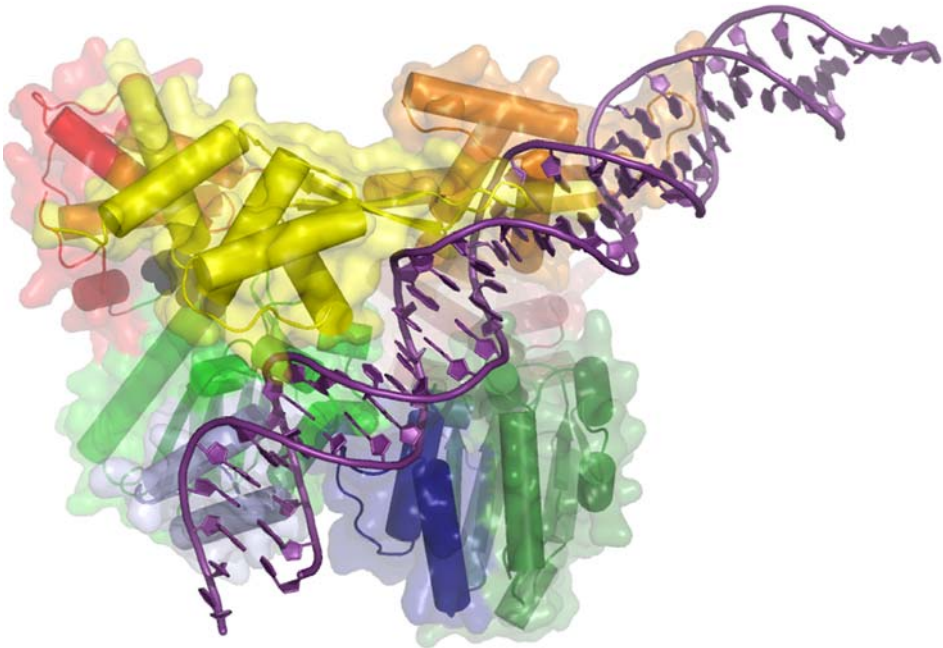
Abstracts of papers presented
at the 2011 meeting on

EUKARYOTIC DNA REPLICATION & GENOME MAINTENANCE

September 6–September 10, 2011



Cold Spring Harbor Laboratory
Cold Spring Harbor, New York

Abstracts of papers presented
at the 2011 meeting on

EUKARYOTIC DNA REPLICATION & GENOME MAINTENANCE

September 6–September 10, 2011

Arranged by

Stephen Bell, *HHMI / Massachusetts Institute of Technology*
Joachim Li, *University of California, San Francisco*
Johannes Walter, *Harvard Medical School*

Cold Spring Harbor Laboratory
Cold Spring Harbor, New York

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Cover: Sulfolobus Orc1-1 and Orc1-3 bound to DNA. Photo courtesy of James Berger.

EUKARYOTIC DNA REPLICATION & GENOME MAINTENANCE

Tuesday, September 6 – Saturday, September 10, 2011

Tuesday	7:30 pm	**1** New Approaches and Views of Replication
Wednesday	9:00 am	**2** Origin Selection and pre-RC Assembly
Wednesday	2:00 pm	**3** Poster Session I
Wednesday	4:30 pm	Wine and Cheese Party *
Wednesday	7:30 pm	**4** Response to Replication Stress and Damage
Thursday	9:00 am	**5** Origin Activation and Timing
Thursday	2:00 pm	**6** Poster Session II
Thursday	7:30 pm	**7** Chromatin, Cancer and Development
Friday	9:00 am	**8** Replisome Activities and Functions
Friday	2:00 pm	**9** Cell Cycle Control and Connections
Friday	6:00 pm	Concert
	7:00 pm	Banquet
Saturday	9:00 am	**10** Postreplicative Repair and Lesion Bypass

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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, September 6—7:30 PM

SESSION 1 NEW APROACHES AND VIEWS OF REPLICATION

Chairpersons: **D. MacAlpine,** Duke University Medical Center, Durham, North Carolina
D. Sherratt, University of Oxford, United Kingdom

WEDNESDAY, September 7—9:00 AM

SESSION 2 ORIGIN SELECTION AND PRE-RC ASSEMBLY

Chairpersons: **J. Blow,** University of Dundee, United Kingdom
M. Debatisse, Institut Curie-CNRS-UPMC, Paris, France

WEDNESDAY, September 7—2:00 PM

SESSION 3 POSTER SESSION I

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Jelena Kusic-Tisma, Branko Tomic, Dragana Stefanovic.
Presenter affiliation: IMGGE, Belgrade, Serbia. 112

WEDNESDAY, September 7—4:30 PM

Wine and Cheese Party

WEDNESDAY, September 7—7:30 PM

SESSION 4 RESPONSE TO REPLICATION STRESS AND DAMAGE

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A. Carr, University of Sussex, Brighton, United Kingdom

Mechanisms for maintaining genome stability at the replication fork
Karlene A. Cimprich.
Presenter affiliation: Stanford University, Stanford, California. 113

ATR autophosphorylation as a molecular switch for checkpoint activation
Shizhou Liu, Bunsyo Shiotani, Mayurika Lahiri, Alexandre Marechal, Xiaohong Yang, Lee Zou.
Presenter affiliation: Massachusetts General Hospital, Charlestown, Massachusetts; Harvard Medical School, Boston, Massachusetts. 114

Dna2 couples DNA replication to the replication checkpoint through activation of Mec1/ATR
Sandeep Kumar, Peter M. Burgers.
Presenter affiliation: Washington University, St. Louis, Missouri. 115

The Mcm6/2 ATPase active site is required for the replication checkpoint
Emily Tsai, Heather MacAlpine, David MacAlpine, Anthony Schwacha.
Presenter affiliation: University of Pittsburgh, Pittsburgh, Pennsylvania. 116

SESSION 5 ORIGIN ACTIVATION AND TIMING

Chairpersons: **J. Berger,** University of California, Berkeley
J. Diffley, Cancer Research UK London Research Institute, South Mimms, United Kingdom

THURSDAY, September 8—2:00 PM

SESSION 6 POSTER SESSION II

SESSION 7 CHROMATIN, CANCER AND DEVELOPMENT

Chairpersons: **N. Francis,** Harvard University, Cambridge, Massachusetts
M. Jasin, Memorial Sloan-Kettering Cancer Center, New York, New York

FRIDAY, September 9—9:00 AM

SESSION 8 REPLISOME ACTIVITIES AND FUNCTIONS

Chairpersons: **M. Botchan,** University of California, Berkeley
E. Fanning, Vanderbilt University, Nashville, Tennessee

A novel function of the DNA replication checkpoint in modulating the architecture of replicating chromosomes.
Thelma Capra, Rachel Jossen, Arianna Colosio, Camilla Frattini, Andres Aguilera, Katsuhiko Shirahige, Marco Foiani, Rodrigo Bermejo.
Presenter affiliation: IFOM (FIRC Institute of Molecular Oncology), Milan, Italy; Institute of Functional Biology and Genomics (IBFG), Salamanca, Spain. 242

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B. Stillman, Cold Spring Harbor Laboratory, New York

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Presenter affiliation: University of North Carolina at Chapel Hill, Chapel Hill, North Carolina. 243

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Presenter affiliation: Indiana University, Bloomington, Indiana. 250

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Zakir Ullah, Christelle de Renty, Melvin L. DePamphilis.
Presenter affiliation: National Institute of Child Health and Human Development, Bethesda, Maryland. 252

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CONCERT
Grace Auditorium

Bella Hristova, violin with piano

FRIDAY, September 9

BANQUET

Cocktails 7:00 PM Dinner 7:45 PM

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SESSION 10 POSTREPLICATIVE REPAIR AND LESION BYPASS

Chairpersons: **H. Ulrich,** Cancer Research UK London Research Institute, South Mimms, United Kingdom
G. Walker, Massachusetts Institute of Technology, Cambridge

THE DROSOPHILA DNA REPLICATION PROGRAM: GENOME-WIDE DISTRIBUTION AND DYNAMICS OF THE MCM COMPLEX.

David M MacAlpine, Sara K Powell, Heather K MacAlpine, Matthew L Eaton, Joseph A Prinz

Duke University, Pharmacology and Cancer Biology, Durham, NC, 27713

We have been exploiting genomic approaches to understand how DNA start sites are selected and regulated in Drosophila melanogaster. As part of the model organism Encylopedia of DNA elements (modENCODE) consortium we have been characterizing the Drosophila replication program at multiple levels including the identification of early activating origins of DNA replication and the identification of binding sites for key initiation factors, such as ORC and MCMs, at near nucleotide resolution. Integration of our replication datasets with hundreds of genomic datasets describing the Drosophila chromatin landscape and transcriptome has allowed us to generate accurate and predictive models of metazoan origin function based only on the surrounding chromatin features.

In addition, our studies of pre-RC localization have provided unique insights into the loading and distribution of the MCM helicase complex throughout the cell cycle. In higher eukaryotes there are many more Mcm2-7 complexes loaded onto chromatin than are required to complete an unperturbed S-phase. These excess Mcm2-7 complexes are thought to be important for the cell to recover from replicative stress. We have found that the Mcm2-7 complex is loaded in limited quantities at ORC binding sites in early G1, but by the G1/S transition there is significantly more Mcm2-7 complex associated with the chromatin. Furthermore, these excess Mcm2-7 complexes have redistributed throughout the chromosome and are no longer limited to sequences proximal to ORC. These redistributed helicases are functional and track with replication fork progression during S-phase. The redistribution of the Mcm2-7 complexes is dependent on cyclin E activity. In the absence of cyclin E, the cells arrest in G1 and we find a limited amount of Mcm2-7 complexes on the chromatin by chromatin fractionation. Inhibition of CDK activity by over expression of dacapo, a p27 homolog, also results in a G1 arrest; however, there are significantly more Mcm2-7 complexes associated with the chromatin. Together these results suggest that there is limited pre-RC assembly at ORC binding sites in the absence of cyclin E and that cyclin E, independent of CDK activity, is important for loading the full complement of Mcm2-7 complexes and the redistribution of the helicases throughout the chromosome. The loading of excess Mcm2-7 complexes and the redistribution of the complex throughout the genome during late G1 may, in part, explain the broad distribution of origin activity observed in metazoan genomes.

INTERROGATION OF DNA REPLICATION USING IPOND AND A PROXIMAL BIOMARKER OF ATR ACTIVATION

Bianca M Sirbu, Edward A Nam, Frank B Couch, David Cortez

Vanderbilt University School of Medicine, Biochemistry, Nashville, TN, 37232

Successful DNA replication and packaging newly synthesized DNA into chromatin is essential to maintain genome integrity. Defects in the DNA template challenge genetic and epigenetic inheritance. Unfortunately, tracking DNA damage responses (DDR), histone deposition, and chromatin maturation at replication forks is difficult in mammalian cells. We developed a technology called iPOND (isolation of proteins on nascent DNA) to analyze proteins at replication forks at high spatial and temporal resolution. Using this methodology, we can examine the timing of histone deposition and chromatin maturation. In addition, iPOND permits an analysis of protein dynamics and post-translational modifications at active and damaged replication forks. A second challenge in studying DDR responses to replication damage is the lack of a proximal biomarker for activation of the ATR signaling pathway. To overcome this limitation, we characterized ATR phosphorylation, identified putative autophosphorylation sites that regulate its activity, and developed a phosphopeptide specific antibody that recognizes an active ATR kinase. I will present our latest data demonstrating the utility of iPOND and the ATR phosphopeptide antibody for analyzing cellular responses to replication stress.

CHROMATIN AND THE LAGGING STRAND

Duncan Smith, Sean McGuffee, Iestyn Whitehouse

Sloan-Kettering Institute, Molecular Biology, New York, NY, 10065

How leading and lagging strand replication are coupled, and the impact of chromatin structure, disassembly and reassembly on replication fork progression, remain important unanswered questions in eukaryotic chromosome biology. To address these questions, we have focused on the mechanistic dissection of lagging strand synthesis in vivo via the characterization of nascent Okazaki fragments.

Working predominantly in S. cerevisiae, we have developed methodologies to label and purify pre-ligation Okazaki fragments, which we have analysed using a combination of biochemical and deep-sequencing techniques. After replication in the context of DNA ligase I downregulation, we observe short fragments with a quantised size distribution that is near identical to the nucleosome repeat length of budding yeast. These fragments are S-phase dependent, can be ligated together after genomic DNA purification, and map almost exclusively to sites predicted to be replicated as the lagging strand, suggesting that they represent completed Okazaki fragments awaiting ligation by ligase I. Deep sequencing millions of purified Okazaki fragments indicates that, contrary to expectations, ligation junctions are not found in internucleosomal linker regions but rather in close proximity to the nucleosome dyad. These data demonstrate that the size and distribution of Okazaki fragments in eukaryotes is dictated by nucleosomes and chromatin structure. Further work will use this system to deconstruct the effects of impaired chromatin assembly and disassembly on Okazaki fragment synthesis and replication fork progression, and vice versa.

Finally, we show that unligated Okazaki fragments provide a record of replication fork movement across the genome. Our technique is superior to pulse labeling approaches and allows a high-resolution spatial and temporal map of replication fork initiation and progression.

MAMMALIAN TELOMERES USE TELOMERE-SPECIFIC, RATHER THAN UNIVERSAL, REPLICATION PROGRAMS

W. C Drosopoulos, S. T Kosiyatrakul, Z. Yan, S. G Calderano, C. L Schildkraut

Albert Einstein College of Medicine, Department of Cell Biology, Bronx, NY, 10461

Mammalian telomeres are specialized structures that cap chromosome termini and consist of hundreds to thousands of tandem TTAGGG repeats complexed with several proteins including telomere-specific shelterins. The majority of telomere DNA is duplicated by conventional semi-conservative DNA replication. Mammalian telomeres challenge the replication machinery due to the combination of their secondary structure-prone repetitive sequence and extensive heterochromatization. We have recently employed single molecule analysis of replicated DNA (SMARD) to analyze mouse telomere replication (Sfeir et al, 2009). In these studies replication of total genomic telomeric DNA was analyzed, which provided only limited information regarding replication programs, i.e., origin location and density, termination site location, fork rate and direction. We have now made key advances in SMARD that allow us to study the replication of specific telomeres of individual chromosomes from both mouse and human cells. Our analysis indicates significant flexibility of the replication programs of specific telomeres. Specifically, we find variation in programs within the same cell type as well as between different cell types. In certain cases, telomere-proximal initiation zones are observed in the subtelomere. Activation of initiation sites in these subtelomeric origin regions has the potential to facilitate the recovery of stalled replication forks in telomere repeats. Furthermore, we have evidence that initiation within telomere repeats also occurs to varying extents. Thus it appears that distinct programs, rather than a universal program, are utilized for the replication of mouse and human telomeres.

Sfeir, A., Kosiyatrakul, S.T., Hockemeyer,D., MacRae,S.L., Karlseder, J., Schildkraut, C.L. and de Lange,T. Cell 138:90–103 (2009)

EXPANDED GAA REPEATS INDUCE ATYPICAL POST-REPLICATIVE DNA JUNCTIONS IN HUMAN CELLS.

Cindy Follonier, Massimo Lopes

Institute of Molecular Cancer Research, University of Zürich, Zürich, 8057, Switzerland

Trinucleotide repeats (TNR) can undergo large deletions or expansions, leading to neurodegenerative diseases like Friedreich's ataxia (GAA), Huntington disease (CAG) or Fragile X (CGG). Expanded TNR have been long suggested to form *in vitro* non-B secondary structures and were shown to stall DNA replication forks in yeast and bacteria. However, the molecular mechanisms leading *in vivo* to DNA replication interference and repeat expansion in human cells have remained elusive. To shed light on genome instability at TNR, we established an experimental system to analyse *in vivo* DNA replication intermediates (RI) across GAA repeats in human cells. SV40-derived plasmids containing GAA repeats in different number and orientation are transfected into 293T or U2OS cells and allowed to replicate. Plasmid RI are recovered and analyzed by a combination of bidimensional (2D)-gels, psoralen crosslinking and electron microscopy (EM).

Our 2D-gel analysis in 293T and U2OS cells reveals that replication forks are only transiently paused by GAA repeats in a length- and orientation-dependent manner. Surprisingly, besides fork pausing, additional unexpected signals are observed, which correlate with the minimal number of GAA repeats known to cause symptoms in Friedreich's ataxia patients. In particular, normal X-shaped molecules formed during plasmid replication and giving rise to the so called "spike" are substituted by the accumulation of one distinct population of X intermediates (X-"spot"). Extensive EM analysis of the total RI population, as well as the molecules eluted from defined position of the 2D gels, has allowed for the first time to visualize at high resolution how sister chromatids are connected shortly behind a moving fork during unperturbed replication. Importantly, in control conditions these junctions happen randomly along the replicated duplexes and do not involve homologous sequences - leading to the formation of the "spike" – whereas expanded GAA repeats stabilize sister chromatid pairing directly at the TNR - leading to the accumulation of the X-"spot".

We will also present key findings from our genetic investigations on the formation and stability of these unusual DNA structures, performed by coupling 2D-gel/EM analysis with siRNA-mediated inactivation of candidate factors previously involved in replication of structured DNA. Altogether, these results strongly suggest that these postreplicative structures could be a molecular determinant of GAA expansion, leading to Friedreich's Ataxia. Similar mechanisms could be involved in the expansion of other TNR, linked to a growing number of human neurodegenerative syndromes.

REPLISOME DYNAMICS IN VIVO

David Sherratt[1], Mark Leake[2], Christian Lesterlin[1], Yichao Dong[1], David Brown[1], Rodrigo Reyes-Lamothe[1]

[1]Oxford University, Biochemistry, Oxford, OX1 3QU, United Kingdom, [2]Oxford University, Physics, Oxford, OX1 3PU, United Kingdom

By using a range of live cell imaging techniques that give single-molecule sensitivity, 3 ms temporal resolution, a few nm spatial precision and the opportunity to measure turnover, we have monitored the assembly of functional E. coli replisomes at replication initiation and tracked the independent progression of sister replisomes and the associated newly replicated DNA derived from a single initiation event. Initiation occurs at activated chromosomal and plasmid origins independently of their cellular position. A strong DnaA-focus associated with the origin appears several minutes before initiation and disappears soon after replication elongation commences. A DnaC focus is associated with a functional origin for a very short time at initiation and we observe an asymmetry in the initial progression of sister forks. Bacteria with two replication origins in a single chromosome initiate replication synchronously at both origins and replication proceeds productively from these spatially separate origins. Replication proteins have <10% of their cellular complement associated with a given fork. Three polymerases associated with a single clamp loader complex are associated with each replisome. Measurement of turnover of replisome components at progressing forks shows that core replisome components are associated with a given fork for a few tens of seconds.

QUANTITATIVE PROTEOMICS REVEALS THE FUNCTIONS OF REPLICATION FACTOR C-LIKE COMPLEXES

Takashi Kubota[1], Shin-ichiro Hiraga[1], Kayo Yamada[2], Angus I Lamond[2], Anne D Donaldson[1]

[1]University of Aberdeen, Institute of Medical Sciences, Aberdeen, AB25 2ZD, United Kingdom, [2]University of Dundee, Wellcome Trust Centre for Gene Regulation and Expression, Dundee, DD1 5EH, United Kingdom

Ctf18 and Elg1 are the major subunits of Replication Factor C-like (RLC) complexes. Yeast cells lacking Ctf18 or Elg1 have multiple problems with genome stability, but the molecular activities of Ctf18-RLC and Elg1-RLC are not well understood. To understand the *in vivo* function of these complexes, we have investigated chromatin abnormalities in *ctf18Δ* and *elg1Δ* mutants using the quantitative proteomic technique of SILAC (Stable Isotope Labeling by Amino acids in Cell culture).
In *ctf18Δ*, 307 of the 491 reported chromosomal proteins were quantitated. The most marked abnormalities occurred when cells were challenged with the replication inhibitor hydroxyurea. Compared to wild-type, hydroxyurea-treated *ctf18Δ* cells exhibited increased chromatin association of replisome progression complex components (including Cdc45, Ctf4 and GINS complex subunits, RPA, and the polymerase processivity clamp PCNA). Very similar defects were observed in an *mrc1Δ* mutant that cannot activate the DNA replication checkpoint. We found that *ctf18Δ* cells are defective for engagement of the replication checkpoint—and that the increase in chromatin-bound replisome proteins in the *ctf18Δ* mutant is caused by inappropriate initiation at late origins, due to loss of the checkpoint.
An *elg1Δ* mutant shows genome stability defects somewhat similar to *ctf18Δ*, but proteomic analysis of *elg1Δ* chromatin revealed a completely different spectrum of abnormalities. 315 chromosomal proteins were quantitated, revealing increased loading of PCNA (and SUMOylated PCNA), the flap endonuclease Rad27, Ctf18-RLC subunits, the DNA-dependent ATPase Mgs1, and the CAF-1 histone chaperone subunit Cac2. The changes in PCNA and Rad27 suggest a role for Elg1-RLC in Okazaki fragment maturation. We found that SUMOylated PCNA on chromatin is also increased in *rad27Δ* and *cdc9-1* mutants, emphasizing the similarity the *elg1Δ* defects to those caused by problems in Okazaki fragment processing. Over-loading of PCNA does not however depend on its SUMOylation, since *elg1Δ* cells lacking the SUMO ligase Siz1 also show increased PCNA on their DNA. Our results indicate that Elg1-RLC mediates PCNA transactions during Okazaki fragment processing on the lagging strand, so that in *elg1Δ* cells PCNA is retained on immature Okazaki fragments, becoming SUMOylated as a consequence.

LOCAL AND ORIGIN SPECIFIC DETERMINANTS MODULATE GLOBAL DNA REPLICATION CONTROLS

Christopher Richardson, Joachim Li

Department of Microbiology and Immunology, University of California San Francisco, San Francisco, CA, 94143-2200

Aberrant re-initiation from replication origins during a single cell cycle can induce high rates of segmental amplification, chromosomal aneuploidy, and possibly other genomic instabilities. Current models for how eukaryotic cells prevent such re-initiation at their many replication origins focus on the global inhibition of replication initiation proteins throughout the cell. Such models, which treat origins as generic and interchangeable elements, cannot account for the diverse efficiencies with which different origins re-initiate when global control mechanisms are disrupted. Moreover, these varied re-initiation efficiencies do not correlate with the well-documented diversity in origin timing and efficiency observed in normal S phase initiation. We now have evidence of a novel mechanism that contributes to the diversity in origin re-initiation efficiency.

To investigate this mechanism in budding yeast, we have focused our studies on the ARS317 origin. ARS317 is the predominant re-initiating origin when the regulation of Cdc6 and Mcm2-7 is disrupted. A 533 bp fragment containing ARS317 retains this preferential re-initiation activity when moved from its endogenous location to other sites in the genome. This fragment can be separated into the ARS317 origin and a novel genetic element that confers preferential re-initiation on the adjacent origin. Strikingly, this element also confers preferential re-initiation on at least two other origins: ARS121 and ARS301. We are unable to detect any change in the replication efficiency or timing of origins adjacent to this element, either in plasmids or in the chromosome, suggesting that its regulatory role is specific to origin re-initiation. Hence, we term this element a Re-Initiation Promoter (RIP). Mutational studies revealed that the RIP contains two essential elements that stimulate re-replication in a distance and orientation dependent manner from an adjacent origin. We are currently investigating how the RIP influences the molecular events at replication origins to permit re-initiation.

Our identification of the RIP introduces a new paradigm in which local elements can modulate the effects of global replication control mechanisms at specific origins. We have preliminary data that other origins in the genome are also under local control. Thus, local control mechanisms may generate a complex genomic landscape of re-replication potential and susceptibility to re-replication induced genomic alterations.

SIGNALING ROLES FOR RIBONUCLEOTIDES IN THE S. CEREVISIAE NUCLEAR GENOME

Thomas A Kunkel, Jessica S Williams, Danielle L Watt, Anders R Clausen, Mercedes E Arana, Alan B Clark

NIEHS, NIH, Laboratory of Molecular Genetics and Laboratory of Strucutural Biology, Research Triangle Park, NC, 27709

During DNA replication, a large number of ribonucletides are introduced into the nuclear genome by the primase that initiates DNA synthesis and by the three replicases, Pols alpha, delta and epsilon. Structural studies indicated that ribonucleotides in DNA alter helix parameters. These facts suggest that ribonucleotides in DNA may act as signals for cellular functions, as has already been demonstrated for mating type switching in fission yeast. In collaboration with several other research groups, we are exploring possible signaling functions for ribonucleotides in the nuclear genome of budding yeast. Evidence will be presented indicating that (1) single and multiple consecutive ribonucleotides in DNA templates impede DNA synthesis by Pols delta and epsilon, (2) ribonucleotides incorporated by Pol epsilon can be proofread, albeit less efficiently than base-base mismatches, (3) a defect in RNase H2, which nicks DNA 5′ of ribonucleotides in DNA, alters the expression of 254 yeast genes, many involved in nucleic acid transactions, (4) unrepaired ribonucleotides in RNase H2-defective strains are recognized and cleaved by topoisomerase 1, which can initiate a mutagenic repair reaction, and (5) a concomitant defect in RNases H1 and H2 renders yeast sensitive to hydroxyurea, and is lethal in combination with a pol2- M655G mutation that increases incorporation of ribonucleotides into DNA. These data demonstrate that ribonucleotides in DNA act as signals for biological processes. We are currently testing the hypothesis that ribonucleotides in DNA may have beneficial signaling functions.

EXTRACHROMOSOMAL MICRODNAS AND CHROMOSOMAL MICRODELETIONS IN NORMAL TISSUES

Yoshiyuki Shibata[1], Pankaj Kumar[1], Ryan Layer[1], Smaranda Willcox[2], Jack D Griffith[2], Anindya Dutta[1]

[1]University of Virginia, Biochemistry and Molecular Genetics, Charlottesville, VA, 22901, [2]University of North Carolina, Lineberger Cancer Center, Chapel Hill, NC, 27514

DNA in normal mammalian somatic cells is present either in chromosomes or mitochondria. Furthermore, other than in specialized tissues like B and T cells, the genomic DNA in normal tissues is usually considered to be an exact copy of germ-line DNA. We hypothesized that errors in DNA replication may lead to the production of extra-chromosomal DNA and/or changes in copy number of segments of the DNA in normal somatic tissue. Here we identify several thousand short double-stranded and single-stranded extrachromosomal circular DNA (microDNA) that are derived from chromosomes in normal mouse brain, heart and liver as well as mouse and human cell lines. These MicroDNAs are 200-400 bases long and are high in GC content. Short direct repeats are often present at the start and end of the corresponding chromosomal sequence suggesting that microDNAs are produced by replication slippage or homologous recombination. Several of the microDNAs overlap with nucleosome-occupied DNA, explaining how such short double-stranded DNA can be bent into a circle. The genomic sources of the microDNAs are enriched in the 5' region of genes, exons and CpG islands. Ultrahighthroughput sequencing of adult brain DNA targeted to chromosomal loci that are enriched sources of microDNA, reveals a small fraction of genomic DNA molecules with micro-deletions that may be generated by the excision of microDNAs by homologous recombination. We have thus identified a new DNA entity in somatic cells and provide evidence that deletions may occur in different genomic loci in somatic tissues, leading to genetic variability between normal somatic cells.

THE DYNAMICS OF REPLICATION LICENSING IN LIVING C. ELEGANS AND XENOPUS EMBRYOS

Remi Sonneville[1], Jolanta Kisielewska[2], Matthieu Querenet[1], Ashley Graig[1], Anton Gartner[1], J. Julian Blow[1]

[1]University of Dundee, Wellcome Trust Centre for Gene Regulation and Expression, Dundee, DD1 5EH, United Kingdom, [2]University of Newcastle, Institute for Cell and Molecular Biosciences, Newcastle-Upon-Tyne, NE2 4HH, United Kingdom

In late mitosis and G1, Mcm2-7 are assembled onto replication origins to license them for initiation. At other cell cycle stages licensing is inhibited, thus ensuring that origins fire only once per cell cycle. Biochemical and genetic approaches have demonstrated that licensing occurs by the stepwise assembly onto origins of ORC, Cdc6, Cdt1 and the Mcm2-7 replicative helicase. The regulation of this system has been well studied in only a few model systems, most notably yeast, Xenopus egg extracts and tissue culture cells. Very little work has been done on normally developing animal cells.

Work in Xenopus egg extracts and mammalian tissue culture cells has suggested that proteolysis of Cdt1 during S phase plays an important role in preventing re-replication. In contrast, we show that during the rapid cell cycles of the early Xenopus embryo the protein levels of Cdt1 and geminin are persistently high. Immunoprecipitation of Cdt1 and geminin complexes and use of functional mutants suggests that licensing activity is regulated in Xenopus embryos by periodic interaction between Cdt1 and geminin rather than their proteolysis.

We have also used video microscopy of C. elegans embryos to provide the first comprehensive view of replication licensing in vivo. MCM2-7 loading in late M phase depends on prior DNA binding of ORC and CDC-6. Surprisingly, CDT-1 and MCM2-7 DNA binding is interdependent, whereas ORC and CDC-6 bind to DNA independently. Using photobleaching experiments we show that MCM-3 chromatin loading is irreversible while CDC-6 and ORC turn over on chromatin in a few seconds. This is consistent with each ORC/CDC-6 complex being able to load multiple MCM2-7 complexes. We show that MCM2-7 chromatin loading further reduces ORC and CDC-6 DNA binding. This dynamic behaviour creates a feedback loop allowing ORC/CDC-6 to repeatedly load MCM2-7 and distribute licensed origins along chromosomal DNA. During S-phase, ORC and CDC-6 are excluded from nuclei by an exportin-dependent process. This exclusion appears to be important because in exportin defective cells re-replication of DNA occurs. These results demonstrate how nucleo-cytoplasmic compartmentalization of licensing factors ensures that DNA replication occurs precisely once per cell cycle.

