrid assay, we have systematically studied the interactions between most of these proteins. We found many novel interactions, including interactions of Noc3p with four ORC subunits, Cdt1p and Mcm6p, further supporting the role of Noc3p as a connector between ORC and other pre-RC proteins. Moreover, we have found novel self-interactions of some ORC subunits and Noc3p.

ORC is the platform for pre-replicative complexes (pre-RCs) at replication origins. It remains unclear as to what happens to ORC and the replication origins after the initiation of DNA replication. We found that the self-interaction of Orc6p occurs at G1 phase, but not S or G2/M phase. Furthermore, with the same cell number equivalent of proteins for each time-point sample loaded in SDS-PAGE, we found that the chromatin-bound ORC remains constant throughout S and G2 phases and increases at late M phase as pre-RCs are formed, suggesting that ORC forms a dimer at the M-to-G1 transition to support pre-RC assembly, and each ORC dimer separates into two monomers to bind both copies of each replication origin after replication initiation.

Noc3p is essential for both ribosome biogenesis and replication initiation. We mapped the Noc3p self-interaction domains to the two coiled-coil motifs (CC1 and CC2) and found that both CC1 and CC2 deletion mutants grow slowly and are defective in S phase entry but not S phase progression, with the CC2 deletion mutant being more defective than the CC1 deletion mutant. CC2 deletion affects ribosome biogenesis strongly at 37°C and only slightly at 30°C, and CC1 deletion does not affect ribosome biogenesis. Therefore, the two Noc3p mutant strains show different degrees of defects in ribosome biogenesis and DNA replication.

Based on these results, we have come up with a model for DNA replication initiation, which involves ORC, Noc3p and MCM dimer formation.

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CUL7 PLAYS A CRUCIAL ROLE IN MITOSIS/CYTOKINESIS CONTROL.

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Cullins are a family of evolutionarily conserved proteins that bind to the small RING finger protein, ROC1, to constitute potentially a large number of distinct E3 ubiquitin ligases. Cullin-7 (CUL7) has been identified as a simian virus 40 large T antigen-binding protein. Disruption of CUL7 gene in mice causes intrauterine growth retardation and perinatal death. CUL7 germlined mutations have been found in patients with autosomal-recessive 3-M and Yakuts short stature syndromes, indicating CUL7 plays a critical role in cell growth. We have found that CUL7 is enriched on mitotic apparatus. Knocking down CUL7 in U2OS cells reduces cell proliferation, and causes tetraploidy, interconnected daughter cells, and cell death during cytokinesis. Further study shows that CUL7 depletion delays chromatids alignment, resulting in a prolonged prometaphase. Additionally, cells depleted of CUL7 show early inactivation of Aurora B at late telophase, a protein which has been reported to stabilize the mid-body. Accordingly, cells depleted of CUL7 is either impaired to form or prematurely disassemble mid-body. These findings suggest that CUL7 is a crucial regulator of mitosis/cytokinesis. The mitotic/citokinesis defects caused by CUL7 loss may attribute to essential activity of Cul7 in mouse development and/or the onset of human 3-M and Yakuts syndromes.

THE ROLE OF GAS2 IN THE OVARY; FOLLICULOGENESIS AND FERTILITY.

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Growth arrest specific gene two (Gas2) was discovered in mouse fibroblasts grown under arresting conditions. The protein contains two functional domains; the Gas2 domain and a calponin homology domain targeted by caspase cleavage. Studies of Gas2 expression in the developing embryo have indicated roles for the gene in chondrocyte and adipocyte differentiation and proliferation, apoptosis of interdigital tissues in the developing embryo, and the development of several organ systems. Gas2 expression has also been seen to change in injured adult tissues, and may be involved in wound healing and remodeling of affected tissues. There is evidence that Gas2 functions as a regulator of apoptosis, participating in cytoskeletal rearrangements of the cell as well as acting as a part of the p53 apoptotic pathway. In order to understand the physiological role of Gas2 in the mammal, we have generated the first mouse in which expression of the Gas2 gene has been knocked out. The mutant mice are capable of surviving to adulthood, but the females display reduced fertility. Histological sections have shown structural differences in the ovary of the mutant mouse. Of interest is the number and ratio of maturing and mature follicles within the ovary. Of the thousands of primordial follicles present in the developing mouse ovary, greater than ninety percent will disappear before they reach maturity. The process of controlled follicular death, called atresia, is regulated by the controlled apoptosis of the granulosa cells of the developing follicle, and the structure of the follicles found in the Gas2 mutant is similar to that which has been observed in both caspase-2 and p27 knockout mice. Mutant ovaries display small sized follicles, and a larger number of immature structures. Superovulated mutant ovaries contain fewer mature structures. Increased apoptosis of the granulosa cells has been confirmed by immunohistological staining of cleaved caspase-3 in ovarian sections taken from our mutant mice. Conversely embryonic fibroblasts isolated from mutant embryos show delayed senescence compared to wild type, however this may further explain the lack of mature and differentiated granulosa cell types in the superovulated ovary. Therefore, it seems that the infertility of the Gas2 mutant mice is due to failed follicular maturation due to excessive apoptosis of the granulosa cell population, though further work is necessary to confirm the mechanism by which Gas2 regulates apoptosis n the maturing follicle.

PCM1-MEDIATED ACCUMULATION OF PLK1 AT CENTROSOME PROMOTES NEDD1-γ-TUBULIN INTERACTION AND CENTROSOME MATURATION

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Proper bipolar spindle formation requires centrosome maturation at G2/M transition, during which the additional pericentriolar material (PCM) proteins are recruited to the centrosome and enhance the microtubule nucleation ability. Polo-like kinase 1 (Plk1) is a highly conserved Ser/Thr kinase that has essential role in the centrosome maturation. However, the mechanism by which Plk1 recruits the crucial component of PCM, γTuRC to the centrosome is poorly understood. Here, we identify Nedd1, a novel centrosomal substrate of Plk1, the accumulation of which at centrosome is largely decreased with Plk1 depletion. The sequential phosphorylation of Nedd1 by Cdk1 and Plk1 promotes its interaction with γ-tubulin, which is important for targeting the γTuRC to the centrosome and for spindle formation. In our study, we also show that Plk1 accumulates at the matrix of centrosome. Plk1 colocalizes with PCM1, a centriolar satellite protein, at centrosome and binds PCM1 directly through its PBD domain. Both PCM1 depletion and overexpression of a truncated mutant disrupt the localization of Plk1 on centrosome. Taken together, we propose that PCM1 is a key factor for targeting Plk1 to the centrosome, which later enhances the interaction of Nedd1 and γ-tubulin and promotes centrosome maturation and spindle assembly.

MAINTAINING GENOMIC STABILITY BY THE SWI/SNF CHROMATIN REMODELING COMPLEX

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Mammalian cells exhibit complex cellular responses to DNA damage, including cell cycle arrest, DNA repair and apoptosis. Defects in any one of these responses can result in carcinogenesis. Absence of the chromatin remodeling complex SWI/SNF is found in many instances of cancer, and we have investigated its role in the DNA damage response. The human carcinoma cell line SW13 is deficient in SWI/SNF due to the lack its two mutually exclusive catalytic subunits, BRG1 and BRM. SW13 cells are very sensitive to UV radiation. In contrast, SW13 cells with ectopic Brg1 expression regain active SWI/SNF and become significantly more resistant to UV radiation. Sensitivity to UV light correlates well with dramatic UV induced apoptosis in SW13 cells, but not in SW13 cells expressing Brg1. Additionally, using Affymetrix Human Genome U133 Plus 2.0 microarray, we have assessed BRG1-mediated alterations in over 47,000 transcripts. We have identified 1496 BRG1 up-regulated genes and 811 repressed genes. Importantly, a large amount of UV inducible genes require BRG1 for proper expression. Brg1 expression in SW13 cells restores expression of p21, ATF3 and Gadd45a, which are involved in DNA damage response and G1 cell cycle arrest. Finally, we show that re-expression of BRG1 in SW13 cells stimulates nucleotide excision repair of UV induced DNA damage. We demonstrate that recruitment of the DNA damage recognition protein XPC, but not DDB2, to sites of UV lesions is significantly disrupted when BRG1 is depleted. Our findings demonstrate that SWI/SNF protects genome stability by orchestrating cell cycle arrest and facilitating DNA damage repair.

OVEREXPRESSION OF P27 T198 PHOSPHOMIMETIC MUTANT PROMOTES BREAST CANCER MOTILITY INDEPENDENT OF CELL CYCLE FUNCTION

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p27 is a cell cycle regulator and an atypical tumor suppressor. However, p27 gene deletions or mutations are rarely observed in human cancers. Instead, p27 is frequently degraded or mislocalized to the cytoplasm in aggressive human cancers. We and others have shown that p27 is phosphorylated at T198 by different PI3K effector kinases (AKT, RSK and SGK) and this leads to p27 accumulation in the cytoplasm due to impaired nuclear import. Recent data suggest that cytoplasmic p27 may acquire cell cycle independent oncogenic function to promote tumor cell migration by inhibiting RhoA-ROCK activation. Until recently, the post-translational modifications that cause p27 to acquire this cytoplasmic gain-of-function were largely unknown. We recently showed that RSK1-mediated phosphorylation of p27 at T198 promotes its binding to RhoA in vitro and PI3K activation in cells is associated with increased p27:RhoA binding. Moreover, the increased cell motility caused by constitutive PI3K effector activation is reversed by p27 knockdown. To better understand the contributions of T157 and T198 phosphorylation with regard to p27's gain of function and ability to promote cell migration and metastasis, we used a p27CK- mutant that fails to bind cyclins and cdks and prepared p27CK- phosphomimetic T157D, T198D or double phosphomimetic mutants. These were introduced into the MDA-MB-231 breast cancer cell line to generate multiple stable clones: p27CK-, p27CK-T157D, p27CK-T198D and p27CK-T157D/T198D. All stable lines show proliferation rates and cell cycle profiles similar to parental cells. Both p27CK-T157D and p27CK-T198D cells show more cytoplasmic p27 localization, greater cell motility and increased matrigel invasion than either p27CK- or vector expressing cells. p27CK-T157D/T198D is predominantly cytoplasmic and confers even greater cell motility and matrigel invasion. The effects of p27 T157D/T198D to alter the actin cytoskeleton and increase local orthotopic tumor invasion and metastasis in MDA-MB-231 xenografts will be presented. These data may illuminate how p27 deregulation contributes to cancer progression and metastasis.