IN VITRO ASSEMBLY OF HUMAN PRE-REPLICATIVE COMPLEX AND ITS REGULATION

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The initiation of eukaryotic DNA replication is a tightly regulated process that is crucial to the maintenance of genome integrity. During G1 phase of the cell cycle, pre-replicative complexes (pre-RC) are assembled at origins of replication through sequential recruitment of origin recognition complex (ORC), Cdc6, Cdt1 and minichromosome maintenance (MCM) complex, the replicative helicase. In metazoans, Geminin interacts with Cdt1, and inhibits the assembly of pre-RC. Here we describe the reconstitution of human pre-RC using purified proteins and DNA containing the lamin B2 origin. We show that HsORC binds to DNA by itself, while its presence greatly enhances the recruitment of HsCdc6 and HsCdt1. HsORC, HsCdc6 and HsCdt1 are all required for maximal association of HsMCM2-7 complex, as well as HsMCM467, a sub-complex of MCM that exhibits helicase activity in vitro. A novel interaction between HsGeminin and HsMCM2-7 was uncovered, and we suggest that HsGeminin negatively regulates the pre-RC formation by inhibiting the DNA binding of HsCdt1, the interaction between HsCdt1 and MCM complexes, and the formation of a salt-resistant complex of MCM2-7 with DNA. The formation of the salt-resistant MCM2-7 complex on DNA requires ATP hydrolysis. These findings demonstrate the key roles of HsGeminin in the regulation of human pre-RC assembly.

STRUCTURE AND MECHANISM IN MCM2-7 LOADING 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 on DNA. The reconstituted reaction depends on all pre-RC proteins, DNA and ATP-hydrolysis for MCM loading. During MCM2-7 loading single-hexameric MCM2-7 gets transformed in a double-hexameric complex.
In the absence of ATP hydrolysis the MCM2-7 loading reaction is blocked, but an initial pre-RC is formed. Using gel-filtration and crosslinking experiments we have shown that this initial pre-RC contains a single hexameric MCM2-7, ORC, Cdc6 and Cdt1. We have obtained a cryo-electron microscopy 3D structure of the same complex and have mapped several subunits using maltose-binding protein fusions. ORC-Cdc6 within the initial pre-RC adopt a shape comparable to clamp loaders. Relatively few contacts are formed between ORC-Cdc6 and Cdt1-MCM2-7, which is consistent with the poor stability of the complex in buffers containing high salt. We will discuss the organisation of the complex and propose a MCM2-7 loading mechanism.

REPLICATORS AS CHROMATIN ORGANIZERS: INSIGHTS FROM REPLICATOR BINDING PROTEIN COMPLEXES AND WHOLE GENOME REPLICATION INITIATION MAPS

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In eukaryotes, replication at distinct chromosomal sites starts at diverse times during the S-phase of the cell cycle and the replication process is precisely orchestrated to insure that each and every genomic locus replicates exactly once per cell cycle. The locations of replication initiation events on chromatin are dynamically regulated by a combination of DNA sequences and chromatin modifications. We aim to understand how replication coordinates with transcription and other chromatin transactions and how such coordination impacts cell cycle progression and genomic stability.

We have recently identified two discrete DNA-protein complexes that regulate where and when replication starts within a replicator, RepP, located at the human beta globin locus. One RepP-associated complex includes a member of the DCAF protein family, interacts with the pre-replication complex and is essential for initiation of DNA replication. The other RepP-associated complex includes chromatin-remodeling proteins, affects replication timing and transcriptional activity in adjacent sequences and mediates an interaction between the RepP replicator and a distal locus control region (LCR). These findings are in line with previous observations suggesting that replicator sequences can affect chromatin structure and gene expression in addition to regulating replication initiation.

To assess how replication associates with gene expression in other loci, we determined the genome-wide distribution of replication initiation events in human cells. The dataset created by those genome wide studies encompasses the locations of replication initiation sites throughout the entire non-repetitive genomes of the analyzed transformed and non-transformed cells. We found that the frequency of replication initiation events increased in genomic regions that were transcribed in moderate levels, but initiation frequency was reduced in genes with high transcription rates. In concordance, high-resolution mapping showed that replication initiation events were excluded from promoter regions and enriched immediately downstream of transcribed promoters. We also found that the frequency of initiation events was affected by chromatin condensation and methylation at CpG sequences. These findings will be discussed in view of an emerging model suggesting a role for replicator sequences in coordinating replication, transcription and chromatin condensation.

GENOME-WIDE MAPPING OF PRE-RC BINDING SITES AND INITIATION SITES IN THE EBV-GENOME REVEALS CONSERVED FEATURES BUT ALSO DIFFERENCES.

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Pre-replicative complexes (pre-RC) are a prerequisite for the activation of origins and are conserved in all eukaryotes. In higher eukaryotes, pre-RCs exhibit very little sequence specificity. We performed a genome-wide analysis of pre-RC proteins and short nascent strand (SNS) sites in the Epstein-Barr virus genome and determined the nucleosome patterns at origins in different phases of the cell cycle. Previous studies revealed that only a single or very few initiation event occur per genome (1). However, approximately 200 pre-RC and correlating SNS sites are detected on the 170 kbp EBV-genome indicating a high plasticity in sites of origin selection. Both, sites of pre-RC formation and origin activation are characterized by nucleosome depletion and positioned flanking nucleosomes. No clear sequence motif emerges from these analyses, although pre-RC formation is slightly favored at CG-pairs, whereas SNS sites are more efficient at A/T-rich regions. We find that the nucleosome occupancy changes at origins over the cell cycle. Interestingly, we also find that the efficiency of pre-RC formation does not correlate with the efficiency of origin activation, but with the mobility of the origin flanking nucleosomes. These findings demonstrate the importance of local nucleosome positioning and mobility at origins as general features, but that additional local characteristics contribute independently to the efficiency of pre-RC formation and activation.

Reference:
1. Norio P & Schildkraut CL (2004); PLoS Biol 2(6):e152.

REPLICATION PROFILES AND FRAGILITY OF COMMON FRAGILE SITE

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We have recently shown (Letessier et al., Nature, *470*, 120, 2011) that the fragility of FRA3B and FRA16D, the most active common fragile sites (CFS) in human lymphocytes, correlates with a striking paucity of replication initiation events in a large core region of the sites. This feature forces forks coming from flanking regions to cover long distances in order to complete replication, leaving the site incompletely replicated at mitosis upon fork slowing. We thus attempted to suppress CFS fragility by treating lymphoblastoid cells with both aphidicolin and RO3307, a CDK1 inhibitor that delays mitotic onset. Under these conditions, we found that breaks at FRA3B and FRA16D are completely prevented. Analysis of the replication dynamics by DNA combing showed that the loss of fragility at FRA3B correlates with a burst of initiation events in the core region of the site. These results (i) indicate that licensed origins are present in the core of CFS, but are prevented to fire by a mechanism yet to be determined, and (ii) confirm our model of CFS fragility.

DNA REPLICATION ORIGINS: CONSERVED FEATURES AND ORGANIZATION IN METAZOANS

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We carried out a genome-scale analysis using pluripotent and differentiated mouse cell lines as well as Drosophila cell lines to characterize DNA replication origins (Oris) in metazoans. Mapping of replication origins was performed using purification of RNA-primed nascent DNA strands followed by analysis by tiling arrays, Q-PCR, high throughput sequencing and DNA combing. We show that in all cell lines most CpG islands (CGI) contain Oris, although methylation is nearly absent in Drosophila, indicating that this epigenetic mark is not crucial for defining the initiation event. Initiation of DNA synthesis starts at the borders of CGIs, resulting in a striking bimodal distribution of nascent strands, suggestive of a dual initiation event. We also found that Oris contain a unique nucleotide skew, characterized by G/T and C/A over-representation at the 5' and 3' of Ori sites, respectively. Repeated GC-rich elements were detected, which are good predictors of Oris, suggesting that common sequence features are part of the elements defining a metazoan Ori.
Our results also show that Oris are in large excess (100 000 in mouse cells) and site–specific. Only one out of 4 -5 is activated in a given cell at each cell cycle, but their activation does not occur at random. Potential replication origins are organized in groups of flexible but site-specific origins that define replicons, where a single origin is activated in each replicon.
This organization provides both site specificity and Ori firing flexibility in each replicon, allowing possible adaptation of DNA replication to environmental cues and cell fates. Indeed, replication origins from mouse embryonic fibroblasts can be reprogrammed by Xenopus mitotic egg extracts, in parallel with the appearance of pluripotency markers and cell colonies.

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IDENTIFICATION AND CHARACTERIZATION OF SHARED CHROMATIN ARCHITECTURE AT *S. POMBE* ORIGINS OF REPLICATION

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Within eukaryotic nuclei, DNA is packaged into nucleosomes to form chromatin. The positions of nucleosomes dictate which DNA sequences are accessible to regulatory proteins involved in transcription, DNA replication, and other processes. We used cryo-grinding to prepare intact nuclei from the fission yeast, *Schizosaccharomyces pombe*. We incubated the nuclei with micrococcal nuclease (MNase), and then identified mono-nucleosome-sized protected DNA fragments by high-throughput sequencing. We wished to use this dataset to learn more about chromatin structure at origins of replication, but origins in *S. pombe* are difficult to map accurately, because *S. pombe*'s ORC complex binds to AT-rich regions but not to specific DNA sequences, and previous attempts to map *S. pombe* origins by microarray analysis of early-S-phase DNA synthesis were accurate only to several kb at best. At this reduced accuracy the chromatin pattern at origins is unresolved and appears as a slight average drop in nucleosome occupancy. To correct for this we developed a chromatin alignment algorithm, ArchAlign, which identifies shared chromatin structural patterns from high-resolution chromatin structural datasets derived from next-generation sequencing approaches such as ours. Alignment of *S. pombe* DNA replication origins on the basis of their chromatin architectures revealed asymmetric localization of Pre-Replication Complex (pre-RC) proteins within a large nucleosome-depleted region. In addition, we identified distinctive groupings of pre-RCs based on their shared nucleosomal patterns, which are significantly, but not completely, associated with telomere-proximity and replication timing.

SEQUENTIAL AND INCREASING ACTIVATION OF REPLICATION ORIGINS ALONG REPLICATION TIMING GRADIENTS IN THE HUMAN GENOME

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Eukaryotic chromosomes replicate from multiple replication origins that fire at different times in S phase. The mechanisms that specify origin position and firing time and coordinate origins to ensure complete genome duplication are unclear. Previous studies proposed either that origins are arranged in temporally coordinated groups or fire independently of each other in a stochastic manner. Here, we have performed a quantitative analysis of human genome DNA replication kinetics using a combination of DNA combing, which reveals local patterns of origin firing and replication fork progression on single DNA molecules, and massive sequencing of newly replicated DNA, which reveals the population-averaged replication timing profile of the entire genome. We show that origins are activated synchronously in large regions of uniform replication timing but more gradually in temporal transition regions and that the rate of origin firing increases as replication progresses. We identify hundreds of U-shaped replication timing domains which coincide with domains of self-interaction of the chromatin fiber. We propose a domino model in which replication forks progressing from early origins stimulate initiation in nearby unreplicated DNA in a manner that explains the shape of the replication timing profile. These results provide a new and fundamental insight into the temporal regulation of mammalian genome replication.

WHOLE GENOME MAPS OF ORIGINS OF REPLICATION IN A NEAR-NORMAL HUMAN LYMPHOBLASTOID CELL LINE.

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Mammalian origins of replication appear to fall into two distinct classes: 1) relatively fixed sites such as the human lamin B2 origin, and 2) broad zones of inefficient sites, such as the CHO DHFR locus that we have studied for the last 30 years. Using genetic strategies, we have shown that critical, non-redundant, replicators do not reside within the DHFR initiation zone itself. In contrast, deletion of the DHFR promoter severely lowers the efficiency of initiation in the origin and allows initiation to spread into the body of the inactive gene. In contrast, deletion of the 3' end of the gene allows transcription forks to invade the downstream origin, completely inhibiting initiation. Therefore, active transcription plays both positive and inhibitory roles in modulating initiation of replication in this locus.

These data suggest a model in which the entire genome is peppered at frequent intervals with inchoate initiation sites whose activities are regulated largely by epigenetic phenomena such as transcription and chromatin architecture. To test this general model, we devised a novel gel-trapping strategy for isolating all of the active initiation sites in complex genomes based on the partially circular character of fragments containing replication bubbles. We have now prepared and sequenced five virtually pure, comprehensive, origin libraries from several human cell lines, including GM06990, a near-normal lymphoblastoid derivative for which considerable transcription and epigenetic data are available via the ENCODE project.

Computational analysis reveals fascinating relationships among replication timing, origin usage, transcription, and covalent histone modifications. Interestingly, most bubble-containing fragments reside next to at least one other bubble-containing fragment, suggesting that most origins to correspond to initiation zones. Furthermore, even bubble-containing fragments exhibiting the highest read-depths are not very efficient, since 2-D gel analysis on log-phase cells all indicate significant amounts of passive replication from these fragments.

A comparison to maps of actively transcribed genes substantiates our previous predications based on studies of the DHFR origin: initiation of replication rarely occurs within the bodies of very actively-transcribed genes; however, transcription in the neighborhood appears to be required to activate origins – perhaps because active promoters establish a permissive chromatin environment for both transcription
replication initiation.

We are identifying significant chromatin modifications that characterize both the local and global environments that facilitate origin activation in human cells, using the data publicly available via ENCODE and other hosts of whole-genome datasets. A model for the epigenetic requirements for origin activation will be presented.

NCC - A NEW TECHNOLOGY TO DISSECT CHROMATIN DYNAMICS AT REPLICATION FORKS

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Inheritance of DNA sequence and its proper organization into chromatin is fundamental for eukaryotic life. The challenge of propagating genetic and epigenetic information is met in S phase and entails genome-wide disruption and restoration of chromatin coupled to faithful copying of DNA. How specific chromatin structures are restored on new DNA and transmitted through mitotic cell division remains a fundamental question in biology. Chromatin restoration involves a complex set of events including nucleosome assembly and remodelling, restoration of marks on DNA and histones, deposition of histone variants and establishment of higher order chromosomal structures including sister-chromatid cohesion. To dissect these fundamental processes and their coordination in time and space with DNA replication, we have developed a novel technology termed nascent chromatin capture (NCC) that provides unique possibility for biochemical and proteomic analysis of chromatin replication in human cells. We will present NCC data on histone marks showing dynamic changes in acetylation and slow restoration of methylation along with large-scale SILAC-based proteomic profiling of nascent chromatin. This comprehensive characterization reveals novel molecular players in chromatin replication and provides exciting insight into chromatin restoration dynamics.

RB-INDEPENDENT TGF-BETA1 CELL CYCLE ARREST AND INHIBITION OF MCM HEXAMER ASSEMBLY IS ABROGATED BY THE REPLICATION ONCOPROTEIN CDT1

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The mechanisms by which TGF-beta1 inhibits cell cycle progression are often disrupted during tumorigenesis, providing growth advantages for such cells. Although growth inhibition by TGF-beta1 is thought to ultimately depend on the antiproliferative function of Rb, TGF-beta1 remains highly effective at inhibiting early G1 progression in the absence of Rb, and, intriguingly, does so without inhibiting c-Myc and Cyclin E-Cdk2. We have identified a previously unknown mechanism by which TGF-beta1 achieves such G1 arrest in the absence of these mediators. TGF-beta1 inhibits assembly of the hexameric MCM helicase by blocking Mcm6 loading, while apparently allowing the five other MCM subunits to load. We also found that a Geminin-Cdt1 complex that is required for MCM hexamer formation, suggested to be an Activator Complex, is also inhibited by TGF-beta1. Importantly, these effects on Geminin-Cdt1 function and MCM hexamer formation play a pivotal mechanistic role in TGF-beta1 arrest, since overexpression of Cdt1, as occurs in some cancers, restores Mcm6 loading and abrogates TGF-beta1 arrest in Rb-lacking cells. Consistent with these observations, the MCM-interacting domain of Cdt1 is also required for this effect. These results demonstrate the existence of a novel TGF-beta1 -inhibitory pathway regulating Geminin-Cdt1 and MCM hexamer assembly that is independent of the well-established Rb and Myc/CycE-Cdk2 pathway. In addition, these findings provide a molecular explanation for the oncogenic nature of Cdt1 that derives from the ability of deregulated Cdt1 to restore MCM hexamer formation and abrogate TGF-beta1 arrest.

MCM10 FUNCTIONS WITH THE 9-1-1 CLAMP IN RESISTANCE TO TOPOISOMERASE I-DNA ADDUCTS.

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Minichromosome maintenance protein 10 (Mcm10) is an essential DNA replication factor. During replication, Mcm10 coordinates DNA unwinding with DNA synthesis, which it accomplishes through several interactions (Ricke and Bielinsky, Mol. Cell 2004, Das-Bradoo *et al.*, MCB 2006). Among other factors, Mcm10 binds to the replication clamp, proliferating cell nuclear antigen (PCNA). The interaction is dependent on Mcm10's PCNA interacting protein motif, or PIP box, and is essential for viability. Importantly, diubiquitination of Mcm10 is a prerequisite for PCNA binding (Das-Bradoo *et al.*, MCB 2006). Molecular modeling studies have predicted, and the crystal structure confirms that PCNA is structurally similar to the checkpoint clamp, Rad9/Hus1/Rad1 (9-1-1) despite low sequence homology. Because of its similarity to PCNA, we investigated whether the checkpoint clamp also interacts with Mcm10. We tested Mcm10 binding to each of the 9-1-1 subunits, Ddc1, Mec3, and Rad17 by yeast two-hybrid assays. Mcm10 interacted with Mec3, but not Rad17 or Ddc1. Mutational analysis revealed that Mec3 bound to Mcm10 through residues in its inter-domain loop, and Mcm10 bound to Mec3 through its N-terminus as well as its PIP box. Further analysis of Mcm10's N-terminus revealed a region between amino acids 100-150 that facilitated the interaction. Importantly, Mcm10 did not require ubiquitination to interact with Mec3. Co-immunoprecipitation experiments showed that all subunits of the 9-1-1 clamp have the ability to pull down Mcm10, and that the interaction is most prevalent during S phase. Overexpression of a Mcm10 mutant, which disrupts the interaction with Mec3, in a temperature sensitive *mcm10-1* background exhibited camptothecin sensitivity at the restrictive temperature. Surprisingly, the same mutant treated with UV, MMS, or HU did not show any sensitivity. These data suggest that Mcm10 may protect the integrity of the replication fork not only through its interaction with PCNA during S phase, but also through its interaction with the checkpoint clamp in the presence of topoisomerase I-DNA adducts. [supported by NIH grant GM074917]

INVESTIGATING THE PROPERTIES OF A REPAIR REPLICATION-FORK IN THE BUDDING YEAST *SACCHAROMYCES CEREVISIAE*

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Genome instability is the hallmark of several human diseases including cancer. Identified forms of genome instability include chromosomal rearrangements such as non-reciprocal translocations, deletions and amplifications. Recently, complex genome rearrangements have also been identified as a feature shared by many cancers and other human diseases. Current research has increasingly focused on endogenous DNA damage originating from such events as replication fork stalling and fork collapse as major sources of genome instability. For example, the FoSTeS (Fork Stalling and Template Switching) model posits that the repair fork originating from a stalled or collapsed fork can trigger complex genome rearrangements by means of template switching of the repair replication-fork. This model has also been termed Microhomology-Mediated Break-induced Replication (MMBIR) because the junctions between translocated segments often have homology as little as 2-4 bp. However, to date the MM-BIR model remains to be rigorously tested in model organisms. Using the budding yeast *Saccharomyces cerevisiae*, we are studying recombination-dependent DNA replication or Break-Induced Replication (BIR) in order to examine the MM-BIR mechanism. A single double-strand break (DSB) induced by HO endonuclease induced in the nonessential terminal region of one chromosome can be repaired by BIR with an ectopic donor sequence, generating a nonreciprocal translocation. We have created a system in which two template switches to ectopic regions separated by ≥ 100 kb results in a functional URA3 gene as part of a complex nonreciprocal translocation. Preliminary results show that these events can occur at a rate of about 2×10^{-5}. We will report experiments to examine the homology requirements for such events and the roles of several DNA helicases and other replication factors.

NEW VIEWS ON STRAND ASYMMETRY IN FISSION YEASTS

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The GC skews (G-C)/(G+C) measure the nucleotid composition asymmetry of a particular DNA strand. GC skew is a classic in silico method for predicting origin and terminus of replication in bacteria and was also used to map replication origins in human genome. For DNA replication the rational is that the leading strand is enriched in G over C, although the processes beyond still remain elusive. We analyzed the skew along the three chromosomes of the fission yeast, Schizosaccharomyces pombe, and found several (<15) abrupt transitions of the skew. Interestingly, all the transitions exhibit a sign (plus to minus) corresponding to replication termination sites. The stronger signal overlaps with the mating-type locus, mat1, known to be flank by two replication fork pauses/termination sites, MPS1 and RTS1. A similar skew distribution profile and number was observed over the S. octosporus chromosomes. The main signals, in both fission yeasts, exhibit a shared synteny revealing an evolutionary constraint. By native 2D-gel experiments we found that the S. octosporus mat1 gene is also flanked by replication fork pauses/arrests, indicating a similar mating type switching strategy. We therefore proposed that in fission yeasts, replication termination sites play a greater role in orchestrating DNA replication than that anticipated earlier.

PHYSICAL AND FUNCTIONAL INTERACTION OF THE HUMAN TIMELESS AND TIPIN PROTEINS WITH DNA POLYMERASE EPSILON AND THE MCM COMPLEX.

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Human Timeless (TIM-1) and Tipin (Timeless-interacting protein) form a tight complex (named "fork-protection complex") during all stages of the cell cycle and are mediators of the S-phase checkpoint promoting ATR-mediated phosphorylation of Chk1 upon DNA damage. Nonetheless, TIM-1 and Tipin are believed to play a role even in unperturbed DNA replication since they are stable components of the replisome progression complex. However, a detailed biochemical analysis of human TIM-1 and Tipin proteins is still missing.
Herein, we report that the recombinant human TIM-1 purified from insect cells enhances the activity of the replicative DNA polymerases (DNA polymerase alpha, delta and epsilon), as measured by enzymatic assays on synthetic primer-template. The most important effect has been observed on DNA polymerase epsilon whose ability to incorporate nucleotides is increased up to 20-fold by recombinant TIM-1 on poly(dA)-oligo(dT) as a primer-template. TIM-1 is able to enhance the synthetic activity of DNA polymerase epsilon even on a duplex DNA molecule made of a 5'-radiolabelled 40-mer synthetic oligonucleotide annealed to an 80-mer template as a primer-template.
Pull-down experiments performed on extracts of insect cells co-infected with baculoviruses expressing TIM-1, Tipin and the four subunits of DNA polymerase epsilon (p261, FLAG-p59, p17 and p12) have revealed that the TIM-1/Tipin complex, or TIM-1 alone, directly interacts with DNA polymerase epsilon.
We have also analysed the physical interaction of TIM-1 and Tipin with the MCM2-7 complex by co-immunoprecipitation experiments carried out on mixtures of the purified recombinant proteins using specific antibodies and have observed that TIM-1 and Tipin both interact with the recombinant MCM2-7 complex. Furthermore, we have found that TIM-1 and Tipin directly interact with MCM 2, 3, 4, 6 and with MCM 3, 4, 6, 7 subunits, respectively, as determined by pull-down experiments performed on extracts of insect cells co-infected with the corresponding baculoviruses. However, we have not observed any effect of TIM-1 or Tipin or the TIM-1/Tipin complex on the MCM467 DNA unwinding activity.
The results of our biochemical analysis support the proposal that the "fork protection complex" might coordinate the polymerase synthetic activity with the helicase function on the leading strand.

STRUCTURAL INSIGHT INTO POLYMERASE-A-PRIMASE HELICASE INTERACTIONS IN THE SV40 PRIMOSOME

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The fundamental steps of eukaryotic chromosomal replication were first elucidated in a simple model system, the cell-free replication of the SV40 genome, which can be reconstituted with SV40 T antigen (Tag) and 10 human proteins. In infected cells, however, viral DNA replication requires host DNA damage signaling, occurs in association with host DNA repair proteins, and uses an "alternative fork" that may mimic a host replication fork recovery mechanism. Three proteins, Tag, human RPA, and human DNA polymerase α-primase (pol-prim), constitute the viral primosome. Interactions among these proteins coordinate parental DNA unwinding with primer synthesis to initiate the leading strand and each Okazaki fragment on the lagging strand. An analogous human primosome that functions at the interface of replication and repair remains unidentified.
To deepen our understanding of the SV40 primosome, we recently determined the structure of a previously unrecognized pol-prim domain (p68N) that docks on Tag, identified the p68N surface that contacts Tag, and demonstrated its vital role in primosome function. Here, we identify the p68N-docking site on Tag by using structure-guided mutagenesis of the Tag helicase surface. A charge-reverse substitution in Tag disrupted both p68N-binding and primosome activity, but did not affect docking with other pol-prim subunits. Unexpectedly, the substitution also disrupted Tag ATPase activity, suggesting a potential link between p68N docking and ATPase activity. To assess this possibility, we examined the primosome activity of Tag with a single residue substitution in the Walker B motif. Although this substitution abolished ATPase activity as expected, it did not reduce pol-prim docking on Tag or primosome activity, indicating that Tag ATPase is dispensable for primosome activity in vitro. Potential implications of our findings for eukaryotic primosome architecture and coordination of helicase and primosome functions during lagging strand synthesis will be discussed.
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EVOLUTION OF DNA REPLICATION PROTEINS ACROSS THE DIVERSITY OF EUKARYOTES

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Research on eukaryotic DNA replication is mainly based on molecular and genetic studies of yeasts and animals which represent only one of the six eukaryotic 'supergroups', the opisthokonts. Most eukaryotes in other supergroups have received little attention with regard to DNA replication. Using comparative genomics and phylogenetic analysis, we have mapped ancient evolution patterns by pinpointing gains, losses and duplication events within over 70 DNA replication gene/protein components across 38 diverse eukaryotes. Replication proteins include components of the pre-replication complex, initiation proteins and replisome constituents. The eukaryotes sampled cover all six eukaryotic supergroups, including 2 amoebozoa, 9 chromalveolates, 4 excavates, 16 opisthokonts, 6 plants and 1 rhizaria. DNA replication genes/proteins have also been analysed in two species with nucleomorphs - residual second nuclei from secondary eukaryotic endosymbiosis events. Our studies have constructed a taxonomic distribution of the core DNA replication proteins across eukaryotes and identified ancient conserved protein components. We have revealed a potential model of the DNA replication machine in the last common ancestor of eukaryotes. This study will help to overcome current animal/fungal bias in DNA replication research and better describe the evolution of functional diversity of eukaryotic DNA replication.

RECONSTITUTION OF ORIGIN-DEPENDENT REPLICATION INITIATION *IN VITRO* USING NUCLEOSOMAL DNA TEMPLATES

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In eukaryotic cells, genomic DNA is compacted and its accessibility regulated by tight association with proteins to form chromatin. The fundamental building block of chromatin is the nucleosome, which is formed by DNA wrapping around a histone octamer, presenting a potential barrier for replication forks. Smooth passage of the replication fork requires disassembly of the nucleosomes prior to the replication and maintenance of a compact genome requires rapid nucleosome re-assembly of the newly synthesized DNA. Although it is clear that nucleosome disassembly/assembly is tightly coupled to passage of the DNA replication fork, how these events influence replication fork function is not fully understood.

Here we report an origin-dependent *in vitro* replication initiation assay that uses nucleosomal DNA templates. Nucleosomes are assembled on long linear DNA templates attached to beads using purified yeast histone octamers, Nap1 and ISW1 complex. Subsequently, these nucleosomal DNA templates were sequentially incubated with G1 and S phase yeast extracts to recapitulate the DNA replication process (1). Coupled with a similar assay that uses non-nucleosomal DNA templates, we are examining the influence of nucleosomes on the DNA replication process. We will report our progress in understanding the influence of histone chaperones known to associate with the replication fork on DNA replication fork progression.

1. Heller, R.C ., Kang, S., Lam, W.M., Chen, S., Chan, C.S., and Bell, S.P. Eukaryotic origin-dependent DNA replication in vitro reveals sequential action of DDK and S-CDK kinases. *Cell*., 2011, **146**: 1-12 (in press)

ACETYLATION OF REPLICATION AND REPAIR PROTEINS REGULATES GENOME FIDELITY

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In eukaryotic nuclear DNA replication, one strand of DNA is synthesized continuously, but the other must be made as short Okazaki fragments that are later joined. The discontinuous process is inherently more complex and the steps involving fragmented intermediates create risks for disruptions of genomic integrity. The RNA primer and first 20-30 nucleotides of DNA of each Okazaki fragment are made by DNA polymerase α, which synthesizes with lower fidelity than DNA polymerase δ (pol δ), which completes the bulk of fragment synthesis to about 150 nucleotides. Therefore, the early part of the fragment is much more likely to have mismatch errors. Each upstream fragment is extended until it displaces a 5' flap in the downstream fragment. Flap endonuclease 1 (FEN1) and Dna2 endonuclease/helicase (Dna2) sequentially coordinate their nuclease activities for efficient resolution of the flap structures. Long patch base excision repair of DNA damage proceeds via a similar mechanism, using many of the same protein components used for Okazaki fragment maturation.
FEN1 can be post-translationally modified by the transcriptional coactivator, p300. Acetylation of FEN1 by p300 inhibits its endonuclease activity impairing flap cleavage, a seemingly undesirable effect. However, in contrast to FEN1, acetylation of Dna2 by p300 stimulates its 5'-3' endonuclease, the 5'-3' helicase and DNA-dependent ATPase activities, allowing for more efficient processing of long flaps. Recent evidence from our laboratory shows that in addition to the two main nucleases, pol δ and RPA are also acetylated by p300. Acetylation of pol δ stimulates its strand displacement function, thereby promoting the creation of longer flaps. On acetylation, RPA shows higher binding affinity to ssDNA promoting longer flaps. Significantly, acetylation of the main repair enzyme, DNA polymerase β (pol β), shifts repair into the long patch mode. Differential regulation of the activities of replication/repair by p300 indicates a mechanism in which the acetylase promotes formation of longer flaps in the cell at the same time as insuring correct processing. Such intentional formation of longer flaps mediated by p300 in an active chromatin environment would increase the re-synthesis patch size, providing increased opportunity for incorrect nucleotide removal during DNA replication and damaged nucleotide removal during DNA repair. Thus we propose acetylation to be a regulatory mechanism, which promotes higher fidelity of DNA transactions in selected, actively expressed parts of the genome.

AN SBF-TO-MBF SWITCH ENABLES RAD53-DEPENDENT ACTIVATION OF G1/S CELL CYCLE REGULATED GENES

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Activation of G1/S cell-cycle transcription is vital for entry into S-phase and initiation of DNA replication. In mammals, cell-cycle-dependent switches between E2F transcription factor complexes at G1/S gene promoters regulate the G1/S transcriptional wave, but the relevance of these switches is not fully understood. Here we show that a switching mechanism between the yeast E2F functional analogues SBF and MBF allows replication stress-induced transcription of G1/S genes. SBF activates transcription of TOS4 and MCD1 during G1, whereas MBF represses expression in S-phase. Consequently, during replication stress, the checkpoint kinase Rad53 is able to induce these and other cell-cycle transcripts via regulation of the MBF co-repressor Nrm1. Finally we link Tos4's important role in the checkpoint response to its interaction to, and probably regulation of, histone-deacetylases. Our data reveal that transcription factor switching couples cell-cycle targets to the checkpoint-transcriptional response and is likely a general fail-safe mechanism to maintain periodic transcription of certain genes.

VARIABLE MODIFICATIONS OF PCNA IN RESPONSE TO DIFFERENT FORMS OF REPLICATIVE STRESS

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Investigating the role of proliferative cell nuclear antigen (PCNA) and its modifications in maintaining genomic stability has yielded important insights into how cells are capable of tolerating DNA damage during S phase. Recent work from our laboratory has described how different forms of replicative stress can activate different site-specific ubiquitination events targeting PCNA. Inhibition of pol δ or pol ε triggers ubiquitination of PCNA at lysine 164, which facilitates recruitment of translesion polymerases. In contrast, defects in the ligation of Okazaki fragments cause PCNA ubiquitination at lysine 107 (Das-Bradoo et al., NCB 2010). These results indicate that inhibition of DNA synthesis results in lysine 164 ubiquitination, whereas errors in the processing of lagging strand fragments trigger lysine 107 ubiquitination. However, it is unclear how ubiquitination at lysine 107 promotes genomic stability. To address this question, we generated and analyzed *pol30-K107* mutants. Although these cells did not display any growth defects or drug sensitivity, we observed significant increases in both PCNA ubiquitination and sumoylation, arguing that these mutants encounter replication stress. We are currently investigating the implications of these modifications and the pathways involved.
To further examine modifications of PCNA following inhibition of lagging strand synthesis, we extended our analysis to *pol1* and *rad27* mutants defective in polymerase α and flap endonuclease function. Interestingly, disruption of *POL1* triggered ubiquitination of PCNA at lysine 164 but *rad27* mutants exhibited lysine 107 ubiquitination. This suggests that cells have the ability to distinguish between replication stress imposed during the initiation vs. processing steps of Okazaki fragment synthesis. Furthermore these results support the notion that ubiquitination of PCNA at lysine 107 is a more general response to defects in Okazaki fragment processing and not solely specific to the inhibition of DNA ligase I. [Supported by NIH grant GM074917 and LLS1023-09]

LOSS OF THE POLζ CATALYTIC SUBUNIT REV3 CAUSES GENOMIC INSTABILITY INDEPENDENTLY OF ITS REGULATORY SUBUNIT REV7 IN HUMAN CELLS

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Translesion synthesis (TLS) is a cellular process by which specialized DNA polymerases synthesize DNA across otherwise replication-blocking lesions. Apart from their role in TLS, additional functions have been found for some TLS polymerases, such as somatic hypermutation and involvement in nucleotide excision repair. An unknown function has long been suspected for TLS polymerase zeta (Polζ) as well. Polζ consists of two subunits: the catalytic subunit Rev3 and a regulatory subunit Rev7. Rev3 in mammals is almost double the size of its yeast counterpart and its inactivation causes embryonic lethality, while transgenic mice lacking other known TLS polymerases are viable. The reason for such an essential role of mammalian Rev3 in embryonic development is currently unclear primarily due to experimental limitations like the lack of a specific antibody against mammalian Rev3, difficulty in expressing REV3 exogenously because of its extremely large protein size (over 350 kDa) and the absence of stable knockdown or knockout cell lines to study long-term effects. Conversely, Rev7 is easier to investigate due to its smaller size (~26 kDa) and availability of reagents. Rev7 is also called mitotic arrest deficient like 2 (MAD2L2) and is known to play multiple roles in addition to TLS. To understand whether such functions are part of Polζ as a protein complex or are specific to Rev7, we carried out a parallel gene suppression study in human HCT116 and HeLa cells using siRNA and lentiviral systems. We found that cellular Rev3 and Rev7 levels increase during metaphase perhaps largely due to increased transcript levels. Surprisingly, the two proteins localize independently of each other in mitotic cells. Rev3 localizes to the chromatin whereas Rev7 is found around the spindle structure. The transient suppression of REV3 but not REV7 results in a significant increase in anaphase bridges (~3 fold) and double-strand chromosomal breaks (~5 fold). Rev7 depletion caused increased prevalence of lagging chromosomes (~5 fold) but no such increase was observed in Rev3-depleted cells. These observations demonstrate that Rev3 and Rev7 work independently of each other during metaphase and the resulting chromosomal breaks may act as a trigger for cell death during embryonic development.

FUNCTIONAL AND STRUCTURAL RELATIONSHIP OF DNA POLYMERASE ζ AND REV1

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Translesion Synthesis (TLS) is the mechanism by which specialized polymerases replicate through DNA damage. Two of these polymerases, Rev1 and DNA polymerase (Pol) ζ, function as a complex to carry out low-fidelity DNA replication resulting in mutagenesis. Rev1 catalytic activity is dispensable for UV-induced mutagenesis, however its interaction with mono-ubiquitinated PCNA and Pol ζ is essential. This suggests that Rev1 may act as a scaffold protein for TLS. We have previously shown that mutations in the PCNA-binding or the ubiquitin-binding domains of Rev1 greatly decrease the efficiency of damage-induced mutagenesis in yeast. We have now identified a conserved regulatory motif (M1) in Rev1 that, when mutated results in a 10-30 fold stimulation of Pol ζ replication activity. Stimulation requires the interaction between Rev1 and Pol ζ, and is absolutely dependent on the presence of PCNA. However, the catalytic activity of Rev1 is not required. We hypothesize that the M1 motif participates in the regulation of damage-induced mutagenesis. The mechanism of Rev1 regulation is currently being investigated.

ASYMMETRIC ASSEMBLY OF T-ANTIGEN AT THE ORIGINS OF POLYOMAVIRUSES

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The central regions of many viral origins contain inverted repeats or near inverted repeats that are responsible for recruiting virally-encoded large T-antigen molecules. These molecules assemble on the DNA as a head-to-head double-ring structure with six T-antigen subunits in each ring. This structure melts the DNA at AT-rich sequences flanking the inverted repeat region. The DNA sequence that appears as an inverted repeat within polyomaviruses is G(A/G)GGC. This sequence binds the origin-binding domain (OBD) within large T-antigen. We've found using isotermal titration calorimetry that the origin-binding domain of murine polyomavirus occupies only three of the four symmetrically-arranged G(A/G)GGC binding sites. Molecular modeling indicates that although the binding sites are symmetrically arranged, some sites are too close together to be simultaneously occupied. Thus, the protein assembly that forms on this symmetrical DNA is intrinsically asymmetric. These data are wholly consistent with our recent crystal structure and binding studies involving the origin of Merkel Cell Virus. They are also consistent with studies in the SV40 system, wherein asymmetrical DNA footprints are seen even though the spacing of GAGGC binding sites allows simultaneous binding of all four protein subunits. Using isothermal titration calorimetry, we've also characterized the affinity of the origin-binding domains for the tandem repeat GAGGC sequences found adjacent to the AT-rich stretch on the early side of the origin. These are termed Site I or Site A depending on the virus. In SV40, the origin-binding domain binds Site I approximately ten times tighter than the central sequences that are arranged as inverted repeats. In most (but not all) viruses these Site I/Site A sequences are dispensable for replication. Their presence on only one side of the origin, however, highlights the essentially asymmetric nature of the T-antigen DNA complex that forms on the origin of polyomaviruses.

REGULATION OF DNA REPLICATION THROUGH SLD3-DPB11 INTERACTION IS CONSERVED FROM YEAST TO HUMANS

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Cyclin dependent kinases (CDK) play crucial roles in promoting DNA replication and preventing re-replication in eukaryotic cells. In budding yeast, CDKs promote DNA replication by phosphorylating two proteins, Sld2 and Sld3, which generates binding sites for pairs of BRCT repeats in the Dpb11 protein. The Sld3-Dpb11-Sld2 complex generated by CDK phosphorylation is required for the assembly and activation of the Cdc45-Mcm2-7-GINS (CMG) replicative helicase. In response to DNA replication stress, the interaction between Sld3 and Dpb11 is blocked by the checkpoint kinase Rad53, which prevents late origin firing.
Dpb11's vertebrate orthologue is the multi-BRCT protein TopBP1 with the BRCT pair 1/2 being orthologous to the Sld3-binding BRCT1/2 of Dpb11. It has recently been shown that each of these BRCT repeats contains a potential phospho-binding pocket. A previously unidentified BRCT domain (BRCT0) was also found at the N-terminus of the protein.
We found that the two key CDK sites in Sld3 are conserved in the human Sld3-related protein, Treslin/ticrr (T969 and S1001), and CDK phosphorylation of these two sites mediates interaction with BRCT0/1/2 repeats in TopBP1. Peptide binding analysis showed that both CDK-sites cooperate in binding to BRCT0/1/2 supporting a model in which each phospho-site binds to one phospho-binding pocket in the BRCT0/1/2 domain. Moreover, mutation of T969 and S1001 to alanine renders Treslin/ticrr incapable of both binding to TopBP1 in cells as well as rescuing replication in cells in the absence of endogenous Treslin.
Finally, we showed that DNA replication stress prevents the interaction between Treslin and TopBP1, similar to Sld3 regulation in yeast. Moreover, the Chk1 checkpoint kinase is required for this process.
In conclusion, our results indicate that Treslin/ticrr is a genuine orthologue of Sld3 and that the Sld3-Dpb11 interaction has remained a critical nexus of S phase regulation through eukaryotic evolution.

THE MECHANISM OF REPLICATION FORK-ASSOCIATED COHESION ESTABLISHMENT FACTORS

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Establishment of sister chromatid cohesion is a process thought to occur as the replication fork passes chromosomal loci bound by the cohesin complex. After fork passage, cohesin holds together pairs of replication products to allow their recognition by the mitotic machinery for segregation into daughter cells. What happens at replication forks to establish sister chromatid cohesion is poorly understood.
We have shown that an essential replication fork-associated acetyltransferase, Eco1, acetylates the cohesin subunit Smc3 during DNA replication to render cohesin resistant against the cohesin destabiliser Wapl. Accordingly, in the absence of Wapl, cohesin acetylation is dispenseable for cohesion establishment. We have therefore turned our attention to a series of additional 'cohesion establishment factors', replication fork-accociated proteins required for efficient cohesion establishment. These include Chl1, Ctf4, Ctf18, Mrc1, Tof1 and Csm3. The action of these proteins could either be to support Eco1 in acetylating cohesin, or they could contribute to an Eco1-independent cohesion establishment reaction. Here we have used genetic and molecular assays to investigate the relationship of these cohesion establishment factors with the cohesin acetylation pathway. This revealed a contribution of all of these factors to efficient cohesin acetylation. However, removal of the cohesin destabiliser Wapl corrected the cohesion defect in most, but not all of the cohesion establishment mutants. As their function cannot be substituted for by Wapl removal, they define a subset of Eco1-independent cohesion establishment factors. Consistently, our genetic analysis revealed pronounced synthetic interactions of these factors with Eco1. We will present our progress in trying to decipher their molecular function at the replication fork and in the process of cohesion establishment.

SINGLE MOLECULE ANALYSIS DEMONSTRATES THAT RPA PHOSPHORYLATION STIMULATES FORK PROGRESSION DURING REPLICATION STRESS

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The RPA2 subunit of heterotrimeric RPA is a target for PI3-like kinases (PIKK; ATR, ATM, and DNA-PK) and cyclin-CDK in response to genotoxic stress. RPA2 modification by these kinases facilitates: i) adaptation of a DNA replication fork to replication stress, and ii) RAD51 recruitment and homologous recombination-mediated DNA repair. To understand the role of RPA2 phosphorylation on replication fork movement during replication stress, we have implemented Single Molecule Analysis of Replicated DNA (SMARD). DNA replication was studied in cells in which endogenous human RPA2 was replaced with RPA2 variants mutated to prevent phosphorylation at the two consensus PIKK sites, or the two cyclin-CDK sites. Using SMARD, we observed that hydroxyurea (HU)-treated cells expressing the PIKK or CDK site mutants each had significantly slower apparent fork rates, and a reduced ability to recover from stress, compared to control cells replaced with wt-RPA2. Knockdown of the PP4R2 regulatory subunit of an RPA2 phosphatase complex also reduced fork movement during replication stress, and during the recovery phase. Thus, either decreasing or increasing RPA phosphorylation from normal levels impedes fork movement during stress. Finally, we find that altered RPA phosphorylation reduces MRE11 association with replication foci during stress. These data suggest that proper RPA phosphorylation acts to recruit MRE11 for the processing of stalled DNA replication forks, stimulating fork movement.

RICE OSRAD51D IS ASSOCIATED WITH FLORAL ORGAN FORMATION AND ENDOSPERM DEVELOPMENT

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Rad51 is one of the important components in homologous recombination (HR) in eukaryotes. In addition to Rad51, mammalian cells contain five Rad51-like proteins (Rad51B, Rad51C, Rad51D, XRCC2, and XRCC3), which play a role as mediators of HR reaction. We identified four rice Rad51 paralogs containing conserved ATPase domain and Walker A and B DNA binding motifs. We found physical interactions among these paralogs in vitro, resulting in the heterotrimeric or heterodimeric complex. We obtained a loss-of-function *osrad51d* T-DNA knockout mutant line and generated RNAi-mediated knockdown lines. Terminal restriction fragment analysis revealed that telomeres in mutants were longer than those of wild type (WT) plant. Homozygous G3 *osrad51d* plants showed normal growth in their vegetative stage. However, the mutant displayed severely impaired reproductive organs and defective anther dehiscence, resulting in sterile flower. *osrad51d* exhibited abnormal pollen development, which led to disrupted pollen viability. Heterozygous G3 mutant plants also showed lower seed filling rate and their mature seeds displayed opaque phenotype composed of abnormal starch granules and multi-layered aleurone layers. Quantitative RT-PCR represented that transcript of seed development related genes reduced in *osrad51d* mutant. The promoter region of OsRad51D contains GCN4 and skn-1 motifs, both of which are required for endosperm development. Using gel retardation assay, we found that RISB, known as one of the important transcription factors in seed development process, binds to OsRad51D promoter in vitro. These results suggest that OsRad51D participates in proper reproductive organ formation and seed development.

SPATIO-TEMPORAL CHARACTERIZATION OF THE REPLICATION PROGRAM ALONG THE WHOLE HUMAN GENOME

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To date, little is known about the molecular mechanisms that govern where and when origins of replication are activated. In order to identify general properties of replication origins, we have mapped active origins along 1% of the human genome in HeLa cells (Cadoret et al, PNAS 2008). Origin density is strongly correlated to genomic landscapes, with clusters of closely spaced origins in GC-rich regions and large GC-poor regions lacking origins. Half of the origins map within or near CpG islands (CGI) and most of the origins overlap transcriptional regulatory elements, providing further evidence of a connection with gene regulation. Finally, half of replication initiation sites identified do not have an open chromatin configuration showing the absence of a direct link with gene regulation.
In order to study replication origins plasticity, we mapped replication origins with the same method along the same regions in the human embryonic carcinoma cell line Ntera2. We also performed whole genome mapping of origins in K562 cells by using deep-sequencing. We defined a class of "constitutive" origins that are found in the three cell lines (~30%) and a class of "tissue specific" origins which are present in only one cell line (~30-40%). Constitutive origins are enriched in CGI whereas tissue specific origins are depleted. Altogether our results show that the spatial program of DNA replication is quite robust but also adapts to cell differentiation.
We have also performed a second analysis which integrates the whole genome temporal map constructed by the group of Stamatoyannopoulos with our whole genome map of replication origins made in the same cell line (K562). We shall discuss new information extracted from this study such as the presence or not of replication origins inside temporal transition regions.

THE APC/C ACTIVATOR CDH1 PROMOTES CHROMOSOMAL STABILITY AND EFFICIENT FORK FIRING AT A SUBSET OF ORIGINS BY REGULATING CDK ACTIVITY

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CDK deregulation in G1 is frequent in cancer cells, and causes chromosomal instability in correlation with defects in origin activation. Accumulative evidences support the model that G1-, and S-CDK upregulation limits the number of licensed origins to fire forks efficiently, resulting in an increased rate of Gross Chromosomal Rearrangements (GCR). To address if all CDK regulators contribute equally to efficient initiation and chromosome stability, we studied the relevance of mitotic cyclin degradation by Cdh1-APC/C in *S. cerevisiae*. Our results show that *Δcdh1* cells have increased fork firing inefficiency at origins, and twice the rate of GCR than *wt* cells. Despite the number of substrates of Cdh1, duplicating the chromosomal copy of the yeast CDK inhibitor *SIC1* is sufficient to suppress both defects indicating that CDK inactivation is the main role of Cdh1 for efficient origin activity and prevention of chromosomal rearrangements. Significantly, origins lose efficiency differentially in *Δcdh1* cells. Indeed, most studied origins maintain normal efficiency (resistant origins), while the rest lose efficiency (sensitive origins) to different degrees. Origin sensitivity is independent on chromosomal location, firing timing, or origin efficiency during normal S phases. Discarding particularities of *Δcdh1* cells, the same origins are resistant or sensitive when we deregulate CDK by depleting Sic1. Thus, it seems some origins (resistant) retain unaffected their normal ability to attract licensing machinery in the presence of increased CDK levels able to partially inhibit other origins (sensitive). However, all origins became sensitive when CDK is highly deregulated, as in cells lacking Cdh1 and Sic1. Supporting the model, the closest active origin (*ARS507*) to the region where we estimate the rate of GCRs at chromosome V, is sensitive, and the rate of GCRs correlates with the extent of inefficiency at *ARS507*. It suggests that those chromosomal regions close to sensitive origins are more prone to break when CDK is deregulated than those regions close to resistant origins. However, causal relationships should exist to validate the model of origin firing inefficiency promoting chromosomal rearrangements. Recent results addressing this issue will be presented.

HUMAN DNA2/BLM AND EXO1 PARTICIPATE IN PARALLEL LONG-RANGE RESECTION PATHWAYS FOR REPAIR OF DSBS DUE TO REPLICATION STRESS

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Recent studies of oncogene induction in human cells have provided strong evidence that DNA replication stress is an early step in cancer development. Such stress is thought to lead to double-stranded breaks (DSBs), hyper-recombination, and chromosome instability. Repair of DSBs during HR proceeds after 5' DNA resection at break sites. After short range resection by MRN/CtIP, one of two pathways mediates a long range resection reaction, yielding a 3' ssDNA overhang suitable for recombination and checkpoint activation. One pathway is mediated by EXO1. The other requires the BLM helicase, but the nuclease has not been identified. Reconstitution experiments implicate human DNA2, a DNA replication helicase/nuclease as the nuclease that functions together with BLM helicase to ensure 5'resection (Nimonkar, 2011). Here, we demonstrate that DNA2 indeed functions in parallel to EXO1 in long-range resection in vivo. In the absence of DNA2 and EXO1, cells have increased sensitivity to DNA damage and are incapable of phosphorylating RPA2 and Chk1 and have reduced RPA and ssDNA foci after S phase-specific DNA damage. Conversely, depletion of DNA2 and EXO1 activates DNA-PK phosphorylation, critical for the NHEJ pathway, presumably to compensate for the loss of the HR pathway. We propose that as in yeast, a DNA2/BLM complex is important for DSB repair in human cells during replication stress.

Nimonkar, A.V. et al., 2011. Genes Dev. 15, 350-62.

REGULATING THE ATR CHECKPOINT THROUGH CLASPIN DEGRADATION

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Maintenance of genomic integrity is critical for cellular survival. Cells must constantly defend against DNA damage that arises from both internal (*e.g.*, reactive oxygen species generated from cellular respiration) and external (*e.g.*, UV radiation) sources. To help safeguard their genomes against such threats, eukaryotes have evolved complex signaling pathways called checkpoints. Upon the generation of single stranded DNA (ssDNA) coated with RPA at resected double strand breaks (DSBs) or stalled replication forks, the Ataxia-Telangectasia Mutated and Rad3-related (ATR) kinase is activated to phosphorylate its substrates. Through the mediator, Claspin, ATR activates the effector kinase Chk1, which promotes replication fork stabilization, DNA repair, and cell cycle arrest until the damage is repaired. Claspin has been shown to be targeted for ubiquitin-mediated degradation in G2/M and G1 phases of the cell cycle, which renders the ATR checkpoint response most robust during S phase.

Here we describe a novel mechanism that controls the stability of Claspin during the DNA damage response. Upon treatment with UV radiation, Claspin levels decline over time. The reduction of Claspin correlates with the attenuation of Chk1 phosphorylation. UV-induced Claspin degradation occurs in S phase and is thus different from the previously described $CRL1^{\beta\text{-}TrCP}$ and APC^{Cdh1}-mediated Claspin degradation in G2/M and G1. We show that this drop in Claspin protein levels is dependent upon the 26S proteasome and ATR, but not on Chk1. While Claspin degradation is induced by UV, it does not occur when DNA replication is inhibited by hydroxyurea, suggesting that specific DNA intermediates and/or repair factors are required. We propose that damage-induced Claspin degradation in S phase may contribute to an ATR-triggered feedback loop that terminates Chk1 activation at the late stage of the checkpoint response, which may be important for efficient checkpoint recovery and cell cycle progression after UV damage.

XENOPUS MCM10 AND ITS ROLE IN DNA REPLICATION

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Chromosomal DNA replication in higher eukaryotes has evolved as a complex regulated process involving a large number of proteins. Both the identity of these replication factors and their regulation are not fully understood in multicellular organisms. Utilizing the Xenopus egg extract system we have analyzed the role of one such replication factor, Mcm10, which has been previously reported as an essential replication initiator. In our investigation we find that Mcm10 is accumulated into the nucleus at the onset of S-phase and its chromatin binding is tightly associated with bulk DNA replication. Mcm10 requires nuclear envelope and functional nuclear pore formation for its import into the nucleus. No Mcm10 chromatin binding was observed when chromatin was isolated from membrane free extract, incapable of making nuclei, or extract treated with wheat germ agglutinin, where no nuclear pore formation occurred. As previously reported, Mcm10 chromatin binding was found to be DNA licensing dependent, i.e. it requires prior loading of Mcm2-7 proteins. But unlike previous reports suggest, Mcm10 chromatin binding requires Cdc7 kinase activity, which primarily phosphorylate Mcm2-7 proteins. Moreover we provide evidence for a strict requirement of CDK-activity as a pre-requisite for Mcm10 chromatin loading. Our results suggest Cdk-dependent chromatin association of Xenopus Mcm10 is an indirect effect involving some upstream Cdk-substrate in mediating its chromatin binding. We did not observe any major effect on bulk DNA replication on Mcm10 depletion from Xenopus egg extract, though there is some effect on stability of replication proteins on chromatin.
We are presently investigating the factors responsible for Mcm10 chromatin loading and the exact role of Xenopus Mcm10 in the replication process.

FUNCTIONAL DISSECTION OF THE YEAST HELICASE ACTIVATING PROTEIN CDC45

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During G1 to S transition, the Mcm2-7 helicase is activated by the S-CDK and DDK dependent recruitment of the helicase activating proteins Cdc45 and GINS. The resulting Cdc45/Mcm2-7/GINS (CMG) complex unwinds double-stranded DNA at the replication fork. Although it is clear that the CMG complex possesses robust helicase activity that is dependent on GINS and Cdc45, the mechanisms by which these proteins stimulate Mcm2-7 helicase activity are unclear. Recruitment of Cdc45 and GINS occurs sequentially with Cdc45 associating with Mcm2-7 in a DDK- and Sld3-dependent manner. Subsequently, S-CDK stimulates the association of GINS along with Sld2 and Dpb11.

To better understand the mechanism of helicase activation, we are dissecting the function of Cdc45 using a combination of *in vivo* and *in vitro* approaches. We have generated a series of deletion and point mutations of Cdc45 based on homology and structural prediction algorithms. We are testing each of the mutants both for the complementation of a CDC45 deletion *in vivo* as well as the ability to complement an *in vitro* replication initiation assay that has been depleted for Cdc45. For any mutant that does not allow DNA replication, we are investigating which of the several biochemical step(s) in the replication reaction that is defective. These Cdc45-dependent events include the initial recruitment of Cdc45 to the origin, subsequent recruitment of GINS, Mcm2-7 helicase activation, or recruitment of replicative polymerases to the replication fork. Our findings will provide important new information about both Cdc45 function and the events that occur during helicase activation.

GRADIENTS OF REPLICATION-ASSOCIATED MUTATIONAL-ASYMMETRY ARE GENERATED BY A SPECIFIC SPATIO-TEMPORAL REPLICATION PROGRAM

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Previous analyses of the nucleotide compositional skew S=(G-C)/(G+C)+(T-A)/(T+A) along the human genome revealed large domains of ~1Mb exhibiting a characteristic N-shaped pattern and covering more than 1/3 of the genome, called N-domains. Sharp upward jumps of the skew profile (S-jumps) at domain borders are associated with earlier replication initiation zones. The striking linear decrease of the skew between these initiation zones raises the following questions: does it result from mutational asymmetries associated with replication? To what extent does this reflect a specific spatio-temporal replication organization?

We have shown that S-jumps that coincide with replication initiation zones result from mutation asymmetries, i.e. different mutation rates on the leading and lagging DNA strands of the replication fork in germline cells. We further showed that along N-domains, these replication-associated mutation asymmetries decrease from maximum values at the left end decreasing to zero at the center and to opposed values at the right end. The skew that would result from these substitution rates acting over long evolutionary times strikingly reproduces the N-shaped pattern: this strongly suggests that this pattern reflects a progressive inversion in replication fork polarity.

We demonstrated that the mean fork polarity can be extracted from the derivative of the timing profile: this profile (e.g. in embryonic stem cells) along N-domains presents a “U-shape” pattern and its derivative is an “N”. This strongly supports that the N shaped skew profile results from a replication fork polarity gradient.

Early replication initiation zones at N-domain borders are enriched in open chromatin marks suggesting that the replication program within these domains is mediated by a gradient of open chromatin conformation. We propose a model in which replication first initiates at N-domain extremities and secondary origins fire coordinately from the borders to the centers in a domino-like manner. Computational simulations of this model generate linear gradients of replication fork polarity and N-shaped skew. Given that replication timing U-domains are widely observed in both human and mouse and that N-domains are present in all studied mammals, this replication program has likely been conserved at least since the mammalian radiation.

FUNCTION OF ALTERNATIVE REPLICATION PROTEIN A IN NORMAL AND TRANSFORMED CELLS

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Both normal and transformed cells must maintain the integrity of their genome for survival. Replication Protein A (RPA) is an essential protein required for both genome maintenance and DNA replication. Preliminary data indicates that human cells have an alternative RPA complex (aRPA) that is antagonistic to the canonical RPA complex in DNA replication. These two complexes differ by one subunit: RPA2 is found in canonical RPA while RPA4 is in aRPA. This subunit switch dramatically changes biological function: both complexes support DNA repair, but aRPA inhibits DNA replication. RPA4 is expressed in normal human tissues but is down-regulated in cancer and in proliferating normal cells. This expression profile supports a role for RPA4 in the regulation of proliferation. It also has prevented the functions of RPA4 from being identified previously because RPA4 is not expressed in cultured cells.