ANALYSIS OF ENDOREPLICATION IN THE *DROSOPHILA* SALIVARY GLAND

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Endocycles are variant cell cycles comprised of DNA Synthesis (S)- and Gap (G)-phases, but lacking mitoses. Endocycles are frequently found in invertebrates, plants and certain mammalin cell-types, where they facilitate post-mitotic growth. Since mitosis is bypassed, mitotic regulators are transcriptionally down regulated, while the same components are used for G-S control as in mitotically dividing cells. DNA replication during endoreplication in *Drosophila* is triggered by Cyclin E (CycE) / Cyclin Dependent Kinase 2 (Cdk2) activity, but this must be extinguished during each G-phase to allow activation of the Anaphase Promoting Complex/Cyclosome (APC/C). The APC/C targets the licensing inhibitor Geminin for proteasomal degradation and thus allows assembly of pre-replication complexes (preRCs) for the next S-phase. However, the mechanism controlling CycE/Cdk2 oscillation during endocycle progression has not been established. We now provide evidence that endocycles in *Drosophila* salivary glands are driven by a molecular oscillator in which E2F1 promotes cycE transcription, CycE/Cdk2 triggers S-phase, and S-phase activates the Cul4-Ddb1-Cdt2 E3 ubiquitin ligase that promotes the destruction of E2F1. The loss of E2F1 during S-phase creates a window of low Cdk2 activity that allows APC activation and preRC formation. Consistent with this mechanism, overexpression of E2F1 accelerated endoreplication, whereas stabilization of E2F1 blocked endocycle progression. This arrest was accompanied by elevated levels of CycE transcript and gradual accumulation of Geminin protein. In addition, we found that the mitotic regulators Cyclin A and Cdk1 accumulate upon overexpression of E2F1. Similar, ectopic expression of Cyclin A and Cdk1 was observed in salivary gland cells mutant for the repressor E2F2. From these observations we conclude that E2F2-mediated repression might be required to prevent mitotic gene expression during endoreplication.

CYCLING TOWARD THE MID BLASTULA TRANSITION

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Embryos of every metazoan phylum undergo rapid cell cycles just prior to gastrulation. In frog and fly, and most organisms, these rapid cycles follow on the heals of fertilization, and run using maternally expressed genes without needing zygotic transcription. More than 25 years ago John Newport began to explore the nature of these early cycles and the remarkable transition called the mid blastula transition (MBT) at which the cycles slow and morphogenesis begins. Our studies in Drosophila, have revealed new views of these keystone cell cycles. I will describe recent work that reveals the basis of the timing of the early cycles, which are streamlined cycles of S phase and mitosis without gap phases, and how cell cycle events change at the MBT. Using genetics and RNAi to manipulate cyclin protein production, we show that the timing of interphase is not determined by a cyclin accumulation clock. Using injection of Geminin, an inhibitor of Cdt1 function that prvents preRC formation, we have entirely eliminated S phase rather than the more usual experimental manipulation in which its progress is inhibited. S phase duration governs interphase length in the early cycles, but does not time cycle progress after the MBT. S phase length dramatically increases at cycle 14, and this first long S phase serves as a transition time at which the numermous changes of the MBT occur. This S phase lengthens because of a reprogramming of DNA replication: replication of satellite sequences, which previously occurred in sync with all the other sequences, is deferred, and sequential “late replication” of each block of satellite prolongs S phase. Maternally provided Cdc25 phosphatase is eliminated during S phase 14 and this elimination results in the pause of cycle 14 in the first G2. If S phase 14 is deleted, cells very rapidly enter mitosis suggesting that the ability of S phase to defer mitosis via checkpoint processes provides a time interval in which the cell cycle regulation is retooled at the MBT. But how is S phase prolonged? Recruitment of HP1, a marker of heterochromatin, to satellite sequences occurs after the onset of S phase 14 suggesting that at selective localization of this protein is not required to consign origins to a late time of replication. By injection of Cdc25 mRNA, we show that the normal prolongation of S phase requires the decline in maternal Cdc25. This leads us to suggest a reinforcing feedback at the MBT in which declining Cdc25 prolongs S phase during which there is further decline of Cdc25 to introduce the G2 phase and the first dependency of the cell cycle on zygotic transcription.

ENDOCYCLE CONTROL: A GROWTH-DEPENDENT TRANSCRIPTIONAL OSCILLATOR

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Endocycles are variant cell cycles comprised of DNA Synthesis (S)- and Gap (G)-phases, but lacking mitoses. Endocycles occur in many invertebrate and plant cells, where they facilitate post-mitotic growth, and are so ubiquitous that they may account for nearly half the earth's biomass. DNA replication in endocycling Drosophila cells is triggered by Cyclin E (CycE) / Cyclin Dependent Kinase 2 (Cdk2) activity, but this must be extinguished during each Gap phase to allow activation of the Anaphase Promoting Complex/Cyclosome (APC/C) and assembly of pre-Replication Complexes (preRCs) for the next S-phase. The mechanism controlling CycE/Cdk2 oscillation during endocycle progression has not been clearly established. We find that endocycles in Drosophila salivary glands are driven by a molecular oscillator in which the E2F1 transcription factor promotes CycE expression, CycE/Cdk2 triggers S-phase, and S-phase activates the Cul4/Cdt2, PCNA dependent E3 ubiquitin ligase that drives the destruction of E2F1. We propose that it is the loss of E2F1 during S-phase that creates the window of low Cdk activity which allows APC activation and preRC formation. Consistent with this mechanism, over-expression of wildtype E2F1 accelerates endocycling, whereas expressing a stabilized variant of E2F1 lacking the PCNA interaction (PIP) motif blocked endocycling. Further, we find that conditions that alter cell growth regulate E2F1 protein levels, thereby making endocycle progression growth-dependent. The regulatory interactions essential to the endocycle oscillator we describe are conserved in animals and plants, suggesting that elements of this mechanism may act in many different types of growth-dependent cell cycles.

CAENORHABDITIS ELEGANS CYCLIN E AND CDK2 CONTROL GLD-1, THE MITOSIS/MEIOSIS DECISION AND GERMLINE STEM CELLS

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Classical cell cycle regulators must be coordinated with cell fate regulators to generate tissues during development and maintain them in adults. Defects in that coordination can shift the balance between proliferation and differentiation with devastating clinical effects. Yet our understanding of molecular mechanisms integrating cell cycle and stem cell controls remains in its infancy. We have found that *Caenorhabditis elegans* CYE-1 (cyclin E) and CDK-2 (Cdk2) shift the mitosis/meiosis balance towards continued mitotic divisions at the expense of meiotic entry, at least in part, by negatively regulating GLD-1, a member of the STAR protein family and a key developmental regulator of meiotic entry. GLD-1 is normally low in mitotically dividing germ cells, but when CYE-1 or CDK-2 is depleted, it becomes abundant. When CYE-1/CDK-2 and GLD-1 are both depleted, the mitosis/meiosis balance is not affected, suggesting that CYE-1 acts through GLD-1. Moreover, GLD-1 appears to be a direct substrate of CYE-1/CDK-2 phosphorylation, revealing a distinct regulatory branch of mitosis/meiosis control. Because GLD-1 also represses cye-1 expression (Biedermann *et al.*, 2009), GLD-1 and CYE-1/CDK-2 form a loop of mutual repression in balancing between mitosis and meiosis. Furthermore, CYE-1/CDK-2 works synergistically with the FBF-1 stem cell regulator to lower GLD-1 and to prevent meiotic entry in the germline stem cell pool. Therefore, the CYE-1/CDK-2 cell cycle regulator emerges as a key developmental regulator with a critical role in stem cell maintenance.

A NOVEL CELL CYCLE PROGRAM DIRECTS DEVELOPMENTALLY REGULATED POLYPLOID MITOSIS IN *DROSOPHILA*

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Within most diploid organisms, truncated endo- or endo-mitotic cell cycles programmed to prematurely exit S- or M-phase generate polyploid cells. As a result of these altered cell cycles, polyploid cells are thought to be incapable of mitosis. Further, studies of artificially-induced polyploidy suggest that polyploid mitosis is error-prone and tumorigenic. However, a small number of classic reports suggest that polyploid cells divide during normal development. We investigated one of these claims[1] in the mosquito (*Culex*) hindgut. Convincingly, we find polyploid cells do in fact undergo multiple mitoses during *Culex* hindgut development. We then identified similar polyploid mitoses in the genetically tractable *Drosophila* hindgut. Using both live and fixed imaging, we defined the parameters of such polyploid divisions. During a brief window of development, polyploid cells undergo two mitoses and cycle together in localized domains to build important absorptive structures. Mitosis of these polyploid progenitors (PPPs) requires conserved cell cycle regulators and Notch signaling. Unlike previous claims of polyploid division, we find PPPs complete S-phase and retain ploidy (8C). Further, by examining when PPPs become polyploid, we found a key cell cycle difference that likely enables PPPs to divide. Unlike most endocycles, which truncate S-phase, PPPs fully replicate prior to undergoing a novel endo- to mitotic cycle switch. Further, we find evidence of diminished mitotic fidelity in polyploid cells. Compared to diploid cells, PPP mitosis length is increased two-fold, and ~10% of PPP anaphases contain lagging chromosomes. This latter observation suggests that polyploidy presents additional challenges during mitosis. Our characterization now permits identification of polyploid mitosis regulators and a means to test the accuracy of polyploid divisions in vivo. Coupled with our previous observation that adult hindgut cells divide following tissue damage[2], our work establishes the hindgut as a valuable system to study previously under-appreciated plasticity in metazoan cell cycles.

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2 Fox, D. T. & Spradling, A. C. Cell Stem Cell 5, 290-297 (2009).