The molecular basis of the different activities of aRPA and its role in normal cells remains poorly understood. Biochemical analysis suggests that altered interactions between aRPA and DNA polymerase alpha prevent aRPA from supporting initiation and Okazaki fragment priming. However, aRPA is able to support DNA repair and DNA synthesis by DNA polymerase delta in the presence of PCNA and RFC. This suggests that both RPA and aRPA function to maintain genome stability and that RPA4 expression prevents cell proliferation. This model is supported by studies that show that constitutive expression of RPA4 is not compatible with cell growth. Current studies are underway to identify the cell distribution of RPA4 expression in normal tissues and how RPA4 expression changes with proliferation. We are also determining how RPA4 levels affect cell proliferation and genome integrity. These studies will provide insights into the cellular functions of aRPA.

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THE C-TERMINUS OF TOPOISOMERASE IIIA IS ESSENTIAL FOR DOUBLE HOLLIDAY JUNCTION RESOLUTION.

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Topoisomerase (Topo) IIIα is an essential component of the double Holliday junction (dHJ) resolving complex in metazoans, along with Blm and Rmi1&2. This important anti-recombinogenic function cannot be performed by Topo IIIβ, the other type IA topoisomerase present in metazoans. The two enzymes share a catalytic core, but diverge in their tail regions. To understand this difference in function, we investigated the role of the unique C-terminus of Topo IIIα. Truncation of the C-terminus has a minimal effect on the type IA relaxation activity of Topo IIIα. However, dHJ resolution is completely abolished. The Topo IIIα C-terminus was also found to strongly interact with both Blm and DNA, which are critical to the resolution reaction. These interactions are greatly reduced in the truncated enzyme. In addition, the *Drosophila* C-terminus contains an insert region not conserved among metazoans. This insert contributes an independent interaction with Blm, which may account for the absence of Rmi1 in *Drosophila*. Therefore, we observe that the C-terminus of Topo IIIα is vital in directing the function of the enzyme during dHJ resolution in the purified complex.

DBF4 CONTROLS DNA REPLICATION, CHROMOSOME SEGREGATION, AND DNA DAMAGE CHECKPOINT SIGNALING.

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Dbf4 is the regulatory subunit of Cdc7 kinase, and is essential for Cdc7 kinase activation and origin firing. We recently reported the molecular basis of a Dbf4 interaction with Cdc5, the only Polo-like kinase in budding yeast. Cdc5 binds to the Dbf4 N-terminus, which is not required for DNA replication. Interestingly, Dbf4 inhibits Cdc5 to prevent exit from mitosis and this is critical when the nuclear spindle is misaligned. Cdc5 inhibition is Cdc7 dependent, suggesting that Dbf4-Cdc7 participates in post-replicative regulation of mitosis. We performed a genome-wide synthetic genetic array (SGA) screen using a *dbf4* mutant unable to bind Cdc5 but otherwise normal for DNA replication. We found that Dbf4 acts in parallel with DNA damage signaling, especially with Rad53 (human Chk2) checkpoint kinase activation. We have now mapped a short Rad53 binding site within Dbf4 and show that the Dbf4-Rad53 interaction is likely mediated by both Rad53 FHA domains. This interaction can be facilitated by eliminating the Cdc5 binding site, probably because Cdc5 binds directly adjacent to the Rad53 binding site. Surprisingly, *dbf4* N-terminal mutants are synthetically lethal with *rad53-1 sml1*Δ (*or rad53*Δ *sml1*Δ) only if they lack the ability to bind *both* Cdc5 and Rad53. Though the biological relevance of the synthetic interactions remains to be fully characterized, our data suggest that Dbf4 acts as a molecular scaffold to coordinate multiple kinases essential for cell cycle progression.

THE STRUCTURAL AND FUNCTIONAL ANALYSIS OF THE ORC6 PROTEIN

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The Origin recognition complex (ORC) is a six-subunit protein important for the DNA replication in eukaryotic cells. ORC is also involved in other cell functions. The smallest metazoan ORC subunit, Orc6, consists of two functional domains. A larger N-terminal domain is directly involved in DNA binding and important for DNA replication. A smaller C-terminal domain of the protein is important for cytokinesis.
Molecular modeling and structural analysis revealed that the N-terminal domain of both *Drosophila* and human Orc6 proteins has an overall fold similar to the transcription factor TFIIB. Amino acids of Orc6 which are directly involved in DNA binding are identified. Alterations of these amino acids abolish DNA binding ability of Orc6 and also result in reduced levels of DNA replication *in vitro* and *in vivo*. We have shown that the expression of human Orc6 in *Drosophila* Orc6 mutant cells rescued DNA replication indicating that two proteins are homologous in replication function. The fusion Orc6 protein containing human N-terminal and *Drosophila* C-terminal domains rescues the lethality associated with the deletion of *orc6* gene in Drosophila. Our data indicate that Orc6 is a one of the DNA binding subunits of metazoan ORC and may position ORC at the origins of DNA replication similar to the role of TFIIB in positioning transcription pre-initiation complex at the promoter.

EFFECT OF WALKER B MUTATION ON RFC, THE EUKARYOTIC CLAMP LOADER

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The processivity of DNA replication polymerases is increased tremendously by sliding clamps, which tether the polymerases to the DNA. Clamp loaders, members of the AAA+ family of ATPases, are required to load the clamps on the replication fork. Clamp loading involves steps such as binding the clamp, opening the clamp, binding the DNA, closing the clamp around the DNA and releasing the clamp and DNA. Some of these steps are associated with ATP binding and others with hydrolysis. P-loop-type NTPases including AAA+ ATPases contain conserved Walker A and Walker B sequence motifs that participate in ATP binding and hydrolysis, respectively. A conserved Glu residue in the Walker B motif (DExx) is believed to activate a water molecule to catalyze ATP hydrolysis, and mutation of this Glu reduces the rate of hydrolysis by as much as a couple of orders of magnitude in some ATPases. We mutated the Walker B Glu in eukaryotic clamp loader, RFC, to Gln in each of the four active ATP sites and characterized ATP binding- and hydrolysis-dependent activities of the quadruple mutant. Fluorescence-based assays were used to measure individual reaction steps required for clamp loading including ATP binding, ATP hydrolysis, clamp binding, clamp opening and DNA binding. Our results show that the Walker B mutations (a) cause a relatively small reduction in ATP hydrolysis activity, which is surprising given the proposed role of Glu in activating a water molecule for catalysis and, (b) affect ATP-binding-dependent activities of RFC, a previously unknown function for this conserved Glu. Thus, the Glu residue may have an important role in mediating ATP-dependent conformational changes in addition to catalyzing ATP hydrolysis.

MULTIPLE RECOMBINATIONAL REPAIR PATHWAYS FACILITATE REPLICATION UNDER DNA DAMAGE CONDITIONS

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DNA lesions generated by endogenous and exogenous agents pose a great challenge for complete genome duplication. Cells employ multiple mechanisms to bypass these lesions or repair the ssDNA gaps left behind by the replisome. Some of these mechanisms require the core recombinational machinery as well as proteins that specialize in coupling impaired replication with recombination. This latter group of proteins includes the two helicases Mph1 and Rad5 that can catalyze fork regression, the Rad51 paralogue Shu complex, and the ubiquitin conjugating enzyme Mms2 that promotes PCNA polyubiquitination. How these proteins function in genetic pathways is not well understood. We showed that Mph1, Shu and Mms2 independently promote the formation of recombination structures during impaired replication, indicating that they can function in separate pathways. We also found that the Smc5/6 complex and Esc2 act in all three pathways to limit the accumulation of toxic recombination structures. Our recent data suggest that the helicase function of Rad5 is involved in the recombinational repair process mediated by the Smc5/6 complex and Mms2, but not those requiring Mph1 and Shu, implying that the helicase activity of Rad5 can function with Mms2 in error-free repair. The presence of multiple pathways for replication-associated recombination repair underlines the importance and complexity of overcoming damage on templates.

A STRATEGY TO IDENTIFY NOVEL INTERACTORS WITH PCNA IN HUMAN CELLS USING BIMOLECULAR FLUORESCENCE COMPLEMENTATION AND 454 SEQUENCING

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A key member of the replication machinery, Proliferating Cell Nuclear Antigen (PCNA) plays an essential role in the cell as a node for many different regulatory pathways including DNA replication and repair, regulating cell cycle progression and sister chromatid cohesion. Although this homotrimeric sliding clamp has no intrinsic activity of its own it is able to recruit a range of other proteins. Many proteins interact with PCNA through the PCNA-interacting Protein (PIP) box motif, allowing them to bind one of the interdomain-connecting loops of the PCNA homotrimer. These motifs are hard to identify in a given protein sequence, due to their sequence degeneracy. The importance of post-translational modification of both PCNA and interactors further complicates interaction studies with PCNA. Thus, functional assays are required to identify novel interactors by directly studying their binding to PCNA.

By implementing a fluorescence–based functional screen of a cDNA library, novel PCNA interactors were implicated by their ability to interact with PCNA *in vivo*. Interactions between PCNA and the cDNA library directly produced fluorescence by bringing together N- and C-terminal fragments of Venus fluorescent protein. Flow cytometry was used to separate cells containing Venus-tagged PCNA interactors from those without on the basis of fluorescence. Genomic extracts made from fluorescing cells, grown in selective media, allowed purification of plasmids containing PCNA interactors. PCNA-interacting members of the original cDNA library were amplified by PCR of the purified plasmids and the amplicons sequenced by 454 sequence-by-synthesis technology.
By studying potentially new responsibilities of PCNA at the DNA replication fork and beyond, we may be able to better understand how such essential processes can go so wrong in diseases such as cancer.

This project has shown the potential of Bimolecular Fluorescence Complementation (BiFC) to directly probe interactions at the human DNA replication fork. Moreover, the use of flow cytometry and 454 sequencing technologies have allowed the rapid screening of large numbers of potential interaction candidates. Selected candidates from the screen will be presented, along with the steps taken to validate their interaction with PCNA and elucidate its biological relevance.

GENOME-WIDE SCREEN FOR MODULATORS OF CELL DEATH INDUCED BY THE NEDD8-ACTIVATING ENZYME INHIBITOR MLN4924

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The NEDD8-activating enzyme (NAE) is an E1 involved in the activation of a large family of ubiquitin E3 ligases termed the cullin-RING ligases (CRLs) through conjugation of the cullin proteins with the ubiquitin-like modifier NEDD8. Polyubiquitination of CRL substrate proteins targets them for degradation by the proteasome. In this way, NAE regulates the stability of proteins required for cancer cell growth and survival. MLN4924 is an investigational small molecule that is a potent and selective inhibitor of NAE in Phase I clinical trials. In order to investigate the primary genetic determinants that confer sensitivity of cells to NAE inhibition, we have performed a genome-wide synthetic lethal RNAi screen using MLN4924 in the A375 melanoma cell line. We have also investigated the biological consequences of NAE inhibition by studying the regulation of protein and transcript levels in MLN4924-treated A375 cells using large-scale quantitative proteomics and gene expression profiling, respectively. The RNAi screen has identified 123 genes whose down-regulation modulates MLN4924-induced cell death, and approximately one-third of these interfere with components of the NEDD8 pathway itself, the cell cycle and apoptotic machinery, and DNA damage-response pathways. Of these genes, 99 were subsequently assessed using high throughput FACS analysis for their contribution to the major phenotype induced by MLN4924. The results emphasize replication, p53, BRCA1/BRCA2, and transcription-coupled repair as being particularly important for MLN4924-induced cell death.

A SPONTANEOUS MUTATION IN CDT1 EXPRESSED IN 129/SV MOUSE STRAIN REVEALS A NOVEL MECHANISM CONTROLLING LICENSING ACTIVITY

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Cdt1 is an essential factor required for the loading of the replicative DNA helicase MCM2/7 allowing duplication of the genome. Cdt1 activity is under the control of multiple pathways, in order to prevent re-replication, genomic instability and oncogenic transformation. First, Geminin binds to and inhibits Cdt1 activity when present in adequate quantity. Also, Cdt1 turnover is regulated by overlapping ubiquitin ligases, SCF^{Skp2} and $Cul4^{DDB1/Cdt2}$, which are responsible for the degradation of Cdt1 during S phase and in response to DNA damage. In addition, Cdt1 chromatin association is cell cycle regulated with low binding observed in mitotic cells. The functional significance of the regulated chromatin association of Cdt1 is unclear. Here, we identified a homozygote deletion in Cdt1 gene found in the 129/Sv mouse strain, which is one of the most used strains for production of transgenic animals. This 6 aminoacids in frame deletion, present in Cdt1 PEST domain, was not detected in any other mouse strain. Characterization of the 129/Sv allele in addition to several other deletion mutants clearly shows that the PEST domain negatively regulates Cdt1 activity. Indeed, PEST mutants exhibited enhanced *in vivo* activity in re-replication assays compared to wild type (WT) Cdt1. Moreover, PEST deletion mutants of Cdt1 transformed NIH 3T3 mouse cells with higher efficiency than WT Cdt1, as revealed by loss of contact inhibition and soft-agar growth assays. Detailed analyses show that the enhanced activity of PEST mutants is independent of Geminin and E3-ligases pathways. However, we found that the PEST mutant is more strongly associated to chromatin compared to the WT protein, specifically in G2/M phases of the cell cycle. This inappropriate high chromatin occupancy of the PEST mutant is expected to stimulate MCM2/7 loading, leading to re-replication. The present study reveals that cell cycle regulated chromatin association of Cdt1 is under the control of its PEST domain and is crucial for proper licensing activity *in vivo*.

THE ROLE OF PCNA UBIQUITYLATION IN POST-REPLICATION REPAIR AND ITS EFFECT ON GENOMIC REPLICATION IN FISSION YEAST

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Post-replication repair (PRR) is a mechanism that enables cells to overcome replication problems caused by unrepaired DNA lesions. Studies in many organisms have demonstrated that, in eukaryotes, the pathway is controlled by the ubiquitylation of the replication clamp protein PCNA. Monoubiquitylation of PCNA activates translesion DNA synthesis, whereas polyubiquitylation is required for the error-free template switching pathway. It remains debatable whether the regulation of PRR is conserved evolutionally; many aspects of replication vary among organisms. In the context of PRR, the extent of induction of PCNA ubiquitylation by DNA damage is highly dependent on organism examined. Our studies in fission yeast show that PCNA ubiqiutylation influences genomic replication without DNA damage treatment. In fission yeast, PCNA ubiquitylation is not strongly induced by DNA damage during S-phase. Instead, the ubiquitylation is likely to be due to lagging DNA strand synthesis as PCNA ubiquitylation is enhanced when lagging DNA strand synthesis (or its maturation) is compromised. Furthermore, the abrogation of PCNA ubiquitylation rescues viability of cells in which one of the lagging strand polymerase subunits is truncated. Based on these observations, we argue that, in fission yeast (and potentially in higher eukaryotes), PCNA ubiquitylation not only regulates PRR but also influence mechanism of replication such as lagging strand DNA synthesis.

THE NUMBER OF MCM LOADED AT ORIGINS REGULATE REPLICATION TIMING IN BUDDING YEAST

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Replication is a well co-ordinated process, which though marked by stochastic events, is completed in a defined period of time. Replication timing is not uniform, in that some parts of the genome replicate earlier than others. Timing of DNA replication correlates with important genomic phenomena such as transcription and chromatin structure. The regions replicating early have origins that fire earlier in S-phase. So the basic question is - What defines the timing of an origin of replication? Origins are defined by the binding of the Origin Recognition Complex (ORC). ORC, along with Cdc6 and Cdt1, loads the MCM replicative helicase complex as a dimer onto origins. We propose that the regulation of timing of origin firing is regulated by the number of MCMs loaded. This model is based on a computational analysis of replication kinetics showing that the observed pattern of origin firing can be explained by a combination of the number of MCMs loaded and the chromatin environment of the origin (Yang et al., 2010). In our model, all MCMs fire with the same low probability, but early origins have more MCMs loaded so that in aggregate they are more likely to fire. Such a model explains the observed replication kinetics, and is consistent with 20-30 fold excess of MCM over ORC in cells. To test this model, we mapped MCM distribution in G1 at 100-bp resolution by Chip-seq. Our results show a significant correlation between MCM levels and the timing of origin firing. However, we also see a subset of origins with high levels of MCM that, nonetheless, fire late. These origins tend to be sub-telomeric, and suggest that chromatin context provides an additional level of timing regulation. Consistent with the model of multiple MCMs being loaded at each origin, we observe that MCMs spread out as much as a kilobase in both directions from the ACS. We speculate that the amount of MCM loaded at an origin may be controlled by the affinity of ORC for the origin, and thus how much time ORC spends bound to the origin loading MCMs. Consistent with this idea, we see a strong correlation between ORC ChIP-seq signal and MCM ChIP-seq signal. Using a biochemical approach to directly measure MCM and ORC binding to specific origins, we are now trying to quantitate the number of MCMs and the affinity of ORC bound at early/efficient origins compared to late/inefficient ones.

S. C. Yang, N. Rhind and J. Bechhoefer (2010) Modeling Genome-Wide Replication Kinetics Reveals a Mechanism for Regulation of Replication Timing. Mol Syst Biol 6:404.

DNA REPLICATION AND GENETIC STABILITY IN *SACCHAROMYCES CEREVISIAE* : A ROLE FOR THE SET1 HISTONE METHYLASE ?

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In *Saccharomyces cerevisiae*, H3 methylation at lysine 4 (H3K4) is mediated by Set1 a large protein that belongs to a complex of eight proteins, most subunits being required to efficiently catalyze methylation of H3K4. A negative effect of SET1 deletion on replication has been described only during meiosis (Sollier et al., 2004). One characteristic of meiotic S phase is its duration that is longer to that in mitotic conditions. The differential origin activity in the meiotic and mitotic cell cycles can account for their different S-phase lengths as described in *Schizosaccharomyces pombe* (Heichinger et al., 2006). We have made the assumption that such a difference could be the basis for the apparent meiotic specificity of Set1 loss on DNA replication. As a decrease in origin firing frequency exists in vegetative cells harboring mutations affecting proteins involved in replication initiation (Liang et al., 1995), we have deleted the SET1 gene in such genetic backgrounds. A clear genetic interaction is observed for the orc5-1 mutation with a consistent growth defect of orc5-1 set1Δ cells even at permissive temperature. This is correlated to a slower progression of DNA replication with genetic evidences for its incompleteness at some temperatures. Fortuitously, the duplication of one chromosome was observed rather frequently in orc5-1 set1Δ cells. Experiments are in progress in order to decipher whether this duplication comes from an elevated chromosomal instability or is selected due to some compensation for the replication defect of orc5-1 set1Δ cells.

THE DOSAGE COMPENSATION COMPLEX (DCC) REGULATES THE TRANSCRIPTION AND REPLICATION PROGRAMS OF THE DROSOPHILA MALE X-CHROMOSOME

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Drosophila melanogaster males possess one X chromosome while females have two. In order to compensate for the difference in gene dosage, transcription of the X chromosome is upregulated two-fold in males. This regulation is mediated by the dosage compensation complex (DCC), which is a ribonucleoprotein complex consisting of five proteins (MSL1, MSL2, MSL3, MOF, and MLE) and two non-coding RNAs (roX1 and roX2). The DCC is specifically targeted to the X-chromosome and up regulates transcription via the MOF dependent acetylation of lysine 16 on histone H4 (H4K16Ac). DNA replication timing assays have shown that the male X chromosome replicates significantly earlier in S-phase than autosomes and the female X chromosome. Previously published data has shown that areas of early replication on the male X chromosome correlate with areas of increased H4K16 acetylation, suggesting that the DNA replication and transcription programs respond to the same chromosomal cues. To investigate the role of the DCC in regulating the replication program of the X chromosome, we have depleted components of the DCC (MSL2 and MOF) by RNAi in male *Drosophila* cell lines and have studied the effects both cytologically and genomically. We find that the DCC and H4K16 hyperacetlyation are necessary for the X chromosome specific advance in replication timing. Analysis of ORC density and inter-early origin distance in both male and female cell lines suggests that the regulation of the X chromosome replication program occurs at the level of origin activation and not origin selection.

ASSEMBLY OF THE HUMAN ORIGIN RECOGNITION COMPLEX OCCURS THROUGH INDEPENDENT NUCLEAR LOCALIZATION OF ITS COMPONENTS

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Initiation of eukaryotic genome duplication begins when a six subunit 'origin recognition complex' (ORC) binds to DNA. However, the mechanism by which this occurs in vivo, and the roles played by individual subunits appear to differ significantly among organisms. Previous studies identified a soluble human ORC(2-5) complex in the nucleus, an ORC(1-5) complex bound to chromatin, and an Orc6 protein that binds weakly, if at all, to other ORC subunits. Here we show that stable ORC(1-6) complexes also can be purified from human cell extracts, and that Orc6 and Orc1 each contain a single nuclear localization signal (NLS) that is essential for nuclear localization but not for ORC assembly. The Orc6 NLS, which is essential for Orc6 function, is facilitated by phosphorylation at its CDK-consensus site and by association with Kpna6/1, nuclear transport proteins that did not co-purify with other ORC subunits. These and other results support a model in which Orc6, Orc1 and ORC(2-5) are transported independently to the nucleus where they can either assemble into ORC(1-6) or function individually.

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DPB11 BINDING TO ORIGIN SSDNA RELEASES THE PROTEIN FROM MCM2-7

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DPB11, an essential Saccharomyces cerevisiae gene, functions both in the initiation of DNA replication and the DNA damage checkpoint. It was recently shown that Dpb11 forms a transient "pre- loading" complex with GINS, Sld2 and Pol ε. This pre-loading complex is recruited to origins in S phase in a manner dependent on CDK activity. CDK phosphorylation of Sld2 and Sld3 promotes their interaction with the C and N terminal BRCT domains, respectively, of Dpb11. These interactions are essential for the CDK-dependent activation of DNA replication in budding yeast. Studies have also indicated that chromosomal replication initiates by a fundamentally similar process in all eukaryotes, with Dpb11 being an evolutionary conserved protein. Though Dpb11 does not travel with the replication fork, it plays an important role in the initiation of replication. We purified Dpb11 and other components of the replication initiation apparatus and studied their interactions *in vitro*. Here we show that Dpb11 binds to the thymine rich single-stranded region of two yeast origins, *ARS1* and *ARS305*. Replacing the thymines in the binding sequence of *ARS305* with adenines completely disrupts Dpb11 binding, suggesting that Dpb11 binds to the thymines of origin ssDNA. We also show a tight, direct interaction between Dpb11 and Mcm2-7. Furthermore, Dpb11 binding to Mcm2-7 is mutually competitive with GINS binding to Mcm2-7. Dpb11 interaction with Mcm2-7 is disrupted in the presence of single stranded origin DNA which binds specifically to Dpb11. We propose that Dpb11 acts as an inhibitor of replication fork helicase activation by blocking the interaction between GINS and Mcm2-7, and origin single-stranded DNA may alleviate this inhibition by removing Dpb11 from Mcm2-7.

COMPARATIVE ANALYSIS OF PRE-REPLICATION COMPLEX PROTEINS IN TRANSFORMED AND NORMAL CELLS

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We examined the abundance of the major protein constituents of the pre-replication complex (pre-RC), both genome-wide and in association with specific replication origins, namely the lamin B2, c-myc, 20mer1 and 20mer2 origins. Several pre-RC protein components, namely ORC1-6, Cdc6, Cdt1, MCM7, as well as additional replication proteins, such as Ku70/86, 14-3-3, Cdc45 and PCNA, were comparatively and quantitatively analyzed in both transformed and normal cells. The results show that these proteins are overexpressed and more abundantly bound to chromatin in the transformed compared to normal cells. Interestingly, the 20mer1, 20mer2, and c-myc origins exhibited a 2- to 3-fold greater origin activity and a 2- to 3-fold greater in vivo association of the pre-RC proteins with these origins in the transformed cells, whereas the origin associated with the housekeeping lamin B2 gene exhibited both similar levels of activity and in vivo association of these pre-RC proteins in both cell types. Overall, the results indicate that cellular transformation is associated with an overexpression and increased chromatin association of the pre-RC proteins examined. This study represents the most systematic comprehensive analysis done to date, using multiple replication proteins and different replication origins, comparing normal and transformed cell lines.

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ANALYSIS OF SUB-CELLULAR LOCALIZATION OF PRE-REPLICATION MACHINERY COMPONENTS DURING THE CELL CYCLE AND LIFE CYCLE OF *TRYPANOSOMA*.

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Tripanosomas are unicellular organisms that early diverged from eukaryote lineage. Data from these organisms might generate clues about unknown mechanisms of replication control. Genome database analysis indicates that the pre-replication machinery of tripanosomas is composed by Orc1/Cdc6 protein and the heterohexamer MCM complex. Data from our lab showed that Orc1/Cdc6 replaces yeast Cdc6 and it is in fact involved in DNA replication. Orc1/Cdc6 is localized at nuclear space during the entire cell cycle of tripanosomas. Moreover, Orc1/Cdc6 is found bound to DNA in all stages of the cell cycle of these organisms, suggesting that Orc1/Cdc6-DNA interaction is not involved with blockage of DNA replication in non-S stages. The life cycle of *Trypanosoma cruzi* involves replicative and non-replicative forms and the molecular bases that warrant the lack of DNA replication in non-replicative/infective forms are unkow. Here we show that Orc1/Cdc6 is localized at nuclear space during the life cycle of *T. cruzi*, even in the non-replicative stages. However, Orc1/Cdc6 does not interact with DNA in the non-replicative ones, which might be an additional way to block DNA replication in these stages. Concomitantly, we are investigating the sub-cellular localization of Mcm subunits in *Trypanosoma* in order to search a possible involvement of these subunits in DNA replication control.

IDENTIFICATION OF ORC1/CDC6-INTERACTING FACTORS IN *TRYPANOSOMA BRUCEI* REVEALS CRITICAL FEATURES OF ORIGIN RECOGNITION COMPLEX ARCHITECTURE.

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Replication of genomes initiates by the formation of a pre-replication complex on sequences termed origins. In eukaryotes, the pre-replication complex is composed of the Origin Recognition Complex (ORC), Cdc6 and the heterohexameric MCM replicative helicase in conjunction with Cdt1. Eukaryotic ORC is considered to be composed of six subunits, named Orc1-6. The monomeric Cdc6 is closely related in sequence to Orc1. However, ORC has been little explored in protists, which provide much of the diversity of the eukaryotic kingdom, and only a single ORC protein has been identified to date in *Trypanosoma brucei*, which is related to both Orc1 and Cdc6, perhaps suggesting a simplified ORC architecture related to that found in archaea. We have identified three highly diverged putative ORC components that act in *T. brucei* nuclear DNA replication. Two of these factors are so diverged that we cannot currently determine if they are orthologues of eukaryotic ORC subunits, or whether they are parasite-specific replication factors. The other we show to be a highly diverged Orc4 orthologue, and demonstrate that this is one of the most widely conserved ORC subunits in protists, revealing that it is a key element of eukaryotic ORC architecture. Additionally, we have examined interactions amongst the *T. brucei* MCM subunits and show that this has the conventional eukaryotic heterohexameric structure, suggesting that pre-RC divergence in *T. brucei* is limited to the earliest steps in origin licensing.

REPLICATION STRESS-INDUCED CHROMOSOME BREAKAGE IS CORRELATED WITH REPLICATION FORK PROGRESSION AND IS PRECEDED BY SINGLE-STRANDED DNA FORMATION

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Increasing evidence suggests that a major cause for chromosome fragility is replication stress-induced destabilization or "collapse" of replication forks. Yet, direct evidence demonstrating that replication fork collapse indeed gives rise to chromosome breakage is still lacking. We have shown previously that a *Saccharomyces cerevisiae* checkpoint-deficient mutant *mec1-1*, after transient exposure to a replication impeding drug hydroxyurea, fails to complete chromosomal replication, accumulates single-stranded DNA (ssDNA) at the replication forks and fragments its chromosomes. Here we test the hypothesis that replication fork collapse causes chromosome breakage in hydroxyurea-challenged *mec1* cells by simultaneous mapping, on a genome-wide scale, replication fork progression via ssDNA detection (Feng et al., 2006) and chromosome breakage sites by a novel double strand break detection method coupled with microarray analysis. We demonstrate that sites of chromosome breakage indeed correlate with replication fork locations. Moreover, ssDNA can be detected prior to chromosome breakage, suggesting that ssDNA accumulation is the common precursor to double strand breaks at collapsed replication forks.

Finally, we show that other forms of replication fork stress such as those by a temperature sensitive mutation *mec1-4* or by aphidicolin (a potent DNA polymerase alpha inhibitor) also give rise to ssDNA production. Thus, we propose that ssDNA formation is a general genomic response to replication stress. We are currently testing if and what nucleases are involved in ssDNA production under different stress conditions.