CHK1 REGULATES STEM CELL PROLIFERATION AND DIFFERENTIATION BY REGULATING EXPRESSION OF THE CDK SPECIFIC INHIBITOR P57/KIP2

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Checkpoint kinase-1 (CHK1) is essential for cell proliferation in mammals. In response to DNA damage, CHK1 regulates the cell division cycle by inhibiting the activities of both CDK1 and CDK2, the two cyclin dependent protein kinases that drive cells into mitosis and S-phase, respectively. Thus, activation of CHK1 inhibits progress through the cell cycle. Here we report a novel role for CHK1 in regulating the transition from cell proliferation to cell differentiation. When deprived of fibroblast growth factor-4 (FGF4), proliferating trophoblast stem (TS) cells differentiate into non-proliferating trophoblast giant (TG) cells that undergo 'endocycles' (multiple rounds of DNA replication without an intervening mitosis) and become polyploid. Previous studies demonstrated that this event rapidly induces expression of p57/KIP2 with concomitant inhibition of CDK1 activity (1). Subsequent studies have revealed that CHK1 activity regulates expression of p57/KIP2. The levels of CHK1 and p57 proteins are inversely correlated; CHK1 protein levels are high in TS cells but suppressed in TG cells, whereas the opposite is true for p57 protein. Inhibition of CHK1 activity in TS cells rapidly induces the accumulation of p57 protein with concurrent differentiation into TG cells. Over expression of wild-type CHK1 in TG cells suppresses expression of p57 protein with the concurrent appearance of mitotic cells, whereas over-expression of a phosphorylation defective CHK1 mutant does not. This is not due to changes in p57 mRNA levels, but to ubiquitin dependent degradation of p57 protein. In vitro, CHK1 phosphorylates p57 only at serine 19; CHK2 does not phosphorylate p57. In vivo, substitution of serine 19 with alanine stabilizes p57 protein whereas substitution with aspartic acid destabilizes it. Therefore, in the absence of induced DNA damage, CHK1 phosphorylates p57 in proliferating TS cells and targets it for degradation. In TG cells where CHK1 expression is suppressed, p57 inhibits CDK activity during G-phase to allow prereplication complex assembly, and then is targeted for degradation by CDK2 during S-phase to allow endocycles to occur. Thus, CHK1, a key intermediate in the DNA damage response pathway, also plays a critical role in regulating p57 dependent cell differentiation in mammals.

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MYC-NICK: A CYTOPLASMIC FORM OF MYC THAT PROMOTES TUBULIN ACETYLATION AND MUSCLE DIFFERENTIATION

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Myc is a BHLH transcription factor that plays a critical role in major biological processes, such as proliferation, growth, apoptosis, and differentiation. Recently, we discovered Myc-nick, a transcriptionally inactive form of Myc that is localized in the cytoplasm. Myc-nick is generated by a proteolytic cleavage of the full-length Myc that is carried out by members of the calpain family of calcium dependant proteases. Myc-nick lacks the C-terminal region (nuclear localization signal, Max dimerization domain, and DNA binding domain) of Myc but contains an intact N-terminus. Ectopic expression of Myc-nick in fibroblasts and epithelial cells promotes changes in cell morphology marked by the induction of cellular protrusions. We found that GCN5 functions is an alpha-tubulin acetyl transferase and it can cooperate with Myc-nick to promote alpha-tubulin acetylation. Since induction of alpha-tubulin acetylation, calpain activation, and decrease in full length Myc all occur during terminal differentiation we investigated the role of Myc-nick in cell differentiation. We found that Myc-nick is augmented during the differentiation of human and mouse primary myoblasts. In addition, we found that while full-length Myc levels diminish, Myc-nick levels remain elevated in adult mouse differentiated muscles, brain and cerebellum. Ectopic expression of Myc-nick accelerates myoblast fusion and the expression of myogenic markers in mouse and human myoblasts and in Rhaddomyosarcomas. Importantly, we found that the expression of Myc-nick renders myc-null fibroblasts competent to MyoD induced transdifferentiation. Based on our results we propose that, while full-length Myc blocks differentiation at the transcriptional level, Myc-nick is involved in promoting differentiation through transcription independent mechanisms.

CDK1-CYCLIN B1-DEPENDENT DESTRUCTION OF MCL-1 DURING MITOTIC ARREST SENSITIZES CELLS TO MICROTUBULE POISONS

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The response of cells to mitotic disruption caused by anti-cancer microtubule poisons involves a balance between cell cycle arrest through the action of the spindle assembly checkpoint, slippage out of mitosis or the induction of cell death by apoptosis. We have studied the regulation of the mitochondrial or intrinsic apoptotic pathway during the cell division cycle and in response to prolonged mitotic arrest induced by microtubule poisons. Previously, we have shown that caspase-9, a critical component of the intrinsic apoptotic pathway, is phosphorylated at an inhibitory site during mitosis by CDK1-cyclin B1. Inhibition of caspase-9 by CDK1-cyclin B restrains apoptosis and promotes cell survival during mitosis [1,2]. This work suggested that the pathway is activated upstream of caspase-9 during prolonged mitotic arrest. We hypothesized that the pathway is initiated by activation of a pro-apoptotic protein or the inhibition of an anti-apoptotic protein during mitotic arrest. Good candidates are members of the Bcl-2 family of apoptotic regulators. We have confirmed that several of these proteins are highly phosphorylated during mitosis. Mcl-1, an inhibitor of apoptosis, is of particular interest. We have found that Mcl-1 is unstable during mitotic arrest and it undergoes proteasome-dependent destruction by a novel Cdc20-dependent mechanism that is directly linked to cell cycle control. Destruction of Mcl-1 requires phosphorylation at a single site targeted by CDK1-cyclin B1, but only occurs after >2 hours of mitotic arrest. Stabilisation of Mcl-1 by mutation of the critical phosphorylation site strongly enhances the resistance of cells to apoptosis induced by microtubule poisons. We propose that that Mcl-1 destruction provides a mechanism that controls apoptosis temporally and thereby distinguishes between normal mitosis and prolonged mitotic arrest. Our results also emphasize the dual role of CDK1-cyclin B1 in driving mitosis and controlling apoptosis during this phase of the cell cycle.

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CELL CYCLE EXIT INDUCED BY SERUM WITHDRAWAL REGULATES RAB11 RECYCLING ENDOSOME, TRAFFICKING RAB8 ACTIVATION, AND THE ASSEMBLY OF THE PRIMARY CILIUM

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The primary cilium organizes critical functions in the postmitotic cell and its presence reflects the quiescent cell and competence for specific signaling pathways. Notably, sensory and signaling pathways are exquisitely organized in primary cilia. Resorption of cilia upon serum addition is linked to the activation of the Aurora A kinase and the HDAC6 tubulin deacetylase. Reformation of the cilia after mitosis requires serum withdrawal, but little is known about the molecular events coupling cell cycle exit to the elaboration of this important and highly conserved signaling organelle.

Here, we define critical molecular steps in ciliary assembly and their control by serum and cell cycle exit. The primary cilium has recently been linked to a potential tumor suppressor function, but the molecular link between the cilium and maintenance of cell cycle arrest is not known.

Among the critical structures in the primary cilium, a highly conserved set protein complexes define the molecular structures of the cilium and the structures allowing the dynamic trafficking of signaling receptors into the primary cilium. These include the intraflagellar transport complexes IFT-A and IFT-B, and proteins defined by mutations in rare human genetic diseases including Bardet-Biedl syndrome. Bardet-Biedl syndrome (BBS) patients have compromised cilia and signaling, leading to retinal degeneration, cystic kidney disease, sensory loss, neurological deficits, and obesity. BBS proteins form the BBSome, a highly conserved seven protein complex, which binds Rabin8, a GEF activating the Rab8 GTPase, required for ciliary assembly. We now describe a detailed molecular pathway linking serum withdrawal to ciliary assembly. We find upon serum withdrawal, Rab11 vesicles associate with microtubules and begin trafficking to the centrosome. Rab11 associates with the Rab8GEF/Rabin8, and via Rab11, Rabin8 also relocalizes to vesicle and is then transported via microtubules to the centrosome. At the centrosome, Rabin8 binds the BBSome, which is required for organized assembly of the ciiary vesicle, a membranous precursor of the primary cilium. Rabin8 activates Rab8, a small GTPase essential for formation of the primary cilium, and over ~100 minutes Rab8 assembles the ciliary membrane. We identify three Rabin8 regulators important for ciliation. First, Rab11-GTP controls Rabin8 membrane association and trafficking through the Rabin8 COOH-terminal region. At the centrosome Rab11 vesicles convert to Rab8 preciliary membranes. Second, BBS1 binds the Rabin8 GEF domain and regulates cilia length, whereas the disease-associated BBS1M390R mutant fails in Rabin8 binding, BBSome assembly, and centrosome/cilia localization. Lastly, TRAPPI/II targets Rabin8 to the centrosome through binding the Rabin8 NH2-terminal domain.

We thus link serum withdrawal to regulated Rab11-dependent cargo delivery to the centrosome for preciliary vesicle formation. Additional mechanisms explaining the link between cell cycle exit and the elaboration of the primary cilium will be presented.

CELL CYCLE EXIT AND RE-ENTRY FROM QUIESCENCE IN BUDDING YEAST

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Pure populations of quiescent cells can be obtained from stationary phase (SP) cultures due to a shift in cell density as they undergo the diauxic shift. These quiescent (Q) cells account for about half the cells in SP cultures and they are all daughter cells. This suggests that there is an asymmetric division upon glucose exhaustion in which daughters adopt a protective Q state at the expense of the mothers, which accumulate reactive oxygen species and show signs of apoptosis (Allen et al. (2006) JCB 174,89.) We find that Ssd1V, a polymorphic RNA binding protein, enables both mothers and daughters to enter the Q state and significantly increases their chronological life span (CLS) in W303. We are investigating the role of Ssd1v in Q cell determination. We are also defining the genes required for entry, maintenance and recovery from the Q state. All quiescent cells exit the cell cycle from G1, so we have studied G1 regulators to see if they are important for entry or maintenance of the Q state. In cycling cells, the genes required for the G1 to S transition are held in a transcriptionally repressed state by Whi5 until Whi5 is phosphorylated and released by cyclin Cln3/Cdk activity. Initiation of S phase requires B type cyclin/Cdk activity, which is inhibited by Sic1 throughout G1. We find that Whi5 is not required for the G1 arrest in SP prototrophs, but whi5- reduces Q cell yield. Sic1 is required for G1 arrest as well as efficient formation and recovery of Q cells. Sic1 mutants remain viable but rapidly lose the ability to form colonies as nutrients are exhausted. Sic1 is highly expressed in Q cells and it persists through the unbudded interval of the Q recovery cycle. Five copies of the CLN3 gene (5XCLN3) also impair Q cell formation and these cells are completely dependent upon the DNA checkpoint kinase Rad53 for viability as nutrients are exhausted. 5XCLN3 is not sufficient to significantly accelerate the recovery cycle. Cln3 mutants produce high yields of Q cells, but they recover from it very slowly, maintaining very high Sic1 levels until after budding resumes.

SPONTANEOUS CANCER CELL QUIESCENCE WITH EPIGENETIC REMODELING ARISING DURING MITOTIC TELOPHASE

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Human cancer cells arise through genetic mutations that drive proliferation. Paradoxically, however, we find that highly mitotic cancer cells continuously produce rare progeny that spontaneously exit the cell cycle into a quiescent state without experimental manipulation. These "G0 cancer cells" are molecularly and functionally quiescent but capable of re-entering the cell cycle and proliferating normally. Spontaneous cancer cell quiescence is associated with the global, coordinate, selective, rapid, and asymmetric loss of multiple nuclear histone marks that occurs specifically during mitotic telophase. Quiescent cancer cells express a specific transcriptional program, which includes upregulation of the HES1 transcription factor that is necessary for maintaining a quiescent state in nonmalignant cells. Furthermore, rare HES1+ G0 cancer cells are present within human breast tumors. These results reveal that highly proliferative cancer cells with extensive genetic mutation can become spontaneously dormant which might have potentially important clinical implications.

COOPERATION OF THE DAM1 AND NDC80 COMPLEXES ENHANCES KINETOCHORE-MICROTUBULE COUPLING AND IS REGULATED BY AURORA B

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During mitosis, kinetochores attach to assembling and disassembling microtubule tips while withstanding tensile forces from the mitotic spindle. This attachment is key to progression through mitosis, and regulation from the spindle assembly checkpoint ensures the detachment of aberrant kinetochore-microtubule attachments. How this attachment is maintained and the mechanism of its regulation remains unclear. Genetic, biochemical, biophysical and structural studies implicate both the Ndc80 and Dam1 complexes as essential points of direct contact between kinetochores and microtubules in budding yeast. However, the distinct contributions of each complex are not known. Using techniques for manipulating and tracking individual molecules in vitro, we demonstrated that the Dam1 complex is a processivity factor for the Ndc80 complex, enhancing its ability to form load-bearing attachments to and track with dynamic microtubule tips. Moreover, the interaction between the Ndc80 and Dam1 complexes is regulated by the yeast Aurora B kinase Ipl1. We show that Ipl1 phosphorylation of Dam1 disrupts both kinetochore-microtubule and intrakinetochore interfaces. Therefore, we provide evidence for a mechanism by which Aurora B resets aberrant kinetochore-microtubule attachments.

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RECONSTITUTION OF DYNAMIC MICROTUBULE ATTACHMENT WITH PURIFIED KINETOCHORE PARTICLES

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Chromosome segregation is directed by the kinetochore, the macromolecular protein complex that assembles onto centromeric DNA and captures spindle microtubules. The simplest characterized kinetochore is in budding yeast where 8 subcomplexes assemble onto the centromere to form a single microtubule-binding site. Conservation of kinetochore proteins suggests that the larger kinetochores of other eukaryotes that bind multiple microtubules are assembled from the repetition of the budding yeast-type kinetochores. A complete mechanistic understanding of kinetochore function requires biophysical and structural studies on purified kinetochores. However, such studies have been impossible to date due to the lack of a robust method for isolating functional kinetochores. To address this, we developed a method to purify functional kinetochore particles via purification of the budding yeast Dsn1 protein. The particles retain the majority of kinetochore proteins and bind microtubules in a manner that depends on conserved microtubule-binding subcomplexes. In optical trap assays, single particles can remain attached to assembling and disassembling microtubule tips for >20 minutes under tensile forces up to 6 pN. This coupling is far more robust than attachments formed from individual subcomplexes, indicating that cooperative interactions between subcomplexes make a major contribution. Taken together, these studies lay the foundation for uncovering the mechanisms that underlie the key mechanical and regulatory functions of kinetochores.

HISTONE H3 THR-3 PHOSPHORYLATION BY HASPIN POSITIONS THE CHROMOSOMAL PASSENGER COMPLEX AT CENTROMERES IN MITOSIS

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The Chromosomal Passenger Complex (CPC), containing INCENP, Survivin, Borealin and the kinase Aurora B, plays key roles in kinetochore assembly, the formation of kinetochore-spindle attachments and cytokinesis. The CPC is found on chromosome arms early in mitosis, but concentrates at inner centromeres during prometaphase, and transfers to the central spindle at anaphase. Both Survivin and Borealin have been proposed as targeting components of the CPC, but the chromatin elements required to concentrate the CPC at inner centromeres are unknown. Phosphorylation of Histone H3 at Thr-3 (H3T3ph), a modification carried out by the kinase Haspin, co-localizes with the CPC at inner centromeres. We find that depletion of Haspin by RNAi decreases the centromeric accumulation of the CPC. Furthermore, recombinant CPC and Survivin bind preferentially to H3T3ph-containing peptides *in vitro*. A Survivin mutant (D70A/D71A) that does not bind preferentially to H3T3ph peptides does not support CPC concentration at inner centromeres in cells. Localization of the Aurora B substrate MCAK to centromeres and spindle assembly checkpoint signaling in taxol are diminished both by Haspin depletion and in cells in which endogenous Survivin is replaced by mutant Survivin (D70A/D71A). CPC formation and other functions of Aurora B, such as Histone H3 Ser-10 and CENP-A Ser-7 phosphorylation, are little changed. These studies suggest that H3T3ph generated by Haspin positions the CPC at centromeres to selectively regulate centromeric targets of Aurora B.

HISTONE H3 PHOSPHORYLATED ON THREONINE 3 BINDS THE CPC TO ACTIVATE THE KINASE AURORA B

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Chromosomes show dramatic structural and functional changes upon entry into M phase to prepare for cell division. Although it has long been known that histones, the major protein component of the chromosomes, become highly phosphorylated at this cell cycle stage, it remains largely unclear whether and how these modifications affect the function of chromosomes during M phase. Here we provide a molecular explanation of how an M phase-specific histone phosphorylation is recognized and translated into dynamic control of mitotic architectures. We reveal that histone H3 containing the M phase-specific phosphorylation of threonine 3 (H3T3ph) recruits a critical regulator of mitosis and meiosis, the chromosomal passenger complex (CPC), to chromosomes to activate its kinase subunit, Aurora B. We demonstrate that the CPC directly binds H3T3ph via its chromatin-targeting subcomplex composed of Survivin, Dasra A and the N-terminal region of INCENP. In addition, we show that in *Xenopus* egg extracts depleted of the H3T3 kinase Haspin, chromosomal recruitment of the CPC and resulting Aurora B activation are severely impaired, causing a decrease in spindle size. Furthermore, constitutive activation of Haspin at the exit from M phase results in failure of the CPC to dissociate from chromosomes, leading to a delay in chromosome decondensation and nuclear re-formation. Our data suggest that H3T3ph acts as a spatiotemporal signal that triggers an Aurora B-dependent reaction cascade on chromosomes during M phase. Thus, this study demonstrates how the mitotic "histone code" can be read to coordinate cell cycle processes.

TAF-Iβ LOCALIZES TO MITOTIC KINETOCHORES IN A BUB1-DEPENDENT MANNER TO HELP RECRUITING SGO1

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To equally segregate duplicated genetic material into daughter cells, sister chromatids have to align at the middle of the mitotic spindle. In metazoan cells, this alignment requires a stable cohesion at the sister centromeres before the final separation in anaphase. To protect centromeric cohesion, Sgo1 is recruited to the inner kinetochores. The recruitment depends on the Bub1 kinase, which phosphorylates at T121 of histone H2A around the entromeric regions. However, the downstream mechanism of this recruitment remains unclear. In this study, we found that TAF-Iβ is recruited to the inner kinetochores from prophase to metaphase. TAF-Iβ and Sgo1 physically interacts with each other and spatially colocalize to the inner kinetochore. Depletion of Sgo1 did not affect TAF-Iβ localization. On the other hand, depletion of TAF-Iβ caused a correlative decrease in centromeric Sgo1. Similar to Sgo1, the kinetochore localization of TAF-Iβ depends on Bub1. We conclude that TAF-Iβ functions downstream of Bub1 and helps to recruit Sgo1 to the kinetochores. TAF-Iβ is a histone binding protein and has a signature earmuff domain shared by NAP family histone chaperones. We are testing the hypothesis that TAF-Iβ may bind directly to the T121-phosphorylated histone H2A and function as the reader of this histone code for sister chromatid cohesion.

BUB1 OVEREXPRESSION DRIVES SPONTANEOUS TUMORIGENESIS THROUGH A MECHANISM THAT IS MITOTIC CHECKPOINT INDEPENDENT BUT INVOLVES MICROTUBULE-KINETOCHORE ATTACHMENT ERRORS

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Bub1 is an essential component of the mitotic checkpoint, a surveillance mechanism that delays anaphase onset until all chromosomes are attached stably to the mitotic spindle. As a master regulator of the mitotic checkpoint, Bub1 has been shown to be required for the binding of several checkpoint proteins to the kinetochore, for proper metaphase congression through correct attachment of microtubles to kinetochores and is thought to contribute to the soluble signaling pathway of the mitotic checkpoint. Moreover, we and others have noted that Bub1 overexpression is a frequent event in a variety of human cancers and a reliable marker for poor clinic outcome. However, the molecular, cellular, and physiological consequences of Bub1 overexpression are not known. The only way to directly test whether the relationship between Bub1 overexpression and the development of tumors is causal is to generate a Bub1 transgenic mouse model. As such, we engineered Bub1 transgenic mice in which Bub1 is overexpressed in a graded fashion. Here we demonstrate that Bub1 overexpression results in aneuploidy in both MEFs and splenocytes. Unlike loss of Bub1 protein, Bub1 overexpressing cells properly inhibit anaphase progression in response to either nocodazole or taxol. Moreover, kinetochores of Bub1 overexpressing cells have a normal complement of BubR1, Cdc20, Mad1, Mad2, and CENP-E. Therefore, Bub1 overexpression does not deregulate mitotic checkpoint signaling or kinetochore assembly. Interestingly, Bub1 overexpression exclusively causes lagging chromosomes in anaphase, indicating that high Bub1 levels disrupt the mechanism that detects and/or resolves aberrant, merotelically attached chromosomes. Finally, consistent with the observed chromosome missegregation and aneuploidy, Bub1 transgenic mice are susceptible to spontaneous tumorigenesis and to Myc-induced lymphomagenesis. These data demonstrate that Bub1 overexpression is causally implicated in tumor development and suggest that merotelic attachments are driving this phenotype. Thus, our results identify Bub1 as a chromosomal instability gene with oncogenic properties and a potential critical target for anti-cancer therapies that may benefit patients otherwise refractory to treatment.