THE HUMAN DNA POLYMERASE THETA MAINTAINS GENOME INTEGRITY DURING UNPERTURBED S-PHASE

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We have recently uncovered in breast cancer unexpected alterations in the expression pattern of POL Q, the gene encoding the recently-discovered alternative DNA polymerase Theta (Pol θ), known thus far to function in vitro in translesion synthesis (TLS) of multiple endogenous DNA damage. Importantly, we have shown that POLQ up-regulation is a source for a replicative stress and genetic instability and we have discovered that POLQ up-regulation is a powerful predictor of the outcome of breast cancer, establishing for the first time a link between negative outcome in cancer and the expression of a DNA polymerase (Lemée et al., PNAS, 2010). The current study builds up on these clinical findings and aims at identifying the physiological function of Pol θ, which remains poorly understood. We hypothesized that, besides its well established in vitro TLS capability, Pol θ holds a more general and important role during the genomic duplication in otherwise unstressed cells. By using siRNA-mediated Pol θ depletion from human cells and MEF knock-out mutant cells, we have evidenced a requirement of Pol θ for cell proliferation and normal S-phase duration in unperturbed cells. We have also found that dynamics of replication factories are also modified in absence of Pol θ. Finally, we observed an earlier S-phase entry in Pol θ depleted cells and a functional link between Pol θ and Cyclin E, a factor believed to modulate origin firing. Collectively, these data suggest a novel function of Pol θ for origin regulation.

RE-REPLICATION DRIVES GENE AMPLIFICATION IN AN EXPERIMENTAL MODEL OF EVOLUTION.

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The duplication or amplification of genes is thought to facilitate evolution by enabling the emergence of novel functions from redundant genes, thereby expanding the phenotypic variation upon which natural selection may act. Phenotypic variation may also be generated more directly from the dosage effects of increased gene copy number. Despite the evolutionary importance of these duplications or amplifications, their source is poorly understood. Recently our lab has demonstrated that re-replication arising from loss of replication control is a potent inducer of gene amplification in *Saccharomyces cerevisiae*. We thus wondered whether rare spontaneous re-replication events arising despite intact replication controls might contribute to gene copy number changes during evolution.

We have developed an evolutionary model for adaptive amplification based on the temperature sensitivity of yeast cells deleted for the Securin gene, *PDS1*. A fraction of the rare survivors that emerge at restrictive temperatures have acquired an amplification of the Separase gene *ESP1*, which is known to suppress the *pds1Δ* temperature sensitive phenotype in high copy. The amplifications are segmental, are ~130-280 kilobases long, and have endpoints that coincide with Ty retrotransposable elements. These features are consistent with amplification structures we previously described for Re-Replication Induced Gene Amplification (RRIGA). We are currently investigating whether the adaptive amplifications completely match RRIGA structures by being oriented in direct repeat at the endogenous chromosomal position.

If spontaneous re-replication contributes significantly to the adaptive amplification of *ESP1*, genetic manipulations designed to alter the frequency of this re-replication should cause a corresponding change in the frequency of the amplifications. To quantify these amplification frequencies, we have developed a flow cytometry assay using fluorescent proteins to report on gene copy number. Consistent with a role for re-replication in the adaptive amplification of *ESP1*, introduction of an additional regulatory block to re-replication results in a reduction in the frequency of gene amplification. Furthermore, preliminary data for strains with disruption of replication controls show significant increases in the frequency of gene amplification. These results suggest that re-replication drives gene amplification in this experimental model of evolution.

SSRP1 DICTATES THE NUMBER AND THE DISTRIBUTION OF REPLICATION ORIGINS

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Timely replication from multiple replication origins is required to maintain genome stability. Formation of DNA replication origins is ensured by stepwise assembly of the ORC complex followed by the binding of Cdc6 and the Mcm2-7 proteins to form the pre-Replication complex. In S. cerevisiae, the ORC complex binds to a specific DNA sequence however in metazoan organisms such as Xenopus laevis and Drosophila, ORC complex assembly onto DNA does not require a defined nucleotide sequence but appears to be restricted to certain regions that have a different distribution between somatic and embryonic cells. In embryonic Xenopus laevis cells replication origins are spaced on average 10-15 Kb apart whereas they are present every 100-150 kb in somatic nuclei. Replication origin distribution is significantly plastic as the configuration can be converted from somatic to embryonic by incubating somatic nuclei in Xenopus mitotic cell free extracts. The mechanisms behind this transition are unknown and might be important to understand how replication origin assembly and distribution is achieved in higher eukaryotes.
We found that SSRP1, a subunit of the FACT chromatin-remodelling factor involved in DNA replication, repair and transcription regulates replication origin assembly and distribution in somatic and embryonic cells.
We showed that depletion of SSRP1 from Xenopus egg extract prevents efficient replication origin assembly due to the impairment of ORC complex binding to chromatin. We also showed that SSRP1 depletion inhibits the remodelling of somatic nuclei incubated in mitotic egg extract. Strikingly, the addition of recombinant SSRP1 to non-mitotic extracts is sufficient to reset the distribution of replication origins of somatic nuclei to the embryonic state by promoting de novo assembly of replication origins. A direct interaction of SSRP1 with Orc1 and nucleosome components appears to be responsible for this process. These observations suggest that SSRP1 is necessary and sufficient to induce replication origin assembly and regulate their distribution on replicating chromatin.

EXPLORING THE ROLES OF THE CHECKPOINT KINASE CHK1 BEYOND THE DNA-DAMAGE RESPONSE

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The metazoan cellular response to DNA damage involves signal transduction cascades leading to activation of the checkpoint kinases Chk1 and Chk2. One of the major roles of these proteins is to delay cell-cycle progression by controlling cyclin-dependent kinase activity to allow time for the cell to repair the damage. Although Chk1 and Chk2 show some functional redundancy, there is a fundamental difference between them: while Chk2-deficient mice are viable, mouse embryos devoid of Chk1 show pre-implantation lethality due to a severe proliferation defect linked to DNA damage in replicating cells. What the essential functions of Chk1 are in the cell remains unclear, mainly because few Chk1 substrates have been identified.

To further investigate Chk1 functions, we used two approaches. The first was based on making educated guesses about which proteins could be involved in the generation of DNA damage that follows Chk1 inactivation. We found that generation of DNA double-strand breaks at replication forks when Chk1 activity is compromised relies on the DNA endonuclease complex Mus81/Eme1. Importantly, we have established that it is Mus81/Eme1-dependent DNA damage – rather than a global increase in replication-fork stalling – that causes incomplete replication in Chk1-deficient cells. Consequently, we found that Mus81/Eme1 depletion alleviates the S-phase progression defects associated with Chk1 deficiency, thereby increasing cell survival. Chk1-mediated protection of replication forks from Mus81/Eme1 even under otherwise unchallenged conditions is therefore vital to prevent uncontrolled fork collapse and ensure proper S-phase progression in human cells.

The second approach involved combining chemical genetics with high-resolution mass spectrometry to identify novel Chk1 substrates and their phosphorylation sites. The list of targets produced reveals the potential impact of Chk1 function not only on processes where Chk1 was already known to be involved, but also on other key cellular events such as transcription, RNA splicing and cell fate determination. We have also refined the consensus sequence for Chk1, which should facilitate the identification of new Chk1 phosphorylation sites. In addition, our identification of KAP1-Ser473 as a new in vivo Chk1 substrate and as a robust readout for Chk1 inhibition could be used to further explore the in vivo effects of Chk1 inhibitors that are being tested on clinical trials.

SELECTIVE BYPASS OF A LAGGING STRAND ROADBLOCK BY THE EUKARYOTIC REPLICATIVE DNA HELICASE

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The eukaryotic replicative DNA helicase, which contains Cdc45, the MCM2-7 ATPase, and GINS (CMG), unwinds DNA via an unknown mechanism. In some models, CMG encircles and translocates along one strand of DNA while excluding the other strand, whereas in others, CMG encircles and translocates along duplex DNA. To elucidate how CMG engages with DNA in S phase, replisomes were confronted with strand-specific DNA roadblocks in *Xenopus* egg extracts. A ssDNA translocase should be arrested by an obstruction on the translocation strand but not the excluded strand, whereas a dsDNA translocase should stall at obstructions on either strand. We found that replisomes bypass large roadblocks on the lagging strand template much more readily than on the leading strand template. Together with other data, our results strongly suggest that CMG is a 3' to 5' ssDNA translocase, consistent with unwinding via “steric exclusion”. Given that MCM2-7 binds dsDNA in G1, the data imply that formation of CMG in S phase involves remodeling of MCM2-7 from a dsDNA to a ssDNA binding mode.

A NOVEL FUNCTION OF ATM DURING THE UNPERTURBED S PHASE: REGULATION OF CDT1 PROTEOLYSIS AND PREVENTION OF REREPLICATION

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Ataxia-telangiectasia mutated (ATM) is a well-known member of the phosphoinositide 3-kinase-related protein kinase family and plays a crucial role in DNA damage responses, especially for DNA double-strand breaks (DSBs). It has been, however, suggested that ATM can be activated not only by DSBs but also by some changes in chromatin architecture, suggesting potential ATM function in unperturbed cell cycle control. Here, we have carefully examined the kinetics of various cell cycle regulators in ATM-silenced and ATM-deficient cells and found that ATM is required for timely degradation of Cdt1, a critical replication licensing factor, during the unperturbed S phase. At least in certain cell types, degradation of $p27^{Kip1}$ was also impaired by ATM inhibition. Intracellular reactive oxygen species were not associated with the observed Cdt1 and $p27^{Kip1}$ deregulation by ATM inhibition. The novel ATM function was dependent on its kinase activity and NBS1. Indeed, we found that ATM is moderately phosphorylated at Ser1981 during the unperturbed S phase. ATM silencing induced partial reduction in levels of Skp2, a component of SCF^{Skp2} ubiquitin ligase that controls Cdt1 degradation. Furthermore, silencing of Skp2 resulted in Cdt1 stabilization like ATM inhibition. In addition, as reported previously, ATM silencing prevented Akt phosphorylation at Ser 473, an indicative of its activation, and Akt silencing led to decrease in Skp2 levels and subsequent Cdt1 stabilization. Therefore, the ATM-Akt-SCF^{Skp2} pathway may partly contribute to the novel ATM function. Finally, ATM inhibition rendered cells hypersensitive to rereplication induction, indicating the importance of the novel ATM function in maintenance of genome stability.

DDK PHOSPHORYLATES CHECKPOINT CLAMP COMPONENT RAD9 AND PROMOTES ITS RELEASE FROM DAMAGED CHROMATIN

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When inappropriate DNA structures arise, they are sensed by DNA structure-dependent checkpoint pathways and subsequently repaired. Recruitment of checkpoint proteins to such structures precedes recruitment of proteins involved in DNA metabolism. Thus, checkpoints can regulate DNA metabolism. We show that fission yeast Rad9, a 9-1-1 heterotrimeric checkpoint-clamp component, is phosphorylated by Hsk1(Cdc7), the Schizosaccharomyces pombe Dbf4-dependent kinase (DDK) homolog, in response to replication-induced DNA damage. Phosphorylation of Rad9 disrupts its interaction with replication protein A (RPA) and is dependent on 9-1-1 chromatin loading, the Rad9-associated protein Rad4/Cut5(TopBP1), and prior phosphorylation by Rad3(ATR). rad9 mutants defective in DDK phosphorylation show wild-type checkpoint responses but abnormal DNA repair protein foci and decreased viability after replication stress. We propose that Rad9 phosphorylation by DDK releases Rad9 from DNA damage sites to facilitate DNA repair.

SWI1 IS INVOLVED IN MAINTENANCE OF TELOMERE LENGTH AND SUB-TELOMERIC HETEROGENEITY

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Although telomere length is associated with cellular lifespan, telomere and sub-telomere structures are highly heterogeneous. Such heterogeneity occurs within a single cell as well as at the population level and has been proposed to have adaptive value for cells to sustain viable and flexible populations. However, the mechanisms involved and the physiological importance of this phenomenon are unclear. We conducted telomere restriction fragment analyses of S. pombe mutants to better understand how DNA replication and repair programs regulate telomere length and heterogeneity. Strikingly, inactivation of Swi1, a component of the replication fork protection complex, leads to accumulation of homogeneous sub-telomeres with minimum length. Swi1 is known to be involved in activation of the replication checkpoint kinase Cds1. However, checkpoint mutants including *cds1*Δ and *chk1*Δ had heterogeneous sub-telomeres similar to wild-type, suggesting that telomere heterogeneity maintenance by Swi1 is independent of checkpoint activation. Since telomere heterogeneity is a feature of cells with active ALT pathways for telomere extension, we have explored the involvement of homologous recombination in the regulation of sub-telomeres. Our investigation shows that *swi1*Δ *rad22*Δ, *swi1*Δ *rhp51*Δ and *swi1*Δ *rhp54*Δ restore sub-telomere heterogeneity, indicating that homologous recombination proteins play a role in the regulation of sub-telomeric length by Swi1. We also investigated the role of Swi1 in telomere repeat length maintenance, and found that *swi1* deletion causes telomere shortening in a manner independent of telomerase. Since Swi1 is known to be involved in replication fork stabilization, we are currently investigating how Swi1 regulates replication fork progression through telomeric repeats. Taken together, our results suggest that Swi1 is involved in multiple regulatory pathways required for maintenance of telomeric and sub-telomeric structures.

SLD-2 IS AN ESSENTIAL CDK SUBSTRATE IN *C.ELEGANS*

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Cyclin-dependent kinase (CDK) activity is essential for replication initiation in eukaryotes. In budding yeast it has been shown that the minimal function of CDK in replication initiation is the phosphorylation of two essential factors, Sld2 and Sld3. This phosphorylation allows their binding to another initiation factor, Dpb11 [1, 2]. Recent work has shown that the orthologue of Sld3 (Treslin/Ticrr) is an essential CDK target required for replication in Xenopus egg extracts and human cells[3], but the importance of CDK phosphorylation of the metazoan orthologue of Sld2 (RecQ4/RecQL4) remains poorly understood.

Unlike yeast Sld2, the vertebrate and insect orthologues of Sld2 (RecQ4/RecQL4) have an additional domain - a RecQ family helicase domain. To understand the evolutionary relationship between Sld2 and RecQ4 proteins we performed a bioinformatic analysis of the Sld2 homology region across eukaryotes. Identification of orthologues from several phyla shows that the Sld2-RecQ helicase fusion gene is ancestral, but there have been multiple loss events of the helicase from the Sld2-like domain throughout evolution, for example in the fungal lineage. Importantly in nematodes, as in yeast, the Sld2 orthologue is a small protein lacking a helicase domain. As a result, the nematode *Caenorhabditis elegans* provides a useful model to specifically analyse Sld2 function. Here we show that *C. elegans* Sld-2 is essential, interacts with Dpb11/Mus-101 and is phosphorylated by CDK both *in vitro* and *in vivo*. Finally we show for the first time outside of fungi that CDK phosphorylation of Sld-2 is essential for its role in replication. This work demonstrates further similarities between yeast and metazoan replication initiation and may establish *C.elegans* as a new system to study the CDK-dependent step of replication initiation in metazoa.

1. Tanaka, S., et al., Nature, 2007. 445(7125): p. 328-32.
2. Zegerman, P. and J.F. Diffley. Nature, 2007. 445(7125): p. 281-5.
3. Kumagai, A., A. Shevchenko, and W.G. Dunphy. J Cell Biol, 2011. 193(6): p. 995-1007.

ANALYSIS OF A LOW COMPLEXITY DNA SEQUENCE THAT REGULATES ORIGIN SELECTION

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In budding yeast, the first step in the process of DNA replication is binding of ORC complex at the ARS consensus sequence (ACS) found within each replication origin. Based on the ACS there are thousands of potential ORC binding sites throughout the yeast genome, however, ORC binds and directs helicase loading at only few hundred of these sites. Previously, we reported that the ACSs that are bound by ORC include additional determinants: these ACSs are asymmetrically positioned within a nucleosome free region (NFR) flanked by an A-rich island towards the downstream end of the NFR and, they are surrounded by positioned nucleosomes.

These findings raise the interesting question of how the non-conserved, low complexity A-rich DNA sequence regulates origin selection and function. I am currently testing two possible, but not necessarily mutually exclusive, functions: (i) the A-rich sequence interacts with ORC facilitating its function at origins; or (ii) the A-rich region regulates nucleosome positioning providing sufficient space for ORC/helicase loading. To address these questions I have created hybrid origins where the A-rich sequence from an ORC-binding ACS and the equivalent region from a non-ORC binding ACS have been swapped. I am analyzing these origins using multiple approaches such as: 1) the *in vitro* ORC binding and ORC-directed helicase loading on the hybrid origins with and without nucleosomes; 2) the *in vitro* nucleosome assembly in the presence and absence of ORC, and 3) the substitution of various endogenous origins with the hybrid origins, which would allow to study the *in vivo* ORC binding and helicase loading, as well as their replication timing and nucleosome positioning profiles. The results of this ongoing characterization will be reported.

These studies will provide significant insights into the role of nucleosomes at *S. cerevisiae* origins and also offer insights into ORC binding at metazoan origins, which exhibit an NFR but lack a defined binding motif.

THE PROTEOME OF REPLICATING CHROMATIN

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Despite years of intense study the organization of the eukaryotic replication machinery is only partially understood and we are far from being able to create a model of the eukaryotic replisome. To address this problem, we used an unbiased approach to identify novel protein complexes assembled on replicating chromatin in *Xenopus laevis* egg extract.

Proteins present on replicating chromatin were released into solution by complete digestion of the DNA in conditions that preserved protein complexes. These complexes were then separated based on their molecular size and shape using gel filtration and glycerol gradient techniques and the proteins present in all fractions were identified by quantitative mass spectrometry. These data were combined to create fractionation profiles for each of the proteins identified, which were compared to identify co-fractionating proteins that may be part of the same complex. The use of two different fractionation techniques allowed independent confirmation of the putative complexes and the estimation of potential complex size.

Proteins forming common chromatin complexes are likely to bind chromatin at a similar time. We therefore combined our data with previously-described timecourse analysis of chromatin bound proteins during DNA replication (Khoudoli *et al.* 2008). Clustering analysis of these data grouped together proteins which are likely to form common complexes.

Using this method we were able to determine previously described replication relevant complexes such as Mcm2-7, RFC, ORC, FACT, cohesins and condensins, verifying our approach. Importantly, we have also identified novel complexes present on chromatin during DNA replication such as that formed by members of the chromosomal passenger complex.

This ongoing project will shed further light on the organization of the replication machinery, allowing a greater understanding of eukaryotic DNA replication.

*(authors contributed equally)

ATM PHYSICALLY AND FUNCTIONALLY INTERACTS WITH PCNA TO REGULATE DNA SYNTHESIS

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Ataxia telangiectasia (A-T) is a pleiotropic disease, with a characteristic hypersensitivity to ionizing radiation (IR) that is caused by biallelic mutations in ATM, a gene encoding a protein kinase critical for the induction of cellular responses to DNA damage, particularly to DNA double strand breaks. A long known characteristic of AT cells is their ability to synthesize DNA even in the presence of IR-induced DNA damage, a phenomenon termed RDS (radio-resistant DNA synthesis). We previously reported that ATM kinase inhibition, but not ATM protein disruption, blocks sister chromatid exchange following DNA damage. We now show that ATM kinase inhibition, but not ATM knockdown, also inhibits DNA synthesis. Investigating a potential physical interaction of ATM with the DNA replication machinery, we found that ATM co-precipitates with PCNA from cellular extracts. Using bacterially purified ATM truncation mutants and in vitro translated PCNA we showed that the interaction is direct and mediated by the carboxyl-terminus of ATM. Indeed, a 20 amino acid region close to the kinase domain is sufficient for strong binding to PCNA. This binding is specific to ATM, as the homologous regions of other PIKK members, including the closely related kinase ATR, did not bind PCNA. ATM was found to bind two regions in PCNA. In order to examine the functional significance of the interaction between ATM and PCNA we tested the ability of ATM to stimulate DNA synthesis by DNA polymerase δ that is implicated in both DNA replication and several DNA repair processes. ATM was observed to stimulate DNA polymerase activity in a PCNA-dependent manner. We are currently studying the physiological role of the observed PCNA-ATM interaction using cell permeable ATM derived peptides, ATM mutants and assays to determine how PCNA modifications affect ATM binding. We will present mechanistic insights into this novel role of ATM in DNA synthesis.

IDENTIFICATION OF THE CHECKPOINT PATHWAYS TRIGGERED BY EXPANDED CAG/CTG REPEATS IN S. CEREVISIAE

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Expansion of CAG/CTG repeats is the cause of a number of neurodegenerative diseases. In addition, expanded CAG/CTG repeats have been shown to form hairpin DNA structures and to be a fragile site on a yeast chromosome. Previously, we demonstrated that an expanded CAG/CTG repeats is sensed by the MRX complex to elicit a cell cycle checkpoint response in budding yeast (Sundararajan and Freudenreich, 2011). In a double-strand-break repair deficient strain, the ultimate Rad53 phosphorylation event in the DNA damage checkpoint cascade could be captured. These results indicated that damage at the expanded repeat happens at a sufficient level to induce a measurable DNA damage checkpoint response. It is not known which type of DNA damage that can occur at a CAG repeat (SSB, DSB, stalled or reversed fork) is primarily responsible for the checkpoint signal, or which subpathway is activated. However, activation of the checkpoint pathway led to cell arrests, increased repeat expansions and cell death. Thus, characterization of the pathway is potentially important for understanding the further repeat expansions and cell death that occurs in patients.

In this study, we investigated which checkpoint pathway is triggered by the expanded repeats. Using a microcolony growth based assay, we compared the sensor, mediator and effector kinase mutants to the wild-type strain strain. The results showed an involvement of all the checkpoint proteins tested so far (mec1Δ rad9Δ, tof1Δ, mrc1Δ, mrc1AQ, rad53Δ). However, some specificity could be observed depending on the size of the expansion (85 or 155 repeats). Rad53 phosphorylation status was also tested in these mutants to validate the checkpoint route. Finally, we analyzed the correlation between the microcolony growth defect and the fragility and instability phenotypes observed for those checkpoint mutants.

Overall, these data clarify the types of checkpoint signals elicited by expanded CAG/CTG repeats, and show that the specificity of the pathway depends on the tract length.

CHARACTERIZATION OF CELL CYCLE DELAYS RESULTING FROM LIMITED HSK1 ACTIVITY

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Schizosaccharomyces pombe Hsk1 is a serine-threonine kinase whose requirement for the G1-S phase transition is conserved from yeast to humans, where it is known more commonly as Cdc7. Hsk1/Cdc7 catalytic activity is regulated during the cell cycle through expression of its regulatory subunit Dfp1/Dbf4, which peaks at the G1/S boundary and remains present until late G2 phase. Results from various organisms have demonstrated that Hsk1/Cdc7 has additional roles outside of S phase initiation. The uses of temperature sensitive (ts) alleles for both hsk1 and cdc7 have demonstrated that decreased kinase activity during an unperturbed S phase leads to prolonged S phase progression and may have a negative impact on genomic stability. Long-term inhibition of Hsk1 activity leads to a cell cycle arrest with an approximate 2C content. This delay is in opposition to the premature entry into mitosis observed with many other mutants of S phase initiation proteins. The nature of the cell cycle delays observed under conditions of limiting Hsk1 activity is unclear because of the synthetic lethality observed when combining hsk1-ts alleles with mutations in known checkpoint proteins.

We have established a chemical genetics system that allows us to control the cellular activity of ATP analogue-sensitive Hsk1 (hsk1-as). Our hsk1-as allele rescues a null and this strain grows with near wild type kinetics in the absence of analogue. Using both flow cytometric analysis and Mcm2 phosphorylation as in vivo indicators of Hsk1 activity, we have shown that we can rapidly and specifically inactivate Hsk1 within *S. pombe*. We are currently using the hsk1-as strain to investigate the requirement for Hsk1 during recovery from hydroxyurea block and to investigate the mechanisms of cell cycle delays resulting from reduced Hsk1 activity.

A PARADIGM SHIFT: SINGLE MOLECULE STUDIES REVEAL FUNCTION OF A THIRD POLYMERASE IN THE REPLISOME

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Recent studies indicate that the *E. coli* replisome contains three polymerases, one more than the number necessary to duplicate the two parental strands. Utilizing single-molecule microscopy, we show that a replisome with a third polymerase has two clearly advantageous features. First, a replisome with only two polymerases performs incomplete lagging strand synthesis, leaving behind single-stranded gaps, while the three polymerase complex fills them near completion. Furthermore, a third polymerase at the replication fork provides an additional contact to the lagging strand, substantially increasing the processivity of the replisome. Together, these beneficial features of a tri-polymerase replisome constitute a molecular basis for the efficiency of genome replication by the bacterial chromosomal replicase.

THE USE OF ZEBRAFISH (*DANIO RERIO*) AS A MODEL SYSTEM FOR THE STUDY OF SPECIALIZED POLYMERASES

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Zebrafish (*Danio rerio*) is an excellent model for the study of basic biological processes such as DNA replication. This vertebrate animal in fact possesses counterparts to all fifteen human polymerases, which allows for the development of a complete model of polymerase functions within human cells. The expression of several of the polymerases (delta, beta, eta, and iota) have been previously been established at stages of development ranging from 2 hours old to 3 years old. The effect of the alkylating agent, methylnitrosourea, on polymerase expression in 1 day old embryos was examined. The study of specialized polymerases is being initiated to determine the biological role of this group of polymerases. As a proof of principle, the decrease of polymerase eta expression is being tested utilizing microinjection of microRNA and morpholinos into fertilized eggs. The embryos develop *ex vivo* and are completely transparent, which allows for the observation of internal structures and the effects of decreased polymerase expression during development can be visualized. Microinjection was followed through fluorescent molecules also allowing the success of the procedure to be determined through the use of a plate reader. The level of polymerase eta expression following treatment of zebrafish embryos with ultraviolet light was completed to determine if polymerase eta is functioning similarly to its human counterpart. Polymerase eta knockdown studies followed by treatment with ultraviolet light were completed to determine its affect of zebrafish survivability. Using zebrafish as a model system for the study of polymerase function is a previously unexplored field. This research lays the foundation to study the roles of polymerases in DNA replication and repair, as well as human diseases such as cancer.

AN *IN SILICO* MATHEMATICAL MODEL OF THE INITIATION OF DNA REPLICATION

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DNA replication initiation is a molecular network guided by the origin recognition complex (ORC) and the activity of a host of associated protein factors. Proper cell proliferation depends on the regulation of and kinetic interactions between the complexes formed by ORC and proteins such as Cdt1, Cdc6, the Mcm2-7 complex, Cdc45 and the Dbf4-Cdc7 kinase complex. To explore the system's behaviour, the approach of mathematical modelling can serve as a valuable analytical tool. Using budding yeast as a model organism, we have developed an ordinary differential equation-based model of the protein-protein interaction network describing replication initiation. Precise quantification of the temporal profile of protein factors is critical and, to this end, we have employed quantitative protein expression analysis methods to determine precise levels of replication factors over the cell cycle. Through subcellular fractionation of samples from synchronized cell cultures, we were able to monitor the fluctuation in chromatin association of a number of the aforementioned proteins. This information was used to infer qualities of the protein network and to build a predictive mathematical model of the process of DNA replication initiation, which can be integrated into existing models of the entire budding yeast cell cycle. Importantly, model robustness was evaluated positively and predictions were concurrent with *in vivo* experimental results as well as outcomes expected from the literature.

WIDESPREAD OCCURRENCE OF SHORT ABORTIVE REPLICATION INTERMEDIATES IN MAMMALIAN CELLS

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We have previously shown that the activation of licensed DNA replication origins (ORIs) in human cells is accompanied by the generation of short DNA fragments that have undergone several rounds of synthesis in a single S-phase (Gómez and Antequera, 2008. GenesDev, 22: 375). We now examined the genomic localization of these re-replicated DNA fragments genome-wide by surveying 0.4% of the mouse genome on the same microarray platform and conditions that we used to identify the most efficient ORIs (Sequeira-Mendes et al., 2009. PLoS Genet. 5: e1000446) and by high-throughput sequencing. Unexpectedly, we found that these short replication intermediates do not derive preferentially from the highly active CpG island-associated ORIs. Instead, they show a widespread occurrence in the mouse genome mainly associated with transcription units and likely identify a subset of differently regulated ORIs. The mapping data and possible functionality of these short abortive replication intermediates will be discussed.

NOVEL APPROACHES TO INVESTIGATE THE *SULFOLBUS ACIDOCALDARIUS* REPLICATION DYNAMICS *IN VIVO*

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As in other archaea, the DNA replication machinery of the hyperthermophilic archaeon *Sulfolobus acidocaldarius* is a simplified, and presumably ancestral, form of the eukaryotic system. DNA replication in this organism is initiated at three distinct origins of replication within a 2.2 Mb circular chromosome. Thus, *Sulfolobus* species represent useful models in which to study the fundamentally conserved aspects of DNA replication.

We have developed novel methods to allow the *in vivo* labeling and detection of newly synthesized DNA in synchronized populations of *S. acidocaldarius*. We have also generated multiple strains that express epitope-tagged replication proteins from their endogenous chromosomal location. These novel tools have been exploited to address the nature and dynamics of the archaeal replisome *in vivo*. More specifically, we have investigated the spatial and temporal constraints of origin firing in individual cells. Additionally, we have monitored the progression of components of the replication machinery along the chromosome during S-phase. Finally, we have employed affinity chromatography approaches to identify novel, but conserved, components of the archaeal replisome.