THE ASTRIN COMPLEX IS TARGETED TO KINETOCHORES THAT ARE UNDER TENSION TO STABILIZE CORRECT ATTACHMENTS

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The kinetochore is a multi-protein assembly that connects mitotic spindle microtubules to chromosomes. The kinetochore transmits a poleward force from microtubules to the DNA to drive chromosome movement, and is also a central point of regulation to ensure that genome is distributed evenly to both daughter cells. In our ongoing effort to define the protein composition of the kinetochore, we have identified a complex comprised of the spindle and kinetochore associated proteins Astrin and SKAP. Astrin and SKAP show interdependent localization and this complex is required for proper chromosome segregation. Interestingly, the localization of the Astrin/SKAP complex is antagonized by Aurora B activity. This differs from previous studies that have suggested that Aurora B is instead required for proper kinetochore assembly and maintenance. Thus, Astrin/SKAP localization is prevented until the presence of tension reduces Aurora B activity at the outer kinetochore. Our phenotypic analysis indicates that the Astrin complex is not required for chromosome congression, but is required to maintain chromosome alignment. In total, Our results suggest that the Astrin/SKAP complex is targeted to sister kinetochores that are under tension to stabilize the attachment of correctly aligned chromosomes.

ORC SUBUNITS Orc2 AND Orc3 ARE REQUIRED FOR STABLE SPINDLE ATTACHMENT AT HUMAN CHROMOSOME KINETOCHORES DURING MITOSIS

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Replication of the human genome prior to each cell division is a highly coordinated process. Accurate inheritance of genetic information requires precise coordination of DNA replication and chromosome segregation during each cell division cycle. Loading of the ORC protein subunits onto replication origins is the earliest step in DNA replication and Orc2 and Orc3 serve as core subunits of the ORC complex. In addition to its role in DNA replication, Orc2 localizes to centrosomes and centromeres during the entire chromosome inheritance cycle. Previous data indicated that Orc2 depletion by RNA interference causes 30% of the cells to arrest in a pseudo-mitotic state, with abnormally condensed chromosome structure and chromosomes not associated with the mitotic microtubule spindles. A similar phenotype is induced when Orc3 is depleted. Immuno-florescence demonstrates that the Orc2-depleted, mitotically arrested cells have many kinetochore proteins correctly localized, including Bub1, BubR1, CENP-F, and NDC80, but the spindle assembly checkpoint protein Mad2 and the anaphase regulator Cdc20 were absent from the kinetochores, even though the microtubule spindles were not attached to the condensed chromosomes. Live cell microscopy using cells with a GFP-tagged Mad2 protein showed, however, that the Mad2 proteins did transiently attach to kinetochores, suggesting that the spindle assembly checkpoint was satisfied. Consistent with this, Cyclin B and Securin, both of which are substrates of APC/C, are dramatically reduced and absent respectively. Live cell images following Orc2 depletion showed that the chromosomes attempt to align in metaphase, and then the cells try to execute anaphase and then chromosomes come off the spindle. The increased distances between sister centromeres in Orc2 depleted chromosome indicated that centromeres have been pulled apart while the sister chromosomes are still associated with each other. Furthermore, the spindle motor protein CENP-E, which provides a connection between the kinetochores and spindles were absent after Orc2 depletion. Co-immuno-precipitation of Orc2/3 with both BubR1 and CENP-E occurs in mitotic cells and co-localization of Orc2 with BubR1 was observed on metaphase chromosomes. We suggest that in the absence of Orc2/3, spindles attach transiently, satisfying the spindle assembly checkpoint, but stable spindle attachment is lost, thereby arresting cells in an irreversible pseudo mitotic state. Since Orc2 and Orc3 participate directly centromere activity and chromosome segregation during mitosis beyond their known roles in the initiation of DNA replication, we suggest that there is an ancient link between origins of DNA replication and chromosome segregation.

LOSS OF PRB CAUSES CENTROMERE DYSFUNCTION AND CHROMOSOMAL INSTABILITY

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Chromosome instability (CIN) is a common feature of tumor cells. By monitoring chromosome segregation, we show that pRB-depletion causes rates of mis-segregation comparable to those seen in CIN tumor cells. The retinoblastoma tumor suppressor is frequently inactivated in human cancers and is best known for its regulation of the G1/S-phase transition. Recent studies have shown that pRB inactivation also slows progression through mitosis and promotes aneuploidy, but reasons for these phenotypes are not well understood. Here we describe the underlying mitotic defects in pRB-deficient cells that cause chromosome mis-segregation. Analysis of mitotic cells reveals that the loss of pRB increases inter-centromeric distance and deforms centromeric structure. These defects promote merotelic attachment resulting in failure of chromosome congression and an increased propensity for lagging chromosomes following mitotic delay. While complete loss of centromere function or chromosome cohesion would have catastrophic consequences, these more moderate defects allow pRB-deficient cells to proliferate but undermine the fidelity of mitosis, leading to whole chromosome gains and losses. These observations explain an important consequence of RB1 inactivation and suggest that subtle defects in centromere function are a frequent source of merotely and CIN in cancer.

A MECHANISM LINKING WHOLE-CHROMOSOME ANEUPLOIDY TO DNA BREAKS

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Cancer is associated with aneuploidy, both structural defects in chromosomes and abnormal numbers of intact chromosomes. The contribution of chromosome breaks to tumorigenesis is well accepted because of the possibility of generating cancer-causing mutations. However, the role of whole chromosome aneuploidy remains a subject of debate, principally because mechanisms by which whole chromosome aneuploidy would affect cancer pathogenesis are poorly understood. We now have evidence supporting one mechanism by which whole chromosome aneuploidy can lead to DNA breaks, via mis-segregation of chromosomes into structures commonly observed in cancer cells called micronuclei. Focusing only on micronuclei that originate from mitotic errors and contain a whole chromosome, we will present evidence for a mechanism whereby whole chromosome micronuclei develop breaks due to aberrant DNA replication and repair. We identify changes in the chromatin state of micronuclei as well as defects in the assembly of nuclear pore complexes and will discuss the implications of these findings for the mechanism of DNA breakage. Together, these results suggest a mechanistic link between errors in mitosis and the generation of DNA breaks.

CEP192 PROMOTES CENTROSOME-DRIVEN SPINDLE ASSEMBLY BY ENGAGING IN ORGANELLE-SPECIFIC AURORA A ACTIVATION

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Accurate chromosome segregation during cell division depends on spatially and temporally controlled protein phosphorylation/dephosphorylation events. The serine/threonine kinase Aurora A (AurA) contributes to multiple aspects of mitosis with key roles in bipolar spindle formation and centrosome function. During spindle assembly promoted by chromatin, AurA is targeted to microtubules by its partner, the microtubule-binding protein TPX2. TPX2 also activates AurA, both allosterically and by protecting the activation loop of its kinase domain from dephosphorylation. The AurA-TPX2 complex participates in setting a proper spindle length, while it is not required for centrosome-mediated spindle assembly. The mechanism underlying AurA recruitment and regulation at centrosomes remains unclear.

We found that a core centrosome protein, Cep192, forms distinct complexes with AurA and acts as its specific cofactor in centrosome-driven spindle assembly. Binding to endogenous Cep192, unlike binding to TPX2, does not, in itself, activate AurA but renders it capable of activation following kinase dimerization. Cep192 targets AurA to centrosomes, where its high local concentration promotes AurA dimerization, which, in the presence of bound Cep192, activates the kinase. This, in turn, drives microtubule nucleation and assembly.

The extent of Cep192/dimerization-induced AurA activation significantly exceeds that resulting from the AurA-TPX2 interaction, thus insuring that more potent AurA activation occurs at centrosomes than in other cellular compartments. This might be critical for the function of centrosomes as dominant microtubule-organizing centers. A “milder” AurA activation mode appears to be employed by TPX2 in chromatin-driven microtubule assembly.

Thus, the role of AurA in the two independent pathways of spindle formation mediated by chromatin and centrosomes relies on spatial and quantitative control of its kinase activity by two distinct cofactors (TPX2 and Cep192 respectively) that utilize different organelle-targeting and AurA regulation strategies.

CEP152 IS REQUIRED FOR PLK4-INDUCED CENTRIOLE DUPLICATION

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When cells divide, it is essential that they segregate their newly duplicated chromosomes into two equal sets. The centrosome plays a fundamental role for nucleating and organizing cytoplasmic microtubules during interphase and promoting the assembly of the mitotic spindle during mitosis. Centrosomes assemble from centrioles that, like DNA, are duplicated once per cell cycle. Aberrant numbers of centrosomes directly promote chromosome missegregation thus contributing to chromosomal instability. Both gain- and loss-of-function studies have identified the Polo-like kinase Plk4/Sak as a crucial regulator of centriole biogenesis. We have identified the novel human centriolar protein, Cep152, the ortholog of Drosophila asterless, Asl, as a direct target and binding partner of Plk4. Cep152 interacts with the cryptic Polo-box of Plk4 via its N-terminal domain. Ablation of Cep152 function by RNAi or expression of a dominant-negative form of Cep152 leads to loss of centrioles, formation of monopolar mitotic spindles and a failure in centriole duplication. Furthermore, we show that Cep152 is required for the Plk4 induced overduplication of centrosomes. Interfering with Plk4 or Cep152 function promotes loss of CPAP, a protein essential for the control of centriole length in Plk4 regulated centriole biogenesis, at the centrosome. The localization of other components of the centriole assembly pathway, hsSas6, CP110 or Cep135 was not impaired by Cep152 siRNA treatment of U2OS cells. Together, these findings demonstrate that Cep152 is an important component in Plk4-induced centriole biogenesis.

AURORA A-DEPENDENT RECRUITMENT OF TACC3 TO SPINDLES REVEALS A COMPLEX CONTAINING CLATHRIN AND A NOVEL +TIP.

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Work over the last decade has shown that chromosome segregation is dependent on a handful of mitotic kinases, among them Aurora A. However, which individual mitotic processes are regulated by Aurora A, and how they are coordinated on a molecular level, is still largely unknown. Aurora A is directed to act on spatially and temporally unique substrates in the cell as it proceeds through mitosis, complicating its study by protein depletion techniques such as RNAi. We have previously shown that point mutations in TPX2 disrupting the activation of Aurora A reveal one specific role for Aurora A, to facilitate spindle pole separation through modulation of microtubule (MT) nucleation and stability. Mass spectrometry-based phosphorylation analyses utilizing these mutants found that the spindle associated protein TACC3 is a TPX2-dependent target of Aurora A phosphorylation. When the Aurora A sites in TACC3 are mutated, TACC3 is no longer targeted to the spindle, and the cells have consequent defects in spindle assembly.

To investigate how phosphorylation of TACC3 targets it to spindles, we quantitatively compared proteins that interact with TACC3 normally with proteins that interact either with a phospho-site-mutated, or Aurora A inhibitor-treated TACC3 protein. This analysis led to the discovery of a clathrin-containing complex that interacts with TACC3 in a phospho-dependent manner. Clathrin heavy chain (CLTC) targets TACC3 to spindles, suggesting an explanation for the observed role of clathrin in MT stability. We also uncovered a novel +TIP protein that associates with TACC3 in a phospho-dependent manner. This protein is required for progression through mitosis and tracks growing MTs in an EB1/EB3-dependent manner *in vitro*.

α-ENDOSULFINE, A NOVEL SUBSTRATE OF GREATWALL KINASE, ENSURES MITOTIC PROGRESSION BY INHIBITING CDK-OPPOSING PP2A-B55Δ IN *XENOPUS* EGG EXTRACTS

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CDK1 is the major cell-cycle controller essential for mitotic phosphorylation. Its activity is modulated through the phosphorylation status of its Tyr15 residue and by cyclin synthesis and degradation. We reported last year that PP2A-B55δ hetero-trimer complex is the major phosphatase for model CDK substrates in *Xenopus* egg extracts and has anti-mitotic activity. PP2A-B55δ activity is high in interphase and completely suppressed in mitosis, exactly opposite to CDK activity. Other groups have reported that Greatwall, a protein kinase required for proper mitosis in flies and frogs, is somehow responsible for the mitotic suppression of PP2A activity. We have discovered that α-Endosulfine and its close relative ARPP-19 are substrates of Greatwall, and find that phosphorylated endosulfine or ARPP-19 specifically inhibit PP2A-B55 *in vitro*. α-Endosulfine is quantitatively phosphorylated in mitotic *Xenopus* extracts and dephosphorylated in interphase. Immunodepletion of α-Endosulfine strongly inhibited mitotic entry in extracts whereas addition of extra (thiophosphorylated) α-Endosulfine accelerated mitotic entry. These results suggest that CDK activation directs an inactivation of the opposing phosphatase activity via a pathway involving Greatwall and α-Endosulfine, helping to sharpen the transition of phosphorylation status from interphase to mitosis. These results account for the phenotype of endosulfine mutants in *Drosophila* (see Von Stetina JR, Tranguch S, Dey SK, Lee LA, Cha B, Drummond-Barbosa D. (2008). Development 135:3697).

BUDDING YEAST WEE1 INHIBITS THE METAPHASE TO ANAPHASE TRANSITION

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Cdk1 promotes the metaphase to anaphase transition by phosphorylating the anaphase-promoting complex (APC), a multisubunit ubiquitin ligase that targets specific mitotic regulators for degradation by the proteasome. The degradation of these proteins is critical for accurate sister chromatid segregation and mitotic exit.

Swe1 (the budding yeast homolog of Wee1) inhibits Cdk1 by phosphorylation on a conserved tyrosine residue. We believe that this inhibitory phosphorylation on Cdk1 plays a role in restraining APC activation in every cell cycle. Deletions of the kinase, Swe1, or the phosphatase, Mih1 (the budding yeast Cdc25) responsible for regulating the levels of Cdk1 inhibitory phosphorylation, both interact genetically with mutations in the APC activators *CDC20* and *CDH1*. Furthermore, we have shown with live imaging that *swe1*Δ cells spend a shorter time in mitosis, while *mih1*Δ cells spend a longer time, indicating *SWE1* may be important in the timing of the metaphase to anaphase transition. Consistent with this role of Swe1, activation of a Swe1-dependent checkpoint causes a prolonged arrest in metaphase.

Overexpression of Swe1 stabilizes mitotic cyclins and Pds1 (securin), and impairs sister chromatid segregation. These effects depend on Swe1 inhibition of Cdk1, and are not due to activation of the DNA damage or spindle checkpoint, which both act to inhibit the metaphase to anaphase transition. If Swe1 is overexpressed in anaphase, after Pds1 has been degraded, we see that Pds1 is re-stabilized, showing that Swe1 can block APC-mediated proteolysis throughout mitosis. Swe1 overexpression reduces APC phosphorylation in vivo, and we are currently testing if this decrease in phosphorylation causes a drop in the in vitro ubiquitination activity of the APC.

We believe the effects of Swe1 are therefore caused by inhibition of Cdk1-dependent phosphorylation of APC subunits, and suggest that Cdk1-dependent phosphorylation of the APC may be essential for mitotic APC activity. Furthermore, our data suggest that *SWE1* plays a role in the normal timing of the metaphase to anaphase transition, in addition to its well-established role regulating mitotic entry.

THE MITOTIC EXIT OSCILLATOR AT WORK

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Mitotic cyclin-dependent kinases (CDKs) promote entry in mitosis. In the budding yeast *Saccharomyces cerevisiae*, phosphatase Cdc14 orchestrates exit from mitosis by inactivating CDKs and reversing CDK-mediated phosphorylation events. During anaphase, Cdc14 is released from its inhibitor Cfi1/Net1 by two successive pathways: FEAR (Cdc*F*ourtheen *E*arly *A*naphase *R*elease) and MEN (*M*itotic *E*xit *N*etwork). Three sets of kinases play a critical role in the Cdc14-release process: the Polo-like kinase Cdc5, the mitotic CDKs themselves, and the most downstream MEN kinase Dbf2. The mechanism that coordinates these different kinases resulting in Cdc14 activation remains unclear. Here we show that Cdc14 release is a two-hit process that requires Cdc5 plus either MEN or CDKs. Once released, Cdc14 triggers a negative feedback loop composed of Cdc5-Cdc14-Cdh1-Cdc5. In the presence of stable levels of mitotic cyclins, this negative feedback generates cycles of Cdc14 release and sequestration. Interestingly this “mitotic oscillator” works also in presence of very high levels of mitotic cyclins. This observation suggests that next to the overall inactivation of mitotic cyclins, the completion of mitosis also relies on the temporally and spatially controlled phopshorylation status of different substrates determined by the mitotic-CDKs/Cdc14 ratio.

A NOVEL TYPE OF POLO-BOX DOMAIN (PBD)-DBF4 INTERACTION CONTROLS MITOTIC EXIT

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Cdc7-Dbf4 is a conserved, two-subunit kinase required for initiating eukaryotic DNA replication. Recent studies have shown that Cdc7-Dbf4 also regulates the mitotic exit network (MEN) and monopolar homolog orientation in meiosis I[1,2]. Both activities likely involve a Cdc7-Dbf4 interaction with Cdc5, the single Polo-like kinase in budding yeast. We previously showed that Dbf4 binds the Cdc5 polo-box domain (PBD) via a ~40 residue N-terminal sequence, which lacks a PBD consensus binding site (S(pS/pT)P/X), and that Dbf4 inhibits Cdc5 function during mitosis[1]. Here we refine the requirements for binding and demonstrate that the PBD-Dbf4 interaction occurs via a distinct PBD surface from that used to bind phospho-proteins. Genetic and biochemical analysis of multiple *dbf4* mutants indicates that Dbf4 inhibits Cdc5 function through direct binding. Surprisingly, mutation of invariant Cdc5 residues required for binding phosphorylated substrates has little effect on yeast viability or growth rate. Instead, *cdc5* mutants defective for binding phospho-proteins exhibit increased resistance to microtubule disruption and have elongated spindles. This study therefore defines the molecular basis for a new mode of PBD binding and reveals that Cdc5 targeting to phosphorylated substrates regulates spindle dynamics. Our data also suggest that Cdc5 binding to phosphorylated substrates is not essential and raises the possibility that essential Cdc5 substrates might be recognized through a Dbf4-like binding site.

1. Miller et al. (2009) PLoS Genetics 5:e1000498
2. Matos et al. (2008) Cell 135:662-78

MECHANISM OF CYTOKINESIS INHIBITION BY THE CHECKPOINT PROTEIN, DMA1.

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Proper cell division requires strict coordination between mitotic exit and cytokinesis. In the event of a mitotic error, cytokinesis must be inhibited to ensure each daughter cell receives the correct genetic material. In the fission yeast, *Schizosaccharomyces pombe*, the checkpoint protein and E3 ubiquitin ligase, Dma1, delays cytokinesis by inhibiting the septation initiation network (SIN) when the mitotic spindle is compromised. To elucidate the mechanism by which Dma1 inhibits the SIN, we screened all SIN components as potential Dma1 substrates. We found that the SIN scaffold protein, Sid4, is ubiquitinated *in vivo* in a Dma1 dependent manner, while we detected no evidence for ubiquitination of any other SIN component. To investigate the role of Sid4 ubiquitination in checkpoint function, we generated a ubiquitination deficient *sid4* allele and our data suggest that Sid4 ubiquitination by Dma1 is required to maintain a checkpoint arrest. Furthermore, Sid4 ubiquitination appears to delay recruitment of the Polo-like kinase and SIN activator, Plo1, to spindle pole bodies (SPBs) during checkpoint activation, providing a mechanistic link between Dma1 activity and cytokinesis inhibition. Collectively, we present a novel mechanism of cytokinesis inhibition by the checkpoint protein, Dma1.

p27^{Kip1} CONTROLS CYTOKINESIS VIA THE REGULATION OF CITRON-KINASE ACTIVITY

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The cell cycle inhibitor p27^{Kip1} (p27) also has cyclin-CDK independent functions. For instance, p27 binds to and prevents the activation of the GTPase RhoA, thereby regulating cell migration and actin cytoskeleton dynamics. To investigate the significance of the cyclin-CDK independent functions of p27 in vivo, we generated a knock-in mouse in which four amino acid substitutions in p27 prevent its interaction with cyclins and CDKs (p27CK-). The p27CK- mutation dominantly causes hyperplastic lesions and tumors in multiple organs compared to wild-type and p27-/- mice, revealing an oncogenic role for p27.
We find that the p27CK- protein causes multinucleation, polyploidy and centrosome amplification in the liver, kidney and primary fibroblasts; a phenotype that resembles that of Skp2-/- mice, in which p27 levels are elevated during the S and G2/M phases of the cell cycle. The p27CK- protein is insensitive to Skp2-mediated degradation, also resulting in elevated levels of the protein during the cell cycle. Thus our data indicates an important role for p27 in G2/M that is independent of cyclin/CDK regulation. Further analysis revealed that the p27CK- protein causes a cytokinesis and abscission defect.
We identified the Rho effector Citron-Kinase as a p27-interacting protein in a mass spectrometry screen. The phenotype caused by p27CK- is similar to that observed upon deletion or inhibition of Citron-Kinase. The interaction of the two proteins was confirmed and the interaction domain of p27 on Citron-Kinase was mapped. Over-expression of this minimal interaction domain was sufficient to suppress the phenotype caused by p27CK-. Finally, we find that by binding to Citron-Kinase, p27 prevents the interaction of Citron-Kinase with RhoA and its activation. Overall, we have identified a novel role for p27 during cytokinesis via the regulation of Citron-Kinase activity.