PREFERENTIAL UNWINDING OF RNA-CONTAINING DISPLACEMENT LOOPS (R-LOOPS) AND G-QUADRUPLEXES BY THE HUMAN DHX9 HELICASE

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Human DHX9 helicase, also known as nuclear DNA helicase II (NDH II) and RNA helicase A (RHA), belongs to the SF2 superfamily of nucleic acid unwinding enzymes. DHX9 melts simple DNA-DNA, RNA-RNA, and DNA-RNA strands with a 3'-5' polarity; despite this little is known about its substrate specificity. Here, we used partial duplex DNA consisting of M13mp18 DNA and oligonucleotide-based replication and recombination intermediates. We show that DHX9 unwinds DNA- and RNA-containing forks, DNA- and RNA-containing displacement loops (D- and R-loops), and also G-quadruplexes. With these substrates, DHX9 behaved similarly as the RecQ helicase WRN. In contrast to WRN, DHX9 melted RNA-hybrids considerably faster than the corresponding DNA-DNA strands. Interestingly, DHX9 stimulated WRN on duplexes that started on one strand with a piece of RNA strongly resembling Okazaki fragments. Moreover, DHX9 preferably unwound R-loops and DNA-based G-quadruplexes indicating that these structures may be naturally occurring. DHX9 also unwound RNA-based G-quadruplexes that are reported to be present in human transcripts. It is believed that an improper dissolution of co-transcriptionally formed D-loops, R-loops, and DNA- or RNA-based G-quadruplexes are potential roadblocks for transcription and thereby enhance transcription associated recombination events. By unwinding these structures, DHX9 may significantly contribute to transcriptional activation and possibly also to the maintenance of genomic stability.

DNA POLYMERASE E HAS DUAL ESSENTIAL ROLES IN ASSEMBLY AND PROGRESSION OF REPLISOME IN FISSION YEAST

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At replication forks, DNA polymerases synthesize the leading and lagging strands on the respective template strands that are unwound by DNA helicase. In eukaryote, the replicative helicase is composed of Cdc45, Mcm2-7 and GINS, forming the CMG complex, while Polε and Polδ are considered to synthesize the leading and lagging strands, respectively, after priming by Polα. All the components of the replisome, containing DNA helicase and DNA polymerases, are assembled at replication origins during the process of initiation. At the onset of S-phase, GINS and Cdc45 are recruited onto the pre-loaded Mcm2-7 to form the CMG complex by aid of initiation factors Sld3, Cut5 and Drc1 in yeasts. It is unclear how assembly of the CMG complex leads to formation of replisome containing DNA polymerases and how DNA polymerases control the movement of replisome.
It has been suggested that the essential role of Polε is the non-catalytic function rather than nucleotide-polymerization, because the C-terminal domain (CTD) but not the N-terminal polymerase domain of the catalytic subunit is essential for cell viability in yeasts. To elucidate the essential function of Polε in assembly and progression of replisome in fission yeast, we depleted Cdc20, the catalytic subunit of Polε, using a promoter shut-off-coupled auxin degron system (*off*-AID). Upon depletion of Cdc20, neither GINS, Cdc45, Cut5 nor Drc1 were recruited to the origin, whereas MCM and Sld3 were localized. These results indicate that Polε is required for assembly of the CMG complex. To elucidate the non-catalytic functions of Polε in progression of replisome, we isolated a temperature-sensitive mutant, carrying mutations in Cdc20 CTD. At the restrictive temperature, CMG components were loaded onto replication origin but they did not migrate from the origin. RPA, Polα and Polδ were localized at the origin, although Polε subunits did not associate with the origin. To distinguish possibilities that migration of the CMG helicase requires either DNA synthesis or Polε, we depleted Polα. Polα-depletion, that greatly decreased DNA synthesis and origin association of Polδ, allowed migration of CMG helicase together with Polε from the origin. These results suggest that the non-catalytic function of Polε is required for progression of CMG helicase, which is independent from DNA synthesis. These results show that Polε plays two essential roles in assembly of CMG complex and its pregression in replisome.

CENTROMERIC RE-REPLICATION IS A POTENT SOURCE OF ANEUPLOIDY

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Eukaryotic cells use multiple overlapping mechanisms to ensure that replication origins initiate DNA replication at most once per cell cycle. In budding yeast, disabling these mechanisms can lead to re-initiation and localized re-replication from select origins. We have previously demonstrated that re-replication on a yeast chromosomal arm strongly induces amplification of the re-replicated segment. Here we describe our investigation of how re-replication of a centromere compromises chromosomal inheritance.

To re-replicate a centromere, we took advantage of strains engineered to conditionally re-initiate replication predominantly from one origin in the genome, *ARS317*. In diploid versions of these strains, we integrated *ARS317* and a copy number reporter ade3-2p near the centromere (*CEN5*) of one homolog of Chromosome 5 (Chr5). After transiently inducing *CEN5* re-replication from *ARS317* in metaphase-arrested cells, we plated single cells for colony outgrowth. Within a colony, cells with no copies of ade3-2p are white, one copy pink, and two or more copies red. We could thus monitor aberrant distribution of the re-replicated Chr5 homolog during subsequent cell division by quantifying colonies with two distinctly colored sectors. *CEN5* re-replication induced a 10-fold stimulation of red/white colonies and a 30-fold stimulation of red/pink colonies, consistent with 2:0 and 2:1 distribution of the re-replicated Chr5 homolog, respectively. The total copy number of Chr5 expected in each sector from these distributions was confirmed by array comparative genomic hybridization (aCGH). Importantly, the absolute frequency of aberrant mitoses induced by *CEN5* re-replication was very high, on the order of 10^{-2} per cell division. In analogous experiments involving Chr16, re-replication of *CEN16* induced similarly high frequencies of aberrant mitoses that resulted in aneuploidy of Chr16. These results demonstrate that centromeric re-replication can be a potent source of chromosome aneuploidy.

We are currently investigating the mechanism of this re-replication induced aneuploidy. During mitosis, the presence of additional copies of centromeres in re-replication bubbles may disrupt the biorientation of sister chromatids and make re-replicated chromatids susceptible to breakage from opposing spindle forces. Thus, as a first step, we are exploring how much of the aneuploidy is due to simple missegregation and how much may be due to more complex events involving chromosome breakage and repair.

GENOME-WIDE ANALYSIS OF DNA REPLICATION DYNAMICS IN *SACCHAROMYCES CEREVISIAE*

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All chromosomes must be completely replicated prior to cell division, a requirement that demands the activation of a sufficient number of appropriately distributed DNA replication origins. Different subsets of *Saccharomyces cerevisiae* origins are active in individual molecules, indicating that their DNA replication has a stochastic component. Appropriate origin distribution and regulation is required because insufficient origin firing generates large replicons and may lead to incomplete replication. As replication forks travel further their chance of stalling is increased, therefore large replicons are prone to DNA damage.

Using deep sequencing we have measured the increase in DNA copy number genome-wide during a synchronous S phase. These data give us the precise dynamics of genome replication in *S. cerevisiae*. Combining this with our mathematical model for genome replication has enabled predictions of individual origin characteristics. Our predictions agree with independent experimental data, offering validation for our model. We have used this method to examine perturbed replication dynamics in a strain with three replication origins inactivated by mutation.

Although our data is collected from large populations of cells, we can use our mathematical model to predict the behaviour of individual molecules and the degree of cell-to-cell variation. For example, we are able to predict the distribution of distances between active origins and the number of replication forks. This work provides a starting point for investigating the mechanisms that cells employ to minimise large replicons.

DEEP SEQUENCING IDENTIFIES A HIERARCHY OF ACTIVE REPLICATION ORIGINS IN *HALOFERAX VOLCANII*

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Replication origins differ in structure, activity and number across the three domains of life. The Archaeal domain includes species that replicate using multiple origins and archaeal replication proteins are similar to the eukaryotic replication machinery. The eukaryotic-like nature of archaeal DNA replication makes it a useful model.

We used deep sequencing to measure genome-wide marker frequency and determine the replication dynamics of the archaeon *Haloferax volcanii*. *H. volcanii* has a high and variable GC-content, making genomic approaches technically challenging. Our successful application of this method illustrates that replication profiling by deep sequencing is a versatile technique that can be applied to a broad range of species.

The chromosomal activity of previously described origins was measured and a third origin was discovered on the main circular chromosome. A distinct hierarchy of chromosomal origin usage was revealed by our replication profiling and is supported by plasmid-based assays. Each characterised origin is adjacent to an orc gene and contains repeated sequence motifs surrounding an A/T-rich duplex unwinding element. Deleting single and multiple chromosomal replication origins demonstrated redundancy in origin function. The perturbed replication dynamics in origin deletion strains were examined using deep sequencing.

* These authors contributed equally to this work.

A NON-CATALYTIC FUNCTION FOR RAD53 SQ/TQ CLUSTER DOMAIN PHOSPHORYLATION DURING UNPERTURBED DNA REPLICATION

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The replication checkpoint is the main pathway involved in stabilizing stalled replication forks to prevent their collapse into recombination-prone structures. While checkpoint mechanisms are widely studied using genotoxic agents, little is known about checkpoint functions during physiological DNA replication.
Here we show that a cluster of budding yeast Mec1/Tel1 (ATR/ATM) phosphorylation sites at the Rad53 (Chk1/2) N-terminus, termed SQ/TQ cluster domain 1 (SCD1), is phosphorylated during normal S-phase. Absence of these sites in the *rad53-4AQ* mutant leads to prolonged replication fork arrest at endogenous stall-prone loci, increased spontaneous histone H2A-S129 phosphorylation, compensatory activation of the Rad9 checkpoint pathway and overall prolonged S-phase progression. Consistently, normal viability of the *rad53-4AQ* mutant relies on *RAD9*, H2A phosphorylation sites and *RAD52*-dependent fork restart mechanisms. These phenotypes differ from a *dun1Δ* deletion and are not caused by defective nucleotide regulation. Nonetheless, they are suppressed by *sml1Δ*, which reduces the incidence of spontaneous replication fork stalling by increasing nucleotide pools. Interestingly, even though SCD1 mutations modestly decrease Rad53 kinase activation after DNA damage, the *rad53-4AQ* phenotype differs from the *rad53-K227A* kinase-deficient mutant, indicating that SCD1 phosphorylation exerts a non-catalytic function in the absence of exogenous DNA damage. Restoring threonine 8, but not any of the other three threonines, as the sole phosphorylation site in this region is sufficient to restore normal DNA replication.
Our data suggest that Rad53-SCD1 phosphorylation is required for a kinase-independent function that assists in the resolution of physiologically stalled replication forks. This implies that limited Mec1 activation occurs during normal S-phases to resolve spontaneous fork stalling without eliciting a full Rad53 kinase-dependent checkpoint.

HUMAN DNA POLYMERASE KAPPA IS REQUIRED FOR THE SIGNALLING AND RECOVERY OF THE ATR-MEDIATED REPLICATION CHECKPOINT

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In response to endogenous and exogenous DNA damage or other forms of replication stress, cells trigger the activation of the intra-S checkpoint signalling cascade resulting in the ATR-mediated phosphorylation of Chk1 protein kinase, thus preventing genomic instability. Many of these types of stress cause functional uncoupling of the MCM helicase and replicative DNA polymerases activities at replication forks resulting in production of long stretches of RPA-bound single-stranded DNA (ssDNA). Long stretches of ssDNA, in turn, are thought to trigger primary checkpoint signalling. By using siRNA-mediated depletion strategies, we demonstrate here the requirement of the human Translesion synthesis (TLS) Y-family DNA polymerase kappa (Pol kappa) for checkpoint signalling after endogenous or exogenous replication stresses as well as checkpoint recovery by suppressing the ssDNA stretches. Besides its established role in TLS, these findings identify an unexpected and novel function of Pol kappa in the replication stress response and indicate that a functional interplay between TLS and replication checkpoint signalling is important to maintain genomic stability.

COMBINING YEAST GENETICS AND GENOMICS TO REVEAL HOW DNA REPLICATION ORIGINS ARE CHOSEN WITHIN THE CONTEXT OF CHROMATIN.

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A key step in DNA replication in eukaryotic cells is recognition of specific chromosomal positions by the Origin Recognition Complex (ORC) as sites for pre-RC assembly during G1-phase and, ultimately, origin activation during S-phase. While metazoan ORC shows no DNA sequence-specificity and origins remain poorly defined, budding yeast ORC shows a preference for a bipartite DNA binding site consisting of conserved A- and B1-elements, and yeast origins are small modular elements that can confer autonomous replication to plasmids. However, while yeast ORC shows a preference for a specific DNA sequence, recent studies reveal that chromatin also plays a role in defining yeast origins, and that the selection step likely has a more significant impact on features of origin activation during S-phase than widely appreciated. In this study we examined the potential contribution of chromatin to origin selection in yeast by surveying the contribution that the established yeast ORC-DNA interface makes to ORC-origin interactions in vivo. Specifically, we compared ORC's affinity for confirmed origins in vivo (in the chromatin context, using ChIP-chip in orc2-1 mutants) and in vitro (using purified DNA and recombinant ORC) using genomic approaches. We found that while the ORC-DNA paradigm holds for many yeast origins, a substantial fraction (~40%) rely on features outside of the established ORC-DNA interface for stable ORC binding in vivo. A fraction of these "chromatin-dependent" origins rely on the Orc1-bromo adjacent homology domain (Orc1BAH), a nucleosome binding module, for ORC binding, yet this domain alone cannot fully explain ORC's affinity for all "chromatin-dependent" origins. Moreover, we observe that, on average, yeast origins that bind ORC efficiently in vivo via "chromatin-mediated" interactions activate earlier in S-phase than origins that bind ORC efficiently through the established ORC-DNA interface. We will discuss these and additional unpublished data aimed at both defining the ORC-chromatin interface in vivo and establishing a clearer understanding of the relationship between origin selection mechanisms in G1-phase and various features of origin firing (i.e. efficiency, timing) in S-phase.

CONTROL OF DNA REPLICATION AND CENTROSOME DUPLICATION BY ORC AND CYCLIN-DEPENDENT PROTEIN KINASES

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In metazoans, equal segregation of genetic material is mainly dependent on normal duplication of both DNA and centrosomes. Like DNA replication, centrosomes are also licensed once per cell cycle to ensure that each daughter cell acquires identical content of replicated DNA. Both DNA replication and centrosome duplication are committed as cells progress through G1 phase and into S phase, but little is known about the cross-talk between the two processes. In the past few years, there is evidence accumulating of the presence of replication proteins at centrosomes. We have previously demonstrated that the human Orc1 protein is present at centrosomes, while its depletion leads to centrosome re-duplication. Furthermore, expression of exogenous Orc1 blocks DNA damage induced centrosome re-duplication. We have found that human Orc1 contains a domain that inhibits the kinase activities of both Cyclin E-CDK2 and Cyclin A-CDK2. We show that Orc1 interacts directly with Cyclin A-CDK2 in a Cy-motif (RxL) dependent manner and this mutant does not block CDK kinase activity, but inhibition of Cyclin E-CDK2 is by a different mechanism. Consistent with this observation, Orc1 inhibits Cyclin A-CDK2, but not Cyclin E-CDK2, phosphorylation of Cdc6, an in vivo substrate of CDKs. We also present evidence that human Orc1 harbors a separate centrosome-targeting domain that shows similarity to the PACT domain present in other centrosome associated proteins. We have named the human Orc1 centrosome-targeting region the OPACT domain. The Cyclin/CDK2 kinase inhibitory domain of Orc1, when tethered to the OPACT domain, localizes to centrosomes and blocks Cyclin E-CDK2-dependent centrosome re-duplication that is induced upon hydoxyurea (HU) treatment of cells. We will discuss the interplay between Orc1 and CDKs in controlling both the initiation of DNA replication and centrosome copy number.

MEC1 DEPENDENT PHOSPHORYLATION OF SGS1 FACILITATES REPLICATION CHECKPOINT ACTIVATION BY RECRUITING RAD53 TO THE STALLED REPLICATION FORK.

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An evolutionarily conserved DNA damage checkpoint coordinates cell cycle progression and DNA repair in response to various types of damage. During S phase the genome is exceptionally sensitive to both exogenous and endogenous DNA damage which leads to replication fork stalling. The DNA replication checkpoint maintains the integrity of stalled replication forks, which is in turn essential for the successful resumption of replication after removal of the insult. In *S. cerevisiae*, robust activation of DNA replication checkpoint requires multiple factors, including Sgs1 (RecQ) and Mec1 (ATR kinase), along with pol epsilon, Mrc1, and the Mec1 activators 9-1-1 and Dpb11. We have examined how RecQ helicase Sgs1 can facilitate the activation of the checkpoint effector kinase Rad53 at stalled replication forks. We found that Sgs1 interacts with N-terminus of Rpa1 through its unstructured acidic-domain N-terminal of the helicase domain. Interestingly this domain contains four potential Mec1/Tel1 target sites. Indeed, we found that Mec1 can phosphorylate the acidic domain in vitro and that mutation of the S/TQ sites to AQ abolishes Mec1-dependent phosphorylation. Surprisingly, the same Sgs1-acidic domain, binds the Rad53 FHA1 domain in a phosphorylation-dependent manner. Similarly, *sgs1Δ* or AQ point mutation in the Sgs1 domain failed to activate Rad53 at stalled replication forks when combined with the *rad24* mutation (9-1-1 loader). Our study suggests that Sgs1 facilitates the activation of the replication checkpoint by attracting Rad53 to the site of a stalled replication fork.

THE INTERACTION BETWEEN RECQL4/MCM10/CTF4 AND CMG COMPLEX IS REGULATED BY CDK AND CDC7 KINASE IN HUMAN CELLS.

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The initiation of DNA replication requires the formation of pre-replicative complex to recruit other initiating factors on replication origins of eukaryotic DNA. The Cdc45-Mcm2-7-GINS complex(called as CMG) is assembled during initiation process and plays an essential role as a replicative DNA helicase. In yeast systems, additional factors such as Dpb11/Cut5, Sld2 and Sld3 appear to function for the assembly of CMG complex. The functions of these proteins are governed by the two major kinases in G1/S phase, CDK and DDK. However, the roles and the control mechanisms of such homolog proteins in mammal systems have remained unclear. In our previous study, we showed that the assembly of CMG complex depends on several factors such as RecQL4, Mcm10 and Ctf4 in human cells using bimolecular fluorescence complementation assay. Here, we studies the molecular interaction between initiating factors which are involved in the assembly of CMG complex in human cells. RecQL4, Mcm10 and Ctf4 directly bind to the components of CMG complex in G1 phase and these interactions are dependent upon both CDK and Cdc7 kinase. RecQL4, Mcm10 and Ctf4 also interact with each others prior to the assembly of CMG complex in human cells. In addition, RecQL4, Mcm10 and Ctf4 is loaded on chromatin, which is regulated by CDK and Cdc7 kinase. The control mechanisms affecting the molecular interaction of those human proteins will be discussed.

STRUCTURE AND DYNAMICS OF THE TERNARY COMPLEXES OF FEN1/PCNA/DNA AND FEN1/RAD9-RAD1-HUS1/DNA

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Processivity clamps such as proliferating cell nuclear antigen (PCNA) and the checkpoint sliding clamp Rad9/Rad1/Hus1(9-1-1) act as versatile scaffolds in the recruitment of proteins involved in replication, cell-cycle control and DNA repair (e.g. replicative and translesion polymerases, NEIL1, flap endonuclease 1 (FEN1) or DNA Ligase I). In this capacity, sliding clamps are at the very heart of many essential cellular activities. Structurally, both PCNA and 9-1-1 are composed of three subunits that come together to form closed ring-shaped structures around duplex DNA. While the PCNA ring is a homotrimer, the 9-1-1 complex is heterotimeric, reflecting the differential involvement of the two clamps with protein partners and their distinct roles in coordinating DNA processing. A trimeric ring can provide multiple binding sites for replication and repair factors. Furthermore, competition among simultaneously bound repair factors can lead to conformational switching and sequential exchange (handoff) of these proteins at the sliding clamp locus. These are key processes in PCNA biology, which are incompletely understood from a structural point of view. Herein, we have chosen an integrative computational and experimental approach to modeling the assemblies of FEN1 with its double-flap DNA substrate and each of the two clamps. Fully atomistic models of the ternary DNA/hFEN1/h9-1-1 and DNA/hFEN1/hPCNA complexes were developed based on the crystal structures of h9-1-1(3GGR), FEN1-DNA(3Q8L), and PCNA-FEN1(1UL1). The models were simulated with molecular dynamics (MD) in explicit solvent for 100ns to expose the conformational dynamics of the systems. Clustering analysis of the MD trajectories revealed the most dominant conformations accessible to the complexes. The cluster centroids were subsequently used in conjunction with single particle electron microscopy (EM) to generate an EM map of the h9-1-1/Fen1/DNA assembly to 18.5 Å resolution. Finally, the atomistic models were refined by flexible fitting into the EM density resulting in a 3D structure of the 9-1-1/Fen1/DNA assembly.

MCM-BP CONTRIBUTES TO NUCLEAR MORPHOLOGY AND CELL CYCLE PROGRESSION IN HUMAN CELLS.

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MCM-BP was discovered in human cells as a protein that is strongly associated with minichromosome maintenance (MCM) proteins. The MCM complex is known to be critical for DNA replication through its DNA helicase activity. However the importance of MCM-BP and its functional contribution to human cells has been unclear. Here we show that depletion of MCM-BP by sustained expression of hairpin RNA (shRNA) results in highly abnormal nuclear morphology and centrosome amplification. The abnormal nuclear morphology was not seen with depletion of other MCM proteins. MCM-BP depletion was also found to result in transient activation of G2 checkpoint signaling coupled with slowed progression through G2. In addition, stable knockdown of MCM-BP resulted in increased RPA foci which are indicative of replication stress. A recent study has suggested that MCM-BP functions as the helicase unloader toward the end of S-phase, such that MCM-BP depletion increases MCM levels on chromatin in late S (Genes Dev. 25, 165). In addition, we have observed that sustained MCM-BP depletion caused an increase in the total cellular levels of MCM proteins in S phase. The increase in MCM levels was observed in both the soluble and chromatin bound fraction and was not attributable to increased transcription. Taken together, the results suggest that MCM-BP makes important contributions to nuclear morphology, efficient cell cycle progression and maintenance of MCM levels during replication.

STRUCTURAL STUDIES OF THE S. CEREVISIAE ORIGIN RECOGNITION COMPLEX

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Replication of DNA initiates at one or more discrete sites called 'origins' via the concerted activities of a series of multi-protein complexes. These ensembles of proteins (also known as pre-replicative complexes) perform several important tasks (1-3), including 1) selecting and preparing origin DNA for initiation, 2) assembling the replicative helicase, which will travel ahead of the replication fork during replication, and 3) regulating the number of initiations. Activation of origins for replication culminates in assembly of the replisome (2).
In eukaryotes, two ensembles, the origin recognition complex (ORC, origin selection) and the mini-chromosome maintenance (MCM, helicase) assembly cooperate with other factors at ~100 bp origin DNA segments to initiate replication (2, 4). As an entry-point into structural analysis of the eukaryotic initiation machinery, we are investigating the origin recognition complex (ORC), a six-protein complex, which must assemble on all origins prior to initiation. Answers to such basic questions as overall architecture, interaction with substrates and roles of nucleotide will set the stage for addressing more intricate issues. Several issues are of special interest. How is origin DNA recognized and strands separated for replication? How is the MCM-helicase loaded onto DNA? And how are initiations regulated?
We report here a small angle X-ray scattering (SAXS) analysis of the isolated yeast ORC ensemble, as well as its complex with DNA derived from the Ars1 origin. The complete yeast particle was prepared from subunits expressed in E. coli. SAXS measurements were carried out on sample concentrations of 1, 2 and 4 mg/ml. Reference data were taken from cytochrome C. Analysis of the scattering curves measured from the ORC entities suggests that our samples are of good quality, free of aggregates and inter-particle interference (5).
SAXS scattering curves were processed using published software and methods to yield molecular mass estimates, radii of gyration (Rg) and Dmax values (longest chord) (6). The molecular mass estimates calculated from the SAXS data (ORC: 448 kD) suggest an ensemble that contains one copy of each ORC subunit. The pair-distribution curve P(r) calculated from the SAXS data reveals the ORC is an elongated complex; its longest chord is 195 Å. Molecular envelopes of the ORC and ORC-DNA complexes, constructed using SAXS data, will be presented.

1. Bell, S., Genes & Development 16, 659-672 (2002).
2. Bell, S.P. and A. Dutta, Annu Rev Biochem 71, 333-74 (2002).
3. Mendez, J. and B. Stillman, Bioessays 25, 1158-67 (2003).
4. Sclafani, R.A. and T.M. Holzen, Annu Rev Genet 41, 237-80 (2007).
5. Putnam, C.D., M. Hammel, G.L. Hura, and J.A. Tainer, Quart. Rev. Biophys. 40, 191-285 (2007).
6. Konarev, P., M. Petoukhov, V. Volkov, and D. Svergun, Journal of Applied Crystallography 39, 277-286 (2006).

CHARACTERIZATION OF A DNA POLYMERASE EPSILON COMPLEX LACKING THE ESSENTIAL SUBUNIT DPB2

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DNA polymerase epsilon is a DNA polymerase which has been proposed to participate in many processes including the synthesis of the leading strand in *Saccharomyces cerevisiae*. Pol epsilon is composed of four subunits and the catalytic subunit is called Pol2. *DPB3* and *DPB4* encode the two smallest subunits and are non-essential genes in yeast. *DPB2* is an essential gene, but the function of Dpb2 is unclear. A genetic screen was carried out to isolate lethal mutations in *DPB2*. One of the isolated alleles, *dpb2-201*, carried two mutations and was co-expressed with Pol2p, Dpb3p, and Dpb4p in *S. cerevisiae*. The over-expressed protein complex was purified by conventional chromatography. The inability of dpb2-201 to interact with other Pol epsilon subunits lead to the purification of a Pol2/Dpb3/Dpb4 complex, which possessed high specific activity using oligodT-polydA as substrate. Primer-extension assays demonstrated that the Pol2/Dpb3/Dpb4 complex have a processivity comparable to wild-type Pol ε. Finally, holoenzyme assays with PCNA, RFC and RPA on a single-primed circular template did not reveal any defect in replication efficiency. In conclusion, Dpb2 does not appear to influence the enzyme activity of Pol epsilon. Instead, we propose that the essential function of Dpb2 is to fulfill a structural role at the origins or the replication fork.

COUPLING MITOSIS TO DNA REPLICATION : THE EMERGING ROLE OF THE HISTONE H4-LYSINE 20 METHYLTRANSFERASE PR-SET7

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In metazoans, functional replication origins do not show defined DNA consensus sequences suggesting the involvement of chromatin determinants in the selection of these origins. We have performed ChIP-qPCR with specific histone modifications antibody at well-characterized replication origins in mammalian cells. Our results show an increase in histone H4 lysine 20 monomethylation (H4K20me1) at some origins during mitosis and early G1, which coincides with onset of the replication licensing. During S phase, H4K20me1 is absent, which coincides with pre-RC inhibition. PR-Set7/Set8/KMT5 is the lysine methyltransferase that catalyses H4K20me1 in metazoan. Previous studies have shown that PR-Set7 is cell-cycle regulated, with high levels during M and early G1 and very low in S phase. We have discovered that, at the onset of S phase, PR-Set7 undergoes an ubiquitin-mediated proteolysis, which depends on its interaction with the sliding-clamp protein PCNA and involves the ubiquitin E3 ligase CRL4-Cdt2. Strikingly, Mutation of the PCNA-interacting domain within PR-Set7 is sufficient to prevent its destruction during S phase, thereby causing the maintenance of H4-K20me1 signal at origins and re-replication of specific genomic loci. Consistent with a role in replication licensing at specific origins, inhibition of PR-Set7 enzymatic activity leads to alterations in pre-RC chromatin recruitment and a reduction in origin firing as measured by DNA molecular combing. Furthermore, tethering PR-Set7 methylase activity to an origin-free genomic locus is sufficient to promote the loading of pre-RC proteins at this locus. We propose a model whereby PR-Set7-mediated lysine methylation would constitute an epigenetic indexing platform that contributes to establish a specific replication program at each round of cell division. We will present our last data and discuss the mechanisms by which PR-Set7 regulates DNA replication and genome stability.

CHARACTERISTICS OF *IN VITRO* DNA REPLICATION SYSTEM USING S PHASE EXTRACT OF *S. CEREVISIAE*.

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Eukaryotic DNA replication is highly regulated to ensure that genomic duplication occurs once per cell cycle. This regulation is mainly achieved by separating the loading and the activation of replicative helicases (MCM complexes). In G1 phase, ORC and Cdc6 load inactive MCM complexes to the replication origin to form pre-Replicative Complexes (pre-RCs). Activities of two kinases, DDK and CDK, subsequently activate origin-bound MCM complexes to initiate DNA replication. High CDK activity inhibits loading of MCM helicases during S phase and prevents re-replication.
We have developed an *in vitro* assay that recapitulates the replication initiation process from a defined, *S. cerevisiae* origin of replication. Replication initiation is achieved by loading helicases onto origin-containing DNAs, treating the helicases with purified DDK and incubating the phosphorylated helicases in S phase extracts in which replication activator proteins (Sld2, Sld3, Cdc45 and Dpb11) were over-expressed. From this assay, we found that DDK- and CDK-dependent act sequentially during the recruitment of the Cdc45 and GINS helicase activating proteins and that the origin recruitment of leading and lagging DNA polymerase have distinct requirements for Mcm10 and origin DNA unwinding. We are currently investigating the effect of MCM mutations that are defective in DDK phosphorylation or ATP binding and hydrolysis on the events of replication initiation.