EXAMINING MECHANISMS THAT DETERMINE THE POSITION OF THE CELL DIVISION PLANE

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The accurate placement of the cell division plane during cell division is essential for error-free genome propagation. I will discuss two mechanisms that may contribute to this process. One involves a spatial Aurora-kinase dependent phosphorylation gradient that extends across the spindle midzone, an interdigitating array of anti-parallel microtubules that forms during anaphase. The second involves PRC1 (MAP65/Ase1), a non-motor microtubule crosslinking protein required for spindle midzone formation and successful cytokinesis in eukaryotes. I will discuss findings from biophysical and structural studies that suggest PRC1 functions as a dynamic tracker of antiparallel microtubule overlap, analogous to +TIP proteins that track plus-ends of growing microtubules. This functional specialization in a non-motor microtubule-associate protein could help 'mark' nano-scale geometric features in self-organizing cytoskeletal networks. Such 'marks' can recruit enzymes, such as kinases, to specific intracellular sites and establish micron-scale spatial patterns that may provide positional cues for proper cell cleavage.

STRUCTURAL ELECTRON MICROSCOPY STUDIES OF THE COP9 SIGNALOSOME IN COMPLEX WITH NEDDYLATED SCF

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Ubiquitination and phosphorylation are arguably the two post translational modifications most important for cell cycle progression. The evolutionary conserved COP9 Signalosome (CSN) acts at the interface between kinase signalling and protein degradation. It is known to associate with kinases which phosphorylate NF-κB and p53 among others. CSN is even better characterised as a regulator of cullin ring E3 ligases (CRL). The rate of CRL mediated ubiquitin transfer is increased by attachment of the ubiquitin-like protein Nedd8 (neddylation). CSN catalytically reverses this modification. Moreover, there is evidence that CSN is not a mere deneddylase in cells but also an assembly platform for its modular CRL substrates such as SCF.

Despite its central role in cell metabolism, CSN was poorly understood at the structural level. In order to address this, we developed an insect cell co-expression system from which we purify large amounts of homogenous, correctly assembled and catalytically active human CSN. We report the first structure of CSN using electron microscopy and single particle analysis and segment the map by localising the catalytic subunit CSN5 and some of its helical-repeat scaffold components. Furthermore, we show structural analysis of CSN in complex with its neddylated substrate SCF and propose a mechanism of Nedd8 chain deconjugation.

CELL CYCLE CONTROL BY REPLICATION-COUPLED PROTEIN DESTRUCTION

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Cell proliferation depends on the timely synthesis and destruction of proteins at specific phases of the cell cycle. There is increasing evidence that several key cell cycle regulatory proteins must be destroyed during S phase to ensure normal cell cycle progression. Recently it was discovered that these proteins harbor a motif called a PIP degron that mediates binding to chromatin-bound PCNA at replication forks and recruits the $CRL4^{Cdt2}$ E3 ubiquitin ligase. These interactions comprise a mechanism for coupling DNA replication with ubiquitylation and subsequent proteolysis by the 26S proteasome. We developed a flow cytometry-based method using *Drosophila* S2 cells that recapitulates S phase-specific protein proteolysis. Using this assay we discovered a PIP degron within *Drosophila* E2f1 and demonstrated that PCNA and $CRL4^{Cdt2}$ are necessary for S phase-specific destruction of E2f1, a member of the activating class of E2F transcription factors that controls cell cycle progression. We hypothesize that S phase-specific destruction provides an important pRb-independent negative regulation of E2f1. We will present new data regarding the mechanism and function of replication-coupled E2f1 destruction.

CRL4^{CDT2} REGULATES G2/M PROGRESSION BY TARGETING THE P53 AND HISTONE H4K20 METHYLTRANSFERASE SET8 FOR DEGRADATION

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Chromatin modifications play central role in various biological processes including cellular proliferation, differentiation, gene expression and DNA repair. The histone methyltransferase PR-Set7/Set8 is a cell-cycle-regulated enzyme that regulates chromosomal structure and stability through the monomethylation of lysine 20 of histone 4 (H4K20). It also monomethylates p53 on K382 to suppress transcriptional activation by p53. CRL4-Cdt2 is a Cullin complex that degrades several proteins involved in maintenance of genome stability, e.g. Cdt1, p21, pol eta (in C. elegans) and E2F1 (in Drosophila). We report that CRL4 is the principal E3 ubiquitin ligase responsible for Set8 proteolytic degradation in the S-phase of the cell-cycle. This activity requires the substrate recognition factor Cdt2/DTL and is dependent on the physical interaction of Set8 with chromatin-bound PCNA. We show that CRL4^{Cdt2} promotes Set8 degradation specifically in early and mid S-phase of the cell cycle and in cells irradiated with UV. Inactivation of the CRL4–Cdt2-PCNA-Set8 degradation machinery stabilizes Set8 in S-phase, inhibits cellular proliferation, and arrests the cells in the G2 phase of the cell cycle with chromatin de-condensation. All these effects are also seen in p53 mutant cell lines. The results indicate that the degradation of Set8 by CRL4-Cdt2 in early and mid S phase is essential for cellular proliferation and passage of cells through late S and G2 phases of the cell-cycle.

CRL4^{CDT2} -DEPENDENT DESTRUCTION OF THE SET8 HISTONE METHYLTRANSFERASE IN S PHASE AND AFTER DNA DAMAGE

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The protein methyltransferase, Set8 (KMT5A /PR-Set7/SETD8), is important for the maintenance of genomic stability and essential for cell viability. Previous studies have shown that Set8 protein levels are cell cycle-regulated, being most abundant during G2 and mitosis, and low during S phase. Concomitant with its accumulation in G2 and M phases, Set8 promotes the monomethylation of histone H4 on lysine 20 (H4K20me1), which is involved in chromatin compaction. While Set8 has a clear function in mitosis, why and how Set8 is down-regulated during S phase is not clear.

Here, we show that Set8 is degraded in a DNA replication-coupled and a DNA damage-induced manner. We identify the E3 ubiquitin ligase, CRL4^{Cdt2}, as a critical regulator of Set8 during S phase and in response to DNA damage. Treatment of cells with siRNAs targeting members of the CRL4^{Cdt2} complex increases the steady state levels of Set8 in unperturbed cells and inhibits its UV-induced destruction. Furthermore, disruption of a specific PCNA interacting protein motif (PIP box) within Set8 abolishes its interaction with CRL4^{Cdt2} and inhibits its degradation. The DNA damage-induced and replication-coupled degradation of Set8 is conserved in *Xenopus* egg extracts, where destruction of Set8 is inhibited either by mutations in its PIP box or by depletion of PCNA or Cdt2. In this cell-free system, ubiquitylated forms of Set8 are detected on chromatin. In cells, Set8 is rapidly degraded during S phase, but when Set8 expression is constitutively induced, proliferation is slowed, cells accumulate in the S and G2 phases, and the replication checkpoint is elicited. When expression of the non-degradable PIP box mutant of Set8 is constitutively induced, a dramatic loss of cells in S phase and accumulation of cells in G2 occurs. Transient expression of this Set8 mutant during recovery from a replication arrest results in an increase in H4K20me1 and recruitment of 53BP1 and L3MBTL1 to chromatin, as well as prolonged Chk1 activation. We propose a model whereby Set8 levels are suppressed during S phase to prevent aberrant monomethylation of H4K20, which interferes with DNA replication.

PHOSPHORYLATION BY CASEIN KINASE I PROMOTES THE TURNOVER OF MDM2 VIA THE SCF$^{\beta TRCP}$ UBIQUITIN LIGASE

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The Mdm2/p53 pathway is compromised in more than 50% of human cancers. The dynamics of the p53/Mdm2 pathway is influenced by DNA damage-induced turnover of the p53 ubiquitin ligase Mdm2 but the underlying molecular mechanisms remain unclear. Here we report that Mdm2 is unstable, and that phosphorylation of Mdm2 by Casein Kinase I (CKI) at multiple sites triggers its interaction with, and subsequent ubiquitination and destruction, by SCFβ-TRCP. Inactivation of either β-TRCP or CKI results in accumulation of Mdm2 and decreased p53 activity, and resistance to apoptosis induced by DNA damaging agents. Furthermore, Mdm2C464A mutant could be efficiently degraded by SCFβ-TRCP. These findings reveal a novel pathway that controls Mdm2 stability independently of its own ubiquitin ligase activity, and thereby controls p53 activity in response to genotoxic stress. Hence, our results provide new insight into the signaling pathways controlling Mdm2 destruction and further suggest that compromised regulation of Mdm2 results in attenuated p53 activity, thereby facilitating tumor progression.

MECHANISMS OF UBIQUITINATION BY THE ANAPHASE-PROMOTING COMPLEX

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The anaphase-promoting complex or cyclosome (APC) is a multisubunit ubiquitin-protein ligase (E3) that catalyzes the ubiquitination of securin and cyclins, thereby promoting their destruction and driving the cell through the metaphase-anaphase transition. Like other E3s, the APC promotes ubiquitination by positioning the substrate adjacent to an E2-ubiquitin conjugate, allowing nucleophilic attack by a substrate lysine on a thioester bond linking the C-terminus of ubiquitin to a cysteine in the E2 active site. Conserved residues in the E2 active site are thought to catalyze this reaction.

Different E2 family members display distinct specificities for substrate lysines in different contexts. In the case of the budding yeast APC, we showed in previous work that the initial monoubiquitination of lysines on the substrate is most efficiently catalyzed by the general E2, Ubc4. A different E2, Ubc1, then interacts with the APC to catalyze rapid ubiquitination of a specific lysine, K48, on pre-attached ubiquitin, resulting in K48-linked polyubiquitin chains that target the substrate to the proteaseome for destruction. In the current work, we addressed the mechanistic basis for the K48 selectivity of Ubc1. We identified polar residues near the Ubc1 active site, as well as a residue in ubiquitin itself, that catalyze K48-specific ubiquitin ligation but are not required for general activity toward other lysines. Our results suggest that the active site of Ubc1, as well as the surface of ubiquitin, contain specificity determinants that channel specific lysines to the central residues involved directly in catalysis. Our studies emphasize the central role of the E2 active site in the catalysis and specificity of protein ubiquitination.

REGULATING THE REGULATOR: THE SAC AND THE APC/C

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We are trying to understand how cells divide to ensure that their two daughter cells receive an identical copy of the genome. 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 and have recently begun to use this to study the enzymology of the APC/C. This has revealed that the checkpoint is intrinsic to the timing of mitosis, and that to maintain checkpoint-arrested cells in mitosis the APC/C targets its own regulator, Cdc20, for destruction. We are particularly interested in how the spindle checkpoint acts on the APC/C, and how the APC/C is able to target some substrates when the checkpoint is active while others are stabilized. Our studies have revealed that some substrates are able to compete with the checkpoint proteins for Cdc20, and that these substrates are recognized in a different manner by the APC/C.

DISSECTION OF ACM1, A PSEUDOSUBSTRATE INHIBITOR OF THE ANAPHASE PROMOTING COMPLEX IN BUDDING YEAST

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The Anaphase Promoting Complex (APC) is a multi-subunit ubiquitin ligase responsible for targeting cell cycle regulators for degradation by the 26S proteasome. The WD40-containing proteins Cdc20 and Cdh1 function as APC co-activators that directly bind substrates via the latter's destruction boxes (DB) and KEN boxes. Recently, Acm1 was identified as an APC^{Cdh1} inhibitor. Although Acm1 interacts with Cdh1 through a DB and a KEN Box, Acm1 is not an APC^{Cdh1} substrate. Rather, it acts as a competitive ("pseudosubstrate") inhibitor of substrate binding to Cdh1. We explored what properties make Acm1 an APC inhibitor rather than an APC substrate. Using alanine-scanning mutagenesis, we identified the Acm1 "A-motif", distinct from its DB and KEN box, which is involved in its binding to Cdh1. The KEN box, DB and the A-motif were all required for APC^{Cdh1} inhibition both in vivo and in vitro. A genetic screen identified Cdh1 WD40 residues that were important for A-motif recognition and Acm1-mediated inhibition. These residues are predicted to lie near those involved in recognition of DBs. We next explored the possibility that an APC substrate could be converted into an APC inhibitor by preventing its ubiquitination. We found that an APC substrate (Hsl1) lacking its ubiquitin-acceptor lysine residues bound tightly to Cdh1 and behaved as a potent APC^{Cdh1} inhibitor. Together these findings suggest that tight Cdh1-binding combined with the absence of ubiquitination of the Cdh1-bound protein leads to APC^{Cdh1} inhibition.

SCF-CYCLIN F-MEDIATED DEGRADATION OF CP110 MAINTAINS CENTROSOME HOMEOSTASIS AND THE FIDELITY OF MITOSIS

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Most F-box proteins are the substrate recognition subunits of SCF (Skp1-Cul1-F-box protein) ubiquitin ligase complexes, which mediate the timely proteolysis of important eukaryotic regulatory proteins. Mammalian genomes encode roughly 70 F-box proteins, but only a handful have established functions. The F-box protein family obtained its name from Cyclin F (also called Fbxo1), in which the F-box motif (the ~40 amino acid domain required for binding to Skp1) was first described. Cyclin F, which is encoded by an essential gene, also contains a cyclin box domain, but in contrast to most cyclins, it does not associate with - nor does it promote the activity of - any cyclin-dependent kinases (CDKs) or other kinases.However, like other cyclins, Cyclin F oscillates during the cell cycle, with protein levels peaking in G2. Despite its essential nature and status as the founding member of the F-box protein family, Cyclin F remains an orphan protein, whose functions are unknown. Using an unbiased screen, we identified CP110, a protein essential for centrosome duplication, as an interactor of Cyclin F. Utilizing a mode of substrate binding distinct from other F-box protein-substrate pairs, CP110 and Cyclin F physically associate on the centrioles, and CP110 is ubiquitylated via the SCFCyclin F ubiquitin ligase complex, leading to degradation during the G2 phase of the cell cycle. siRNA-mediated depletion of Cyclin F induces centriole amplification and mitotic abnormalities, such as multipolar spindles and asymmetric bipolar spindles. These phenotypes were reverted by co-silencing CP110 and were recapitulated by expressing a stable mutant of CP110 that is unable to bind Cyclin F. Cyclin F knockout (KO) MEFs show higher levels of CP110 and centrosomal amplification, as compared to WT MEFs. Finally, expression in cultured cells of a stable CP110 mutant also promotes the formation of micronuclei, a hallmark of chromosome instability. We propose that SCFCyclin F–mediated degradation of CP110 is required for the fidelity of mitosis and genome integrity.

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VISITOR INFORMATION

EMERGENCY	CSHL	BANBURY
Fire	**(9) 742-3300**	**(9) 692-4747**
Ambulance	**(9) 742-3300**	**(9) 692-4747**
Poison	**(9) 542-2323**	**(9) 542-2323**
Police	**(9) 911**	**(9) 549-8800**
Safety-Security	**Extension 8870**	

Emergency Room **Huntington Hospital** 270 Park Avenue, Huntington	**631-351-2300** **(1037)**
Dentists Dr. William Berg Dr. Robert Zeman	 **631-271-2310** **631-271-8090**
Doctor MediCenter 234 W. Jericho Tpke., Huntington Station	**631-423-5400** (**1034**)
Drugs - 24 hours, 7 days Rite-Aid 391 W. Main Street, Huntington	**631-549-9400** (**1039**)

Free Speed Dial
Dial the four numbers (********) from any **tan house phone** to place a free call.

GENERAL INFORMATION

Books, Gifts, Snacks, Clothing, Newspapers
BOOKSTORE 367-8837 (hours posted on door)
Located in Grace Auditorium, lower level.

Photocopiers, Journals, Periodicals, Books, Newspapers
Photocopying – Main Library
Hours: 8:00 a.m. – 9:00 p.m. Mon-Fri
10:00 a.m. – 6:00 p.m. Saturday
Helpful tips - Obtain PIN from Meetings & Courses Office to enter Library after hours. See Library staff for photocopier code.

Computers, E-mail, Internet access
Grace Auditorium
Upper level: E-mail only
Lower level: Word processing and printing.
STMP server address: mail.optonline.net
To access your E-mail, you must know the name of your home server.

Dining, Bar
Blackford Hall
Breakfast 7:30–9:00, Lunch 11:30–1:30, Dinner 5:30–7:00
Bar 5:00 p.m. until late
Helpful tip - If there is a line at the upper dining area, try the lower dining room

Messages, Mail, Faxes

Message Board, Grace, lower level

Swimming, Tennis, Jogging, Hiking

June–Sept. Lifeguard on duty at the beach. 12:00 noon–6:00 p.m. Two tennis courts open daily.

Russell Fitness Center

Dolan Hall, west wing, lower level
PIN#: **Press 64355 (then enter #)**

Concierge

On duty daily at Meetings & Courses Office.
After hours – From tan house phones, dial x8870 for assistance

Pay Phones, House Phones

Grace, lower level; Cabin Complex; Blackford Hall; Dolan Hall, foyer

CSHL's Green Campus

Cold Spring Harbor Laboratory is pledged to operate in an environmentally responsible fashion wherever possible. In the past, we have removed underground oil tanks, remediated asbestos in historic buildings, and taken substantial measures to ensure the pristine quality of the waters of the harbor. Water used for irrigation comes from natural springs and wells on the property itself. Lawns, trees, and planting beds are managed organically whenever possible. And trees are planted to replace those felled for construction projects.

Two areas in which the Laboratory has focused recent efforts have been those of waste management and energy conservation. The Laboratory currently recycles most waste. Scrap metal, electronics, construction debris, batteries, fluorescent light bulbs, toner cartridges, and waste oil are all recycled. For general waste, the Laboratory uses a "single stream waste management" system, removing recyclable materials and sending the remaining combustible trash to a cogeneration plant where it is burned to provide electricity, an approach considered among the most energy efficient, while providing a high yield of recyclable materials.

Equal attention has been paid to energy conservation. Most lighting fixtures have been replaced with high efficiency fluorescent fixtures, and thousands of incandescent bulbs throughout campus have been replaced with compact fluorescents. The Laboratory has also embarked on a project that will replace all building management systems on campus, reducing heating and cooling costs by as much as twenty-five per cent.

Cold Spring Harbor Laboratory continues to explore new ways in which we can reduce our environmental footprint, including encouraging our visitors and employees to use reusable containers, conserve energy, and suggest areas in which the Laboratory's efforts can be improved. This book, for example, is printed on recycled paper.

1-800 Access Numbers

AT&T	**9-1-800-321-0288**
MCI	**9-1-800-674-7000**

Local Interest

Fish Hatchery	631-692-6768
Sagamore Hill	516-922-4447
Whaling Museum	631-367-3418
Heckscher Museum	631-351-3250
CSHL DNA Learning Center	x 5170

New York City

Helpful tip -

Take Syosset Taxi to Syosset Train Station ($8.00 per person, 15 minute ride), then catch Long Island Railroad to Penn Station (33rd Street & 7th Avenue). Train ride about one hour.

TRANSPORTATION

Limo, Taxi

Syosset Limousine	516-364-9681 (**1031**)
Super Shuttle	800-957-4533 (**1033**)
To head west of CSHL - Syosset train station	
Syosset Taxi	516-921-2141 (**1030**)
To head east of CSHL - Huntington Village	
Orange & White Taxi	631-271-3600 (**1032**)
Executive Limo	631-696-8000 (**1047**)

Trains

Long Island Rail Road	822-LIRR
Schedules available from the Meetings & Courses Office.	
Amtrak	800-872-7245
MetroNorth	800-638-7646
New Jersey Transit	201-762-5100

Ferries

Bridgeport / Port Jefferson	631-473-0286 **(1036)**
Orient Point/ New London	631-323-2525 **(1038)**

Car Rentals

Avis	631-271-9300
Enterprise	631-424-8300
Hertz	631-427-6106

Airlines

American	800-433-7300
America West	800-237-9292
British Airways	800-247-9297
Continental	800-525-0280
Delta	800-221-1212
Japan Airlines	800-525-3663
Jet Blue	800-538-2583
KLM	800-374-7747
Lufthansa	800-645-3880
Northwest	800-225-2525
United	800-241-6522
US Airways	800-428-4322