MCM10 IS REQUIRED FOR ORIGIN DNA UNWINDING BY CMG COMPLEX IN FISSION YEAST

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DNA helicase activity is essential for initiation of DNA replication and progression of replication forks. Minichromosome maintenance complex composed of Mcm2-7 is a key component of the helicase, although assembly of other factors is required for activation of the helicase. In G1 phase, Mcm2-7 complex is loaded onto replication origins to form pre-RC, which does not have a helicase activity. Upon activation of CDK and DDK at the onset of S phase, GINS and Cdc45 are loaded onto pre-RC to form the CMG complex, which acts as a helicase at replication forks. This process requires several other factors such as Sld3 and Cut5 that do not migrate with forks and are specifically required for the initiation process. It is still unknown how assembled replication factors are converted into replication forks containing the active helicase. Mcm10, a conserved replication factor, has strong genetic and physical interactions with Mcm2-7. These facts suggest that Mcm10 functions in close relation with Mcm2-7, although the actual role in the initiation process has not been well understood.

To investigate the function of Mcm10 in replication initiation, we depleted Mcm10 protein from fission yeast cells, using a promoter shut-off-coupled auxin-inducible degron system (*off-AID*), and analyzed origin localization of replication factors. Under Mcm10-depleted conditions, Mcm6, Psf2 (GINS) and Cdc45 were localized at replication origins, indicating that Mcm10 is not required for assembly of the CMG components. In contrast, localization of Rpa2, a subunit of ssDNA binding protein (RPA), and Pol1, the catalytic subunit of DNA polymerase α, at the origin greatly decreased, suggesting that replication origin was not unwound. Interestingly, initiation specific factors such as Sld3 accumulated, suggesting that protein assembly was not converted into those at replication forks. These results show that Mcm10 plays an essential role in origin DNA unwinding. We will discuss the function of Mcm10 that is required for the origin unwinding.

REGULATION OF THE ORIGIN FIRING PROGRAM BY RIF1 IN FISSION YEAST

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Complete and accurate DNA replication is crucial to the maintenance of genetic integrity of all organisms. In eukaryotic cells, this event is initiated at multiple chromosome loci called replication origins. DNA replication proceeds in two stages; the formation of pre-RC mediated mainly by ORC, Cdc6, Cdt1 and Mcm and subsequent firing of origins triggered by Cdc7-Dbf4 and CDK-mediated phosphorylation events. However, the mechanisms that determine where on the genome and when during S-phase are still elusive.
Recently, we identified Rif1 (Rap1 Interacting Factor 1) as a suppresser of lethality of hsk1 (Cdc7) deletion in fission yeast. Analysis of the replication initiation profile by BrIP-Chip assay indicates that some replication origins that are normally dormant in the presence of HU are fired in the *rif1Δ* mutant. At the same time, some active, early-firing origins are suppressed in the same mutant, suggesting that the origin firing pattern is dramatically altered in the absence of Rif1. ChIP-chip assays revealed that Rif1 binds not only at the telomeres but also at the arm segments. In contrast to binding to telomere, binding of Rif1 to the arm is independent of Taz1. We are now analyzing the functional domains of Rif1 protein as well as cell cycle regulation of its localization and modification. Based on the data presented, we will discuss how Rif1 may regulate the origin firing program of fission yeast.

UNCONVENTIONAL RESPONSES TO DNA DAMAGE AND REPLICATION STRESS IN TETRAHYMENA.

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Three processes are required to faithfully transmit intact chromosomes: DNA replication, DNA repair and chromosome segregation. While key regulatory proteins and enzymes in these pathways are conserved in eukaryotes, ciliated protozoa must maintain genic balance in a nucleus with chromosomes that lack centromeres (the macronucleus). We previously showed that DNA damage/replication stress inducing agents (methylmethanesulphonate (MMS) and hydroxyurea (HU)) trigger an intra-S phase checkpoint response in Tetrahymena that arrests DNA replication in the diploid (mitotic) micronucleus and polyploid (amitotic) macronucleus. This process requires ATR (ataxia-telangectasia-related) and a novel protein, Tif1p, to coordinate DNA replication, macronuclear division and cytokinesis. The temporal link between macronuclear and cell division, and amitosis itself, suggest that the Tetrahymena checkpoint responses differ from haploid/diploid mononuclear eukaryotes.

To better understand the S phase checkpoint response in Tetrahymena, we synchronized cells by centrifugal elutriation and compared their fate following exposure to HU or MMS during G1 or mid (macronuclear) S phase. Three major differences were uncovered that distinguish Tetrahymena from yeast/mammalian cells. First, instead of simply arresting or slowing down replication, the addition of HU or MMS to mid-S phase Tetrahymena led to a progressive reduction in DNA content, essentially attempting to re-establish a G1 DNA dosage. This occurred without (a) the completion of S phase, (b) macronuclear division, (c) cell division, or (d) elimination of DNA in the form of macronuclear extrusion bodies. Second, whereas activation of the yeast/mammalian intra-S phase checkpoint leads to the reversible phosphorylation of the replicative helicase, MCM2-7, Mcm6p was actively degraded in HU-treated Tetrahymena. Tetrahymena Orc1p was similarly degraded. MMS did not promote Mcm6p or Orc1p degradation, suggesting that these agents trigger different checkpoint cascades in Tetrahymena. Third, de novo synthesis/replenishment of Tetrahymena Orc1p or Mcm6p was not required for DNA replication during the initial S phase following HU removal. Recovering cells underwent apparently normal nuclear/cell division. In contrast, partial depletion of the ORC1 gene in the polyploid macronucleus (by gene replacement) failed to trigger a checkpoint response and resulted in high levels of aberrant macronuclear division. The collective data suggest that the recovery from HU-induced stress corresponds to a unique cell cycle in Tetrahymena, in which replication initiation and elongation is largely independent of ORC and MCM2-7.

RECONSTITUTION AND BIOCHEMICAL CHARACTERIZATION OF THE PRE-RC FORMATION IN *S. CEREVISIAE* USING PURIFIED, RECOMBINANT PROTEINS

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Chromosomal DNA replication occurs only once per cell cycle. In eukaryotic cells, the pre-replicative complex (pre-RC) assembles at both early and late origins in G1 phase and is essential for replication licensing prior to S phase. Pre-RC formation in *S. cerevisiae* requires ORC, Cdc6, and Cdt1 to recruit MCM hetero-hexamers onto replication origins called ARS's. Although purification of each component is of great importance to conduct quantitative biochemical analyses on pre-RC formation *in vitro*, recombinant MCM hexamer consisting of Mcm2/3/4/5/6/7 (Mcm2-7) was a challenge to purify as a form active in pre-RC formation. To date, Mcm2-7 complexes active in pre-RC assembly have only been purified from *S. cerevisiae* cells. In this study, we developed methods to purify from insect cells infected with recombinant baculoviruses the *S. cerevisiae* Mcm2-7 complexed with Cdt1 to near homogeneity. Mcm2-7 and Cdt1 co-migrated during every fractionation step and eluted at a molecular mass of ~700 kDa upon gel-filtration, consistent with the theoretical mass of Mcm2-7•Cdt1 heptamer. The purified Mcm2-7•Cdt1 complex retained pre-RC formation activity. Loaded Mcm2-7 formed a ~1300 kDa complex, corresponding to an $(Mcm2\text{-}7)_2$ double hexamer, and could slide along dsDNA. Mcm2-7 in the purified complex could be loaded onto representative early and late origins at an equal efficiency. Analyses using mutant ARS's identified sequences important for Mcm2-7 loading and showed that pre-RC assembly is dependent upon DNA sequences present in active origins of DNA replication.

IDENTIFICATION OF ATM/ATR PATHWAY INHIBITORS THAT SENSITIZE P53-DEFICIENT CELLS TO DNA-DAMAGING AGENTS YET DO NOT DIRECTLY SUPPRESS ATR KINASE CATALYTIC ACTIVITY *IN VITRO*

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Many human cancers have p53 functional deficiency and are resistant to DNA-damaging agents. There is thus considerable interest in cancer therapies that sensitize p53-deficient cells to DNA damage. Such sensitization can be achieved by inhibition of key DNA damage response proteins such as ATR or ATM. To better understand ATR activation mechanisms and discover biological probes for this pathway, we performed a phenotype-based chemical genetic screen for inhibitors of hydroxyurea-induced phosphorylation of Chk1, a key ATR substrate. After subsequent biological screens, we selected 4 compounds that inhibited ATR and ATM pathways; 3 were known bioactive agents and 1 was a diversity-oriented-synthetic product. These compounds sensitized p53-deficient cancer cells to diverse DNA-damaging agents. Xenograft experiments were performed on one compound which showed that it suppressed p53-deficient tumor growth synergistically with cisplatin. Importantly, unlike typical ATM/ATR inhibitors, these compounds did not suppress ATR kinase catalytic activity in *in vitro* assays. Thus, these compounds may act on previously uncharacterized component(s) of DNA damage response pathways. To identify molecular targets of one of these compounds, "MARPIN" (ATM and ATR pathway inhibitor), we defined the active site of this small molecule through structure-activity relationship analysis, resulting in synthesis of inactive derivatives of MARPIN. The identification of proteins that specifically bind MARPIN, but not its inactive derivatives, is underway. This phenotype-based chemical genetic screen identified novel ATM/ATR pathway inhibitors that are mechanistically distinct from kinase catalytic inhibitors, and these compounds could serve as mechanistic probes to identify novel druggable targets in DNA damage response pathways.

REGULATION OF CDK INHIBITOR XIC1 UBIQUITINATION AND PROTEOLYSIS BY PCNA-CRL4^{CDT2}, CDK, AND XDRP1.

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The activities of vertebrate cyclin-dependent kinase (CDK) inhibitors of the Cip/Kip-type are modulated by ubiquitination and protein turnover, but how the turnover of these proteins is coordinated during the molecular events of DNA replication initiation is not well-characterized. The Xenopus Cip/Kip-type CDK inhibitor, Xic1/Kix1, shares sequence homology and functional similarities to both mammalian p27 and p21. We study the regulation of Xic1 turnover using the Xenopus interphase egg extract as a model system and have found that Xic1 is recruited to sites of DNA replication initiation through its association with Proliferating Cell Nuclear Antigen (PCNA) and is ubiquitinated by the CRL4^{Cdt2} ubiquitin ligase at lysine residues proximal to the PIP box and within the Cdt2 binding domain.Our recent findings indicate that Xic1 is hyper-phosphorylated at 4-6 CDK sites and stabilized during mitosis and is dephosphorylated upon the exit from mitosis and the transition into interphase by a PP2A-like phosphatase. Specific phosphorylation of Xic1 at residue T172 negatively regulates its association with PCNA and a phosphomimetic T172E mutant of Xic1 is significantly stabilized in interphase. These studies suggest that CDK phosphorylation may be an important regulator of both Xic1 binding to PCNA and its turnover by CRL4^{Cdt2}.Following the ubiquitination of Xic1 by CRL4^{Cdt2}, Xic1 is targeted for degradation by the 26S proteasome, but the molecular steps from CDK inhibitor ubiquitination to degradation by the proteasome are unclear. Using the Xenopus extract, we have studied the degradation of ubiquitinated Xic1 and have found that Xic1 proteolysis appears to be dependent upon XDrp1, a Xenopus homolog of the Rad23-like adapter protein, Dsk2. Our studies also suggest the involvement of Xenopus Rpn10 and chromatin in the proteolysis of Xic1 during DNA replication.

IDENTIFICATION OF TWO UBIQUITIN LIGASES THAT CONTROL CDC6 DEGRADATION IN G1 AND AT THE G1/S TRANSITION

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DNA replication takes place once per cell cycle to maintain genomic integrity. Cdc6, a component of pre-replicative complex (pre-RC), is an unstable protein whose degradation is mediated by the SCF^{Cdc4} complex at the G1/S transition and during G2/M in budding yeast. Cdc6 degradation may be an important mechanism to prevent re-replication. However, stabilized Cdc6 alone is not sufficient to cause re-replication in budding yeast. By contrast, over-expression of Cdc18, the fission yeast homologue of Cdc6, does lead to re-replication. One possible explanation for this difference is that there are multiple regulatory mechanisms for Cdc6 proteolysis in budding yeast. There are at least three modes of Cdc6 degradation; the pathway that targets Cdc6 for degradation in G1 is not dependent on the SCF^{Cdc4} complex and has not been identified. Here, we show that in addition to the SCF^{Cdc4} complex, both the Hect-domain ubiquitin ligase Tom1 and the SCF^{Dia2} complex also target Cdc6 for ubiquitin-dependent degradation during the cell cycle. Tom1 and Dia2 physically associate with Cdc6. Cdc6 ubiquitination is dependent on each ligase both *in vivo* and *in vitro*. Cdc6 is stabilized in *tom1Δ* and *dia2Δ* mutants in G1 as well as at the G1/S transition. Importantly, Cdc6 stabilization is dramatically increased in *cdc4-1 dia2Δ* and *cdc4-1 tom1Δ* double mutants and the *cdc4-1 tom1Δ dia2Δ* triple mutant at the G1/S transition. Hydroxyurea- and MMS-sensitivity is also exacerbated in double and triple mutants, indicating that all three E3 ligases function independently to control Cdc6 proteolysis. We find that Cdc6 proteolysis requires both the TPR (tetratricopeptide) and LRR (leucine rich repeat) domains of Dia2, suggesting that the chromatin association of Dia2 through the TPR domain may be important for Dia2-mediated Cdc6 degradation. We also find that Cdc6 remains associated with chromatin in *tom1Δ* and *dia2Δ* mutants in S and G2/M. Taken together, these findings suggest that three independent E3 ligases contribute to the degradation of Cdc6 at the G1/S transition and both Tom1 and Dia2 also control Cdc6 degradation in G1.

MUTUALLY EXCLUSIVE FUNCTIONS OF HUMAN SLX4 IN REPAIRING DNA DAMAGE INDUCED BY INTERSTRAND CROSSLINKING AGENTS AND CAMPTOTHECIN

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Mitomycin C (MMC)-induced interstrand crosslinks (ICLs) and camptothecin (CPT)-induced damage which freezes Topoisomerase I (TOPI) on the DNA, result in replication fork stalling and collapse. Although CPT and MMC-derivatives are used as chemotherapeutic agents, the underlying mechanisms of repairing CPT and MMC induced DNA damage are not fully understood. Implicated in MMC and CPT-induced damage repair is SLX4, a multidomain scaffold protein, which interacts with at least three different nucleases: XPF-ERCC1, MUS81-EME1, and SLX1. Together with SLX1, SLX4 shows Holliday junction resolvase activity in vitro, but the biological consequences of the SLX4-nucleases interactions are not yet clear. Depletion of SLX4 in human cells leads to enhanced sensitivity to DNA crosslinking agents and CPT. Consistent with this, biallelic mutations of SLX4 have been identified in patients with Fanconi anemia, a rare recessive genetic disorder characterized by genome instability. In the present study, using a patient cell line that is null for SLX4, we dissected and characterized the multiple functions of SLX4 responsible for conferring resistance to MMC, CPT and poly ADP-ribose polymerase (PARP) inhibitor which measures the homologous recombination (HR) competence. We have constructed an extensive array of SLX4 mutants and expressed them in the SLX4 null patient cell line to assess the sensitivity to MMC, CPT and PARP inhibitor. We found that there are mutually exclusive domains of SLX4 responsible for repairing ICL damage and TOPI-DNA adducts. The SLX4 mutant with a SAP domain deletion (SLX4ΔSAP) is as competent for rescuing crosslink DNA damage as wild type SLX4, but is not able to repair CPT induced DNA damage. SLX4ΔSAP co-immunoprecipitates with XPF/ERCC1, but not with MUS81, indicating that the activity of MUS81 is critical for repairing CPT induced DNA damage, but is dispensable for ICL repair. On the other hand, the tandem UBZ domains and XPF interacting domain are critical for conferring resistance to MMC, but are not necessary for repairing CPT induced DNA damage. Complementation of null cell lines with a mutant lacking the SBD domain, which is critical for SLX1 interaction, rescued CPT sensitivity and partially rescued sensitivity to MMC. This implies that under physiological conditions, the Holliday junction resolvase activity of SLX4 is required to repair ICL damage. Lastly, the SLX4 mutants that rescue CPT-induced DNA damage also confer resistance to PARP inhibitor, suggesting that the HR activity of SLX4 is required for repairing CPT induced DNA damage but is not necessary for ICL repair.

FACT AND ATR COORDINATELY PROMOTE THE PROGRESSION OF UNPERTURBED DNA REPLICATION FORKS IN XENOPUS EGG EXTRACTS.

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FACT is an evolutionally conserved chromatin remodeling factor, which facilitates gene transcription by destabilizing nucleosomes in the path of RNA polymerases. In addition, FACT has been reported to be a component of the replication progression complex (RPC) and to be involved in an efficient DNA replication. However, the exact function of FACT in DNA replication is poorly understood. Hence, we analyzed the function of FACT for the replication of sperm chromatin in Xenopus egg extract, which allows us to examine DNA replication in the absence of transcription. The immunoprecipitation using antibody against a subunit of FACT showed that FACT associated with AND-1 and Claspin even in the extracts as well as on replicating chromatin, suggesting its possible involvement in the checkpoint system. To clarify the role of FACT in DNA replication, we immunodepleted FACT from the extracts, then analyzed the chromatin-binding of replication proteins by immunoblotting and also measured the replication activity by dNTP incorporation. Depletion of FACT did not affect the timing of initiation of DNA replication, nor the binding of replication fork proteins except the increased binding of Mcm10. However, dNTP incorporation was significantly decreased in FACT-depleted extracts compared with mock-depleted extracts. Direct observation of replicated DNA tracks by DNA combing techniques revealed that replication forks did not stall but the velocity of the fork was remarkably reduced in the FACT depleted extracts. Inhibition of ATR by caffeine further shortened the lengths of replication tracks in FACT-depleted extracts as well as mock-depleted extracts, while adding back of recombinant FACT after the initiation of the replication promptly restored the speed of forks in FACT-depleted extracts. These results suggest that the depletion of FACT does not suppress the fork progression through the repression of ATR, and illuminate the importance of FACT for the efficient progression of the replication fork but not RPC formation or maintenance.

ELG1, THE MAJOR SUBUNIT OF AN ALTERNATIVE RFC COMPLEX, INTERACTS WITH SUMO-PROCESSING PROTEINS

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PCNA is a homotrimeric ring with important roles in DNA replication and repair. During replication, PCNA is loaded and unloaded by the RFC complex, which is composed of five subunits (Rfc1-5). Three additional complexes that share with RFC the small subunits (Rfc2-5) and contain alternative large subunits, were found in yeast and other eukaryotes. We have recently reported that one of these, the Elg1-RFC complex, interacts with SUMOylated PCNA and may play a role in its unloading during DNA repair.

Here we report that a yeast-two-hybrid screen with the N terminus of Elg1 (which interacts with SUMOylated PCNA) uncovered interactions with proteins that belong to the SUMO pathway, including Slx5 and Slx8, which form an E3 ubiquitin ligase that ubiquitinates SUMOylated proteins. Mutations in SLX5 result in a genomic instability phenotype, similar to those of elg1 mutants. The physical interaction between the N terminus of Elg1 and Slx5 is mediated by poly-SUMO chains but not by PCNA modifications, and requires Siz2, but not Siz1, activity. Thus our results highlight the many important roles played by Elg1, some of which are PCNA-dependent and some PCNA-independent.

DNA BINDING PROPERTIES OF HSORC4

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DNA binding properties of ORC indicated that it has a little sequence specificity which is in consistency with lack of consensus sequence in eukaryotic origins of replication. Nevertheless, origin transfer studies show that they are genetically determined and consist of functionally interchangeable modules. One of such modules in human laminB2 origin of replication demonstrates ability to adopt unorthodox structure partly composed of intramolecular triplex . Formation of this structure was stimulated by addition of HsORC4 protein, one of ORC's subunits that exhibit independent DNA binding activity in vitro.
To explore potential significance of unorthodox DNA structures within replication origins we tested DNA binding properties of HsORC4 using EMSA. We show that HsORC4 recognizes and directly binds short triple stranded 40mers, while it's binding to corresponding single or double stranded 40mer could not be detected under the same conditions. In competition with fragment from laminB2 origin for HsORC4 binding the short and long triple stranded DNA were the far best competitor compared to its single and double stranded counterparts. Systematical deletion of origin fragment used in competition binding experiments demonstrate that 70bp fragment recognized by HsORC4 still has the ability to assume alternative DNA structure
We conclude that HsOrc4 preferentially recognize and binds alternative DNA structure. Such feature could play part in origin selection through directing ORC to DNA sequence prone to adopt unorthodox structure.

MECHANISMS FOR MAINTAINING GENOME STABILITY AT THE REPLICATION FORK

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Maintenance of genome stability is of critical importance to the cell, and the integrity of the genome can be threatened when DNA is damaged by either endogenous or environmental agents. DNA is particularly susceptible to damage during DNA replication, when replication forks can stall at DNA lesions. As a result, the cell has finely tuned processes to allow the replication of damaged DNA, to repair the DNA damage and to stabilize/restart stalled replication forks. A failure to carry out these processes can lead to the loss of genomic integrity by interfering with proper repair of DNA, and by leading to the collapse of stalled forks into double-strand breaks. We carried out a genome-wide screen to identify proteins involved in preventing DNA damage in the absence and presence of replication stress. Our analysis revealed unexpected connections between known cellular pathways and the prevention of DNA damage. Our recent progress on the proteins identified in this screen and their roles in maintaining genome stability will be described here. Furthermore, we will describe recent studies regarding the role of other proteins known to promote the replication of damaged DNA either by direct bypass or via restart of DNA replication.

ATR AUTOPHOSPHORYLATION AS A MOLECULAR SWITCH FOR CHECKPOINT ACTIVATION

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The ataxia telangiectasia-mutated and Rad3-related (ATR) kinase is a master checkpoint regulator safeguarding the genome. Upon DNA damage, the ATR-ATRIP complex is recruited to sites of DNA damage by RPA-coated single-stranded DNA and activated by an elusive process. Here, we show that ATR is transformed into a hyperphosphorylated state after DNA damage, and that a single autophosphorylation event at Thr 1989 is crucial for ATR activation. Phosphorylation of Thr 1989 relies on RPA, ATRIP, and ATR kinase activity, but unexpectedly not on the ATR stimulator TopBP1. Recruitment of ATR-ATRIP to RPA-ssDNA leads to congregation of ATR-ATRIP complexes and promotes Thr 1989 phosphorylation in trans. Phosphorylated Thr 1989 is directly recognized by TopBP1 via the BRCT domains 7 and 8, enabling TopBP1 to engage ATR-ATRIP, to stimulate the ATR kinase, and to facilitate ATR substrate recognition. Thus, ATR autophosphorylation on RPA-ssDNA is a molecular switch to launch robust checkpoint response.

DNA2 COUPLES DNA REPLICATION TO THE REPLICATION CHECKPOINT THROUGH ACTIVATION OF MEC1/ATR

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In response to replication stress the DNA replication checkpoint is activated and it slows down cell cycle progression. In budding yeast the principal component of this pathway is Mec1/Ddc2 (human ATR/ATRIP). Mec1 kinase activity is activated by specific activators that function in a cell-cycle specific manner. Earlier studies from our lab have identified Ddc1 (human Rad9, a subunit of 9-1-1 checkpoint clamp) as a G1-specific activator of Mec1, whereas in G2/M both 9-1-1 and Dpb11 (human TopBP1) activate Mec1 in response to DNA damage. However, our studies of these activators pointed out for the existence of additional activator(s) of Mec1 during S phase. Here, we propose that Dna2 serves as a S-phase specific activator of Mec1. Dna2 is an essential nuclease/helicase that is involved in Okazaki fragment processing and in DNA end resection at dsDNA breaks. In this study we show that Dna2 is able to activate Mec1 kinase activity *in vitro*. Activation requires the presence of two conserved aromatic amino acids in the unstructured N-terminus of Dna2. Our genetic data show that the 9-1-1/Dpb11 circuitry and Dna2 are partially redundant for the replication checkpoint.

THE MCM6/2 ATPASE ACTIVE SITE IS REQUIRED FOR THE REPLICATION CHECKPOINT.

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Mcm2-7 is the catalytic core of the eukaryotic replicative helicase. It is composed of six distinct and essential evolutionarily conserved subunits that are each AAA+ ATPases. Unlike other hexameric helicases, considerable evidence suggests that each of the six subunits is functionally distinct. ATPase active sites are formed at subunit interfaces, with one subunit providing the Walker A and Walker B motifs, and the opposing subunit contributing the arginine finger motif. Work from our lab and others indicates that the active site formed between Mcm7 and Mcm4 (i.e., the Mcm7/4 site) is predominately involved in helicase activity, and that the Mcm2/5 interface forms a structural discontinuity that is the site of regulation by the replication proteins GINS and Cdc45. The possible functions of the remaining ATPase active sites are unknown. Using Saccharomyces cerevisiae, we study the in vivo role of the Mcm6/2 ATPase active site using a viable mutant, which carries substitutions in the conserved residues, DE→NQ in the Walker B motif (mcm2DENQ). This allele is specifically defective for the replication checkpoint and shares a variety of phenotypes with mutations in Mrc1. In combination with a deletion of Rad9 (to eliminate checkpoint contributions from the DNA damage response) and moderate concentrations of the DNA damage agent MMS, mcm2DENQ mutants are inviable, lose the normal block to cell cycle progression, and largely eliminate the phosphorylation of the downstream kinase Rad53 (Chk2). However under these conditions Mrc1 is still phosphorylated, indicating that DNA damage is sensed and the checkpoint cascade is still activated in mcm2DENQ. Similar to mutations in Mrc1, ChIP-seq analysis of BrdU incorporation in G1 synchronized cells arrested in the presence of hydroxyurea indicates that the mcm2DENQ mutant loses the repression of late origin activation. Interestingly we observe a subset of late origins that do not activate in the mcm2DENQ mutant comparing to mrc1Δ, suggesting the possibility of different functional classes of late origins. As recent work has demonstrated that physical interaction between the C-terminal of Mcm6 and Mrc1 is necessary for checkpoint function (Komata et. al., 2009 MCB 18:5008), our data is consistent with the Mcm6/2 active site being an integral member of the replication checkpoint cascade. This possibility suggests that the funneling of the checkpoint cascade through Mcm2-7 may provide a novel mechanism to ensure control of the replicative helicase in the presence of DNA damage.

VISUALIZING COLLAPSE: WHAT HAPPENS WHEN FORKS GO BAD?

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Replication fork collapse results when checkpoint-deficient cells are treated with hydroxyurea (HU). But what really happens? Using novel imaging techniques we have probed the roles of intra-S phase checkpoint proteins Cds1 and its adapter Mrc1 in fission yeast in HU-treated cells.

First, we were surprised to observe substantial amounts of DNA synthesis during and following the HU block in both *cds1Δ* and *mrc1Δ*, monitored by incorporation of nucleoside analogues. This was not seen in bulk DNA FACS profiles. Seemingly paradoxical, this result was clarified by analysis of chromatinized DNA fibers. We observed EdU incorporation in *cds1Δ* cells as punctate tracts – not uniform labeling. In contrast, wild type cells produced short, dense tracts of replication during HU and uniform synthesis following release. This different approach detected replication fork proteins associated near tips of replicated DNA, suggesting a model for the roles of Cds1 and Mrc1 activities during HU arrest and restart.

Next, we used live single cell imaging of cells held in a microfluidics chamber. We investigated how asynchronous cultures respond to HU arrest by monitoring the distribution and accumulation of fluorescently tagged RPA and RAD52(Rad22). Both *cds1Δ* and *mrc1Δ* cells acquired high levels of RPA and RAD52 in foci during the HU block, suggesting substantial accumulation of single strand DNA and DNA damage. Moreover, these foci remained unresolved over hours, pointing to long-term damage. This is consistent with both helicase uncoupling from existing forks, and late origin firing. Wild type cells did not substantially recruit RPA or RAD52 until HU release and restart, a transient effect. Unexpectedly, we find that a fraction of checkpoint-deficient cells never show foci; these cells may account for the fraction of *cds1Δ* cells that survive HU treatment.

Finally, we have found that checkpoint-deficient cells accumulate phospho-H2A signal and Chk1 phosphorylation following release, pointing to the production of extensive damage during recovery from HU. We verified that the DNA damage in *cds1Δ* cells is dependent upon the Mus81 endonuclease. Nevertheless, Chk1 is activated in *mus81Δ* cells. These data give us unique insights into replication fork architecture during HU arrest and restart, defining replication fork collapse as a concept.

REPLICATION FORKS RESTARTED BY HOMOLOGOUS RECOMBINATION DISPLAY A HIGH FREQUENCY OF ERRORS AT INVERTED REPEATS

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Replication restart by HR at the sites of a collapsed replication fork promotes cell survival, but at the expense of increasing the potential for genome rearrangement. We have previously shown that, when a replication fork collapses within a small (~900bp) inverted repeat, homologous recombination is required to restart the collapsed fork. In the majority of cases, the restart event is effective and promotes cell survival. However, the presence of a nearby repeat (homologous sequence) results in an inappropriate template exchange in a few percent of events, producing dicentric and acentric giant palindromic chromosomes.
We also studied the consequence of replication fork arrest within a small (~5kb) palindrome. We observed an increase in the percentage of formation of dicentric and acentric palindromic chromosomes to approximately 20% (1:5 restart events). We have been studying the reason why these events occur so frequently when replication forks collapse within a palindrome. This has lead us to observe that, when replication forks collapse and restart at a unique sequence, they are prone to generate errors when they replicate through small inverted repeats or small palindromes.
We conclude that two distinct mechanisms can result in gross chromosomal rearrangements: 1. When replication forks collapse within a repeated sequence, incorrect homologous recombination can occur that results in repeat fusion. 2. The restarted replication fork is itself error prone, and is particularly sensitive to making errors when replicating closely spaced inverted repeats.

THE ACCUMULATION OF NICKED DNA INDUCES PCNA UBIQUITINATION, HOMOLOGOUS RECOMBINATION AND SINGLE STRAND ANNEALING

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DNA ligase I (LIG1) is an essential protein involved in the ligation of Okazaki fragments. We have shown that cells trigger a novel ubiquitination pathway that targets proliferating cell nuclear antigen (PCNA) at lysine (K) 107 in *S. cerevisiae cdc9* (LIG1) mutants. Most importantly, PCNA ubiquitination at K107 is crucial for the activation of the S phase checkpoint kinase Rad53 in *cdc9* mutants (Das-Bradoo *et al*, NCB 2010). Furthermore, PCNA ubiquitination at K107 occurs independently of PCNA-K164, a known ubiquitination site in response to UV-irradiation. To determine which defects trigger PCNA ubiquitination in LIG1 mutants, we complemented *cdc9-1* cells with either wild type or mutant Cdc9. PCNA ubiquitination was not detected in *cdc9-1* cells that were complemented with wild type *CDC9*. This suggested that PCNA ubiquitination was not due to nonspecific secondary defects. We further observed that PCNA was ubiquitinated in *cdc9-1* cells that were complemented with two different catalytically inactive LIG1 mutants. Mutations at K419A and K598A in *cdc9-1* cells result in the accumulation of "clean" (3'OH and 5'PO4 ends) and "dirty" (3'OH and 5'AMP ends) nicks, respectively (Ellenberger and Tomkinson, Annu. Rev. Biochem. 2008). Lastly, a *cdc9* mutant that was unable to interact with PCNA did not induce PCNA ubiquitination, consistent with the notion that PCNA ubiquitination is triggered by the accumulation of nicks and not due to the absence of the PCNA-Cdc9 interaction.
To better understand how cells cope with the accumulation of nicks during Okazaki fragment maturation and what the molecular function of PCNA ubiquitination might be, we utilized *cdc9-1* in a genome-wide synthetic lethality screen. Confirming previous reports, genes in the *RAD52* epistasis group were synthetically lethal with *cdc9-1*, arguing that homologous recombination plays a crucial role in the survival of *cdc9-1* cells. Unexpectedly, we also identified genes in the single strand annealing (SSA) pathway (*RAD59*, *RAD1* and *RAD10*) that were required to maintain cell growth at the semipermissive temperature. We propose a model by which Okazaki fragments anneal to the nascent leading strand via SSA in order to continue DNA synthesis, bypassing the requirement for LIG1. [supported by NIH grant GM074917 & LLS1023-09]

SCF^{DIA2} AS A MEDIATOR OF CHECKPOINT RECOVERY FROM REPLICATION STRESS

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Cells sophisticatedly control DNA replication in response to replication stress and DNA damage in order to safeguard genome integrity. The ubiquitin-proteasome pathway is one mechanism of control in DNA replication. The SCF^{Dia2} (Skp1/Cdc53/Rbx1/F-Box) is an ubiquitin-ligase complex of *Saccharomyces cerevisiae* that regulates DNA replication. Cells lacking the *DIA2* gene (dia2Δ) exhibit chromosome breakage and rearrangement. Not surprisingly, *dia2*Δ cells are sensitive to DNA damaging agents and defective in replicating DNA in the presence of alkylating agent MMS. The F-box protein Dia2 is stabilized when S-phase checkpoint is activated in response to replication stress. However, the role of Dia2 in the checkpoint remains unknown. To investigate further, we performed a genetic suppressor screen to identify potential ubiquitination substrates of Dia2. The screen identified an allele of *MRC1* (mediator of replication checkpoint), consistent with a previous study that identified Mrc1 as a substrate of Dia2. Until now, the biological significance of Dia2 targeting Mrc1 for degradation remained elusive. Our studies with this *mrc1* loss-of-function allele provide a plausible explanation for the degradation. The suppressor allele ($mrc1^{CS}$) is truncated at the C-terminus and checkpoint-defective. Unlike the previously described checkpoint-defective phosphomutant $mrc1^{AQ}$, $mrc1^{CS}$ retains all the checkpoint-activated phosphorylation sites. Kinetic studies show that $Mrc1^{CS}$ is phosphorylated at a slower rate and the activation of downstream effector Rad53 is compromised in the $mrc1^{CS}$ mutant. Both $mrc1^{CS}$ and $mrc1^{AQ}$ suppress the hypersensitivity of *dia2*Δ to MMS and accelerate *dia2*Δ progression through DNA replication in response to MMS. Furthermore, Dia2 is required for the degradation of checkpoint-activated Mrc1. Taken together, we propose that in response to MMS, Dia2 is stabilized to mediate degradation of Mrc1 for checkpoint recovery.

IRREVERSIBLE FORK COLLAPSE IN ATR-DEFICIENT CELLS IS DEPENDENT ON PLK1

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The ATR-CHK1 axis stabilizes stalled replication forks and prevents their collapse into DNA double strand breaks (DSBs). Previous studies have suggested that fork collapse is rendered irreversible through the loss of ATR-CHK1-dependent processes that facilitate homologous recombination and reestablish the replication fork structure. In this study, we demonstrate that the irreversibility of replication failure in ATR-suppressed cells is driven by a PLK1-mediated process that prevents replication reinitiation after RAD51-dependent reformation of the fork structure. Following acute DNA polymerase inhibition (6 hour aphidicolin treatment), ATR-deleted cells exhibited a five-fold decrease in their ability to restart previously fired replication forks when compared to control cells. The failure of ATR-depleted cells to restart replication was associated with proteasome-dependent loss of chromatin-bound PCNA, but not Claspin, suggesting that in an ATR-depleted environment Claspin degradation is not the primary cause of failed restart. Notably, partial inhibition of CDK2 or suppression of Aurora A or PLK1 activity permitted replication reinitiation in ATR-deleted cells. Furthermore, recruitment of the activated form of PLK1 (phospho-T210) to chromatin was prevented by each of these inhibitory treatments, implying that PLK1-dependent events are the most proximal to replication suppression. The ability of PLK1 suppression to rescue replication in ATR-deficient cells was not caused by a change in the frequency of replication fork collapse into DSBs, since DSB formation was equivalent in the absence or presence of PLK1 activity. Accordingly, the replication reinitiation fostered by PLK1 inhibition occurred in a RAD51-dependent manner, indicating that the PLK1 pathway limits replication recovery upon reformation of a productive replication fork structure. Taken together, these results indicate that, following fork collapse, ATR is not necessary for replication restart and that PLK1 opposes replisome reassembly. The mechanism underlying replisome inhibition and the implications of these findings on checkpoint adaptation will be discussed.

THE PHOSPHORYLATION NETWORK FOR EFFICIENT ACTIVATION OF THE DNA REPLICATION CHECKPOINT IN FISSION YEAST

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Protein phosphorylation is the hallmark of checkpoint activation. Hundreds of phosphorylation targets of checkpoint kinases have been identified recently by genome-wide investigations. However, the complete picture of a phosphorylation network required for activation of a checkpoint pathway has not been available. The DNA replication checkpoint in the fission yeast *Schizosaccharomyces pombe* contains two major protein kinases, the sensor kinase Rad3 and the effector kinase Cds1, with the latter mediating most of the checkpoint functions. By large-scale mutational analyses and immunoblottings with phospho-specific antibodies, we found that when DNA replication is arrested in fission yeast, efficient activation of Cds1 requires five phosphorylations that cooperate in a parallel or a sequential manner. Phosphorylation of a threonine residue (Thr^{11}) in Cds1 by Rad3 occurs at a basal level in the absence of three other parallel Rad3-dependent phosphorylations on the mediator Mrc1 and Rad9 in the checkpoint clamp complex. However, the three parallel Rad3-dependent phosphorylations are all required for efficient phosphorylation of Thr^{11} in Cds1 by Rad3. Phosphorylation of Thr^{11} has been shown previously to promote autophosphorylation of Thr^{328} in the kinase domain of Cds1, which directly activates the enzyme, leading to full activation of the checkpoint pathway. Interestingly, phosphorylation of Mrc1 by Rad3 is independent of Rad9 phosphorylation, suggesting that activation of the sensor kinase Rad3 in the replication checkpoint of fission yeast may involve a different mechanism that does not require the recruitment of Cut5 (TopBP1 in fission yeast) mediated by phosphorylated Rad9. *In vivo* genetic analysis complemented with *in vitro* biochemical methods are powerful means of dissecting molecular details of a complex biological system. The clear picture of the phosphorylation network required for efficient activation of Cds1 *in vivo* can serve as a roadmap for *in vitro* biochemical studies of the replication checkpoint pathway in fission yeast.

MECHANISMS OF ORIGIN MELTING BY THE DnaA REPLICATION INITIATOR

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The initiation of DNA replication requires the melting of chromosomal origins to provide a template for replisomal polymerases. In bacteria, the DnaA initiator plays a key role in this process, forming a large nucleoprotein complex that opens DNA through a complex and poorly understood mechanism. Using structure-guided mutagenesis, biochemical, and genetic approaches, we establish an unexpected link between the duplex DNA-binding domain of DnaA and the ability of the protein to both self-assemble into a helical oligomer and engage single-stranded DNA in an ATP-dependent manner. Intersubunit crosstalk between this domain and DnaA's AAA+ ATPase region regulates this link, and is required for both origin unwinding in vitro and initiator function *in vivo*. Our findings indicate that DnaA utilizes two distinct higher-order conformations for engaging single- and double-stranded DNA, and that these states play distinct roles in controlling DNA melting and the progression of initiation.

CDT1 COORDINATES MCM2-7 RING OPENING WITH ORIGIN LICENSING

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Cdt1 is essential, along with ORC and Cdc6, for loading the Mcm2-7 replicative helicase onto double stranded origin DNA during G1 phase of the cell cycle. In metazoans, many of the mechanisms limiting replication to once per cell cycle work through Cdt1. Thus, understanding how Cdt1 contributes to origin licensing is important for understanding eukaryotic DNA replication. We have found that Cdt1 forms a stable complex with soluble Mcm2-7. This complex contains a single molecule of Cdt1, which interacts with both Mcm2 and Mcm6. Using biochemical approaches and electron microscopy, we show that Mcm2-7 forms a continuous ring, but Cdt1 binding destabilises the interaction between the Mcm2 and Mcm5 subunits. To ascertain the significance of this ring opening, we examined a series of Cdt1 mutants for their ability to open the Mcm2-7 ring and to load the complex. We will describe one mutant, *cdt1-C1*, that can still interact with Mcm2-7, but cannot destabilise the Mcm2/5 interface. Cdt1-C1 can still be recruited to origins *in vitro*, suggesting it can still interact with ORC. Cdt1-C1, however, cannot load Mcm2-7 onto origin DNA *in vitr*o, and cannot complement loss of Cdt1 *in vivo*. This indicates that Mcm2-7 ring opening by Cdt1 is essential for origin licensing.

MULTIPLE CDT1 MOLECULES ACT AT EACH ORIGIN TO LOAD REPLICATION-COMPETENT MCM2-7 HELICASES

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Eukaryotic origins of replication are selected by loading a head-to-head double hexamer of the Mcm2-7 replicative helicase around origin DNA. Cdt1 plays an essential but transient role during this event, however, its mechanism of action is largely unknown. Through analysis of Cdt1 mutations, we demonstrate that Cdt1 performs multiple functions during helicase loading. A C-terminal region of Cdt1 binds Mcm2-7, and this interaction is required for efficient origin recruitment of both proteins. We show that ORC and Cdc6 recruit multiple Cdt1 molecules to the origin during helicase loading, and disruption of this multi-Cdt1 intermediate prevents helicase loading. Although dispensable for stable Mcm2-7 loading, the essential N-terminal domain of Cdt1 is required to load Mcm2-7 complexes that are competent for association with helicase-activating proteins and replication initiation. Our data support a model in which origin-bound ORC and Cdc6 recruit two Cdt1 molecules to initiate double-hexamer formation prior to helicase loading and demonstrate that Cdt1 influences the replication competence of loaded Mcm2-7.

ESSENTIAL ROLE OF DNA POLYMERASE EPSILON AT THE INITIATION STEP OF CHROMOSOMAL DNA REPLICATION IN BUDDING YEAST

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DNA polymerase epsilon (Pol epsilon), one of three eukaryotic replicative DNA polymerases, synthesizes the leading strand at the replication forks. It compromises four subunits, Pol2, Dpb2, Dpb3 and Dpb4. Pol2 is a 256-kDa catalytic subunit, of which the N-terminal half is responsible for DNA polymerase activity and the C-terminal half interacts with other Pol epsilon subunits. Surprisingly, the N-terminal half of Pol2 is dispensable while the C-terminal half is essential for cell growth and DNA replication in yeast cells. The essential function of the C-terminal half had not been elucidated.

We will show that the Pol epsilon functions for recruiting GINS to replication origins to form an active replicative helicase, as a component of the pre-Loading complex (pre-LC). The pre-LC contains Dpb11, Sld2, GINS, and Pol epsilon, and is formed when Dpb11 binds to the N-terminal part of Sld2 in a CDK-dependent manner. Among Pol epsilon subunits, Dpb2 binds to GINS and the C-terminal half of Pol2 binds to the middle part of Sld2. While Sld2 lacking the middle part does not support the cell growth, the same construct fused to the C-terminal half of Pol2 functions as Sld2. On the other hand, while the C-terminal half of Pol2 that lacks the ability to bind to Sld2 does not support the cell growth, the same Pol2 construct fused to Sld2 supports the cell growth. Thus, the binding between Sld2 and Pol2 is essential for their functions. Moreover, the Sld2-Dpb2 fused protein bypasses the requirement of the C-terminal half of Pol2, probably because the fused protein directly recruits GINS to Dpb11 and then to replication origins. Taken all together, we propose that Pol epsilon plays an essential role of a hub of GINS and Sld2 for formation of the pre-LC. We will further propose a general model for the initiation of chromosomal DNA replication in eukaryotic cells.

SLD2 AND SLD3 INHIBIT GINS BINDING TO MCM2-7, AND THIS INHIBITION IS ALLEVIATED BY ORIGIN SINGLE-STRANDED DNA

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Sld2 and Sld3 are required for the initiation of DNA replication, but these proteins do not travel with the replication fork. Sld2 and Sld3 are phosphorylated by CDK, activating the association of Sld2 and Sld3 with Dpb11. The CDK-phosphorylated state of Sld2 is mimicked by the mutation Sld2T84D. Sld3 binds to Cdc45 throughout the cell cycle. Aside from these functions, little is known about how these proteins trigger the initiation of DNA replication. We purified components of the replication initiation machinery and studied their interactions *in vitro*. We found that Sld2, Sld2T84D, or Sld3 binds tightly to the Mcm2-7 complex. Binding of Sld2 or Sld3 for Mcm2-7 is mutually competitive. Furthermore, binding of Sld2, Sld2T84D, or Sld3 to Mcm2-7 inhibits the interaction between GINS and Mcm2-7. Moreover, Sld2 or Sld2T84D in combination with Sld3 additively inhibits the interaction between GINS and Mcm2-7. In the presence of origin single-stranded DNA, Sld2T84D or Sld3 is released from the Mcm2-7 complex, while Sld2 remains bound to Mcm2-7. Origin single-stranded DNA also allows GINS to bind to Mcm2-7 in the presence of Sld2T84D and Sld3, but not Sld2. These data suggest that CDK-activation of Sld2 is required for GINS interaction with Mcm2-7. We propose that when budding yeast origin DNA is melted prior to the onset of DNA replication, the exposed single-stranded DNA releases CDK-phosphorylated Sld2 and Sld3 from Mcm2-7, allowing GINS to bind to Mcm2-7. Thus, origin single-stranded DNA may act as a trigger to help assemble the Cdc45-Mcm2-7-GINS (CMG) complex, thereby activating the replication fork helicase.

REPLICATION TIMING MAPS DEFINE A DISCRETE UNIT OF LARGE-SCALE CHROMOSOME ORGANIZATION

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Replication timing has been anecdotally linked to many developmentally regulated chromosome functions but causality has been difficult to address largely due to the lack of systems that can elicit changes in replication timing. Toward the development of such systems, we have generated genome-wide replication-timing profiles for over 75 human and mouse cell lines, embryonic stem cell (ESC) differentiation intermediates, ESC knockout lines, trans-differentiation models and cells from diseased patients (replicationdomain.org). Changes in replication timing during development, as well as abnormalities in diseased tissue, collectively affect at least half the genome and consistently occur in units of 400-800 kb. Replication timing profiles align closely to genome-wide maps of long-range chromatin interactions, suggesting that replication domains are spatially segregated regions of chromatin folding. During ESC differentiation, genes subject to replication-timing changes become difficult to reprogram back to the pluripotent state, demonstrating an epigenetic canalization linked to replication timing shifts. These same regions undergo a large-scale chromatin reorganization that is restricted to the domain of replication timing change, more localized than sub-nuclear position changes, and independent of changes in transcription or general nuclease accessibility. Together, our findings strongly support the hypothesis that replication-timing domains delineate developmentally regulated structural and functional units of higher order chromosome architecture.

REGULATION OF REPLICATION PROGRAM IN FISSION YEAST AND HUMAN CELLS

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The location and timing of origin firing are under dynamic regulation during cell cycle and also during development in higher eukaryotes. Many factors including chromatin structure, checkpoint signaling and nuclear architecture may regulate this process. The conserved Cdc7 kinase triggers the firing from selected pre-RCs during S phase, but precise mechanisms of how Cdc7 selects the origins to be fired are not known. In mammalian cells, genome-wide replication timing program may be dictated by "replication (timing) domains" which were recently defined in various cell types. However, factors that may regulate the formation of "replication domain" are still elusive.
Search for bypass mutants of Hsk1 kinase (Cdc7 homologue) in fission yeast led to identification of a number of genes, deletion of which enables permits the growth of *hsk1*Δ at 30°C. Among them, Mrc1, a checkpoint adapter protein, suppresses the firing in both checkpoint-dependent and -independent manners. Mrc1 binds selectively early-firing origins in a manner independent of Hsk1 and Cdc45, and physically interacts with Hsk1. This led us to propose that Mrc1 may serve as a mark for early-firing origins in fission yeast.
Rif1 was identified as the most vigorous suppressor of *hsk1*Δ. *rif1*Δ cells are resistant to genotoxic agents and, therefore, the suppression is checkpoint-independent. Some dormant origins are fired early and some early-firing origins are suppressed in *rif1*Δ, indicating that Rif1 regulates the origin firing program both negatively and positively. Human Rif1 is also a chromatin binding protein and in human cells depleted of Rif1, the bulk S phase progression is not affected but the structure of replication timing domains is disrupted; i.e. inversion of the replication timing, consolidation of replication domains, and homogenization of replication timing. Furthermore, Cdc7-mediated phosphorylation and chromatin loading of Cdc45/PCNA are significantly enhanced in early S phase, while Cdc7 kinase activity is not affected in Rif1-depleted cells. These data strongly indicate that Rif1 is a key factor that regulates selection of origins to be fired and formation of replication timing domains in fission yeast and human cells.
ChIP-chip assays revealed that fission yeast Rif1 binds specific arm segments, which do not necessarily overlap with pre-RC sites. On the basis of these and other results, we would like to discuss possible mechanisms of Rif1-mediated regulation of replication domains.

REPLICATION TIMING CONTROL BY TAZ1 THAT BINDS TO TELOMERIC REPEATS PROXIMAL TO INTERNAL LATE REPLICATION ORIGINS.

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Replication of chromosome DNA is initiated from a number of distinct loci, called replication origins. The replication origins are not activated simultaneously at the beginning of S phase, but fire at the distinct timings during S phase. In fission yeast, pre-replicative complexes (pre-RCs) are formed at all the origins, but Sld3 is loaded dependently on DDK only onto the early origins in early S phase. To uncover the mechanism of replication timing control, we looked for a *cis*-acting element that confers specific replication timing on replication origins. By inserting the fragments derived from the chromosomal late origin into the proximal of the early origin on the chromosome, we identified a Replication Timing Control (RTC) element that represses the initiation of the early origin. By deletions and base substitutions of the fragment, we determined the sequence essential for the RTC activity. The essential sequence contains two copies of telomeric repeats. We found that telomeric repeats exist near 20 late origins located in the internal regions of chromosomes. Disruption of the telomeric repeats resulted in early firing from the associated late origins, indicating telomeric repeats play essential role in the timing control. Then, we asked whether Taz1, the fission yeast homologue of telomeric repeat binding protein TRF1/TRF2, is required for the timing control. In *taz1*Δ strain, late origins associated with telomeric repeats fired in early S phase, whereas those without the repeats did not change. In addition, a cluster of late origins in 50-kb long sub-telomeres extensively replicated in early S phase in *taz1*Δ. Our results demonstrate that Taz1 that binds to telomeric repeats associated with the internal late origins plays a crucial role in replication timing control in fission yeast.

FORKHEAD TRANSCRIPTION FACTORS ESTABLISH ORIGIN TIMING AND LONG-RANGE ORIGIN CLUSTERING IN S. CEREVISIAE

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Transcription factors have been implicated as modulators of replication origin efficiency and timing of activation, however, the mechanisms and significance of this regulation remain relatively obscure. We have identified the yeast Forkhead transcription factors, Fkh1 and Fkh2, as global determinants of replication origin timing. In their absence, most early origins fail to initiate promptly, while most late origins are advanced. These effects on origins are independent of proximal changes in transcription associated with deletion of FKH1 and FKH2. Remarkably, the early-firing of centromere-proximal origins is independent of Fkh1 and Fkh2, whereas late-replicating telomere-proximal origins are further delayed in their absence, suggesting a mechanism involving chromosome architecture. Furthermore, Forkhead-activated and Forkhead-repressed origins frequently occur in discreet groups along chromosomes consistent with replication timing domains. Accordingly, we show that normally early origins that are deregulated by elimination of Fkh1 and Fkh2 fail, in G1-phase, to associate with the initiation factor Cdc45 and to cluster with other origins destined to fire early, suggesting that Fkh1 and Fkh2 are responsible for the selective recruitment of origins to emergent replication factories. Fkh1 and Fkh2 co-immunoprecipitate with ORC, providing a potential mechanism for origin clustering. The conservation of Forkhead box (Fox) transcription factors in higher eukaryotes suggests that analogous mechanisms act in multicellular organisms and are particularly intriguing considering the involvement of Fox proteins in development and replication timing changes that occur during development.

COMPETITION FOR LIMITING REPLICATION INITIATION FACTORS EXECUTES THE TEMPORAL PROGRAMME OF ORIGIN FIRING IN BUDDING YEAST

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Eukaryotic chromosomes are replicated from multiple origins that initiate throughout the S-phase of the cell cycle. The distribution and timing of these initiation events are developmentally and epigenetically regulated but why all origins do not fire simultaneously at the beginning of S-phase is not known. Replication initiation requires two kinase activities: cyclin-dependent kinase (CDK) and Dbf4-dependent kinase (DDK). Although both of these kinases are activated at the G1-S phase transition, they are continually required throughout S-phase for all initiation events. Here we show that the two essential CDK substrates Sld3 and Sld2 and their binding partner Dpb11, together with the DDK subunit Dbf4 are in low abundance and limiting for replication initiation in the budding yeast, Saccharomyces cerevisiae. Over-expression of these factors is sufficient to allow normally late firing origins of replication to initiate early. Increased dosage of these factors together with deletion of the histone deacetylase RPD3 promotes the firing of heterochromatic, dormant origins indicating that chromatin context controls the accessibility of these limiting factors at origins. Using this over-expression system, we demonstrate that the normal programme of origin firing prevents deoxyribonucleotide depletion and controls S-phase length in budding yeast. Finally we show that deregulation of the temporal programme of origin firing causes loss of viability. These results explain how the competition for limiting DDK kinase and CDK-targets at origins controls replication initiation kinetics during S-phase and establishes a unique system with which to investigate the biological roles of the temporal programme of origin firing.

PARTIAL COMPLEMENTATION OF A DNA LIGASE I DEFICIENCY BY DNA LIGASE III: IMPACT ON SURVIVAL AND TELOMERE SISTER FUSIONS

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Whilst DNA ligase I (LigI) plays a central role in the joining of strand interruptions during replication and repair, it has been shown that Okazaki fragments are ligated in cells with altered LigI activity, suggesting that in such cells an alternative pathway can process unligated replication intermediates. We provide, for the first time, evidence that DNA ligase III (LigIII) and XRCC1, which are involved as a complex in single strand break repair, are required for the proliferation of LigI-depleted cells. We show that in cells with either dysfunctional LigI activity or in cells depleted in LigI that both LigIII and XRCC1 are retained on the chromatin and colocalize to late-S phase replication foci. These results suggest that in cells with dysfunctional LigI, LigIII could contribute to Okazaki fragment ligation when LigI functions are compromised. One of the hallmarks of LigI- and LigIII-deficiency is the increase in the incidence of interstitial-SCEs (I-SCE). We tested the effect of the depletion of LigI or LigIII individually or together on the incidence of both (I-SCE) and telomere-SCEs (T-SCEs). The observed synergistic increase in I-SCEs and T-SCEs frequencies in the LigI-LigIII depleted cells suggest that both LigI and LigIII efficiently complement each other for the suppression of SCEs at both telomeric and non-telomeric DNA. The depletion of LigI resulted in an increase in the frequency of sister telomere fusions. We also demonstrate that LigI, but not LigIII, suppresses telomere sister fusions. Our working model is that compromised Okazaki fragment ligation during telomere replication in LigI-deficient cells is not fully complemented by LigIII, and the remaining single strand breaks could render the lagging strand telomere more prone to producing double strand breaks in interstitial lagging strand sequences. Broken lagging strand telomeres will be fused by NHEJ with the newly replicated leading chromatid and give rise to the dramatic increase in sister telomere fusions observed in LigI-deficient cells.
In conclusion, our observations show that the LigIII/XRCC1 complex is required to promote the viability of LigI deficient cells and that LigI and LigIII efficiently cooperate to inhibit high frequencies of SCEs. However, LigI has a specific role in the suppression of sister telomere fusions, revealing its importance for telomere maintenance.

ROLE OF MCM4 IN REPLICATION DYNAMICS AND COMMON FRAGILE SITES STABILITY

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Common fragile sites (CFS) are loci that recurrently exhibit chromosome instability, visible as gaps, constrictions and breaks on metaphase chromosomes, following perturbation of DNA synthesis. CFS are increasingly recognized to be preferential targets for oncogene-induced DNA damage in pre-neoplastic lesions and hotspots for chromosomal rearrangements in various cancers. Moreover, some CFS lie within tumour suppressor genes, which could contribute to cancer development.
A recent study has shown that CFS fragility relies on the paucity of replication initiation events along with late replication completion (1). We have therefore decided to study the impact of mutations altering proteins involved in replication initiation on CFS stability. We have used embryonic mouse fibroblasts (MEFs) harbouring an hypomorphic allele of Mcm4 named Mcm4^{Chaos3} (2). MCM4 is an essential subunit of the replication licensing factor MCM, a heterohexameric complex conserved in all eukaryotes. Mcm4^{Chaos3} encodes an apparently unstable MCM4 which destabilizes the whole MCM complex. Mcm4$^{Chaos3/Chaos3}$ MEFs are highly sensitive to perturbations of DNA replication, and mutant mice develop tumours. In particular, 80% of Mcm4$^{Chaos3/Chaos3}$ females succumb to mammary adenocarcinomas.
We have analyzed the replication dynamics in Mcm4$^{Chaos3/Chaos3}$ cells using molecular combing, which allows a reliable measurement of replication forks speed as well as initiation density. Despite a decreased loading of the MCM complex onto the chromatin by more than 50%, neither global replication speed nor initiation density were modified. In addition, the ability of these cells to activate latent replication origins following partial inhibition of DNA synthesis was not compromised. As Mcm4$^{Chaos3/Chaos3}$ cells exhibit a higher frequency of chromosome breaks following treatment with DNA replication inhibitors, we reasoned that Mcm4^{Chaos3} mutation could specifically impact loci such as CFS. We have established the pattern of CFS in MEFs, and are now investigating if CFS are more prone to breakage in Mcm4$^{Chaos3/Chaos3}$ cells.

(1) Letessier et al. Nature (2011); (2) Shima et al. Nat Genet (2007).

TOBACCO SINGLE-STRAND BINDING PROTEIN GTBP1 PROTECTS TELOMERE FROM IMPROPER INTERCHROMOSOMAL HOMOLOGOUS RECOMBINATION.

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Extreme ends of linear eukaryotic chromosomes are maintained by array of DNA-protein complexes called telomeres. Sequence specific telomeric DNA binding protein complex caps telomeres, distinguishing them from DNA damage sites. Here, we studied a single-strand specific telomere binding protein NtGTBP1 in tobacco, human HnRNP A1 homolog. In gel retardation assays, NtGTBP1 bound specifically to plant single-strand telomere repeats (TTTAGGG). CHIP assays showed that NtGTBP1 was co-immunoprecipitated with telomere repeats in tobacco suspension culture, indicating telomere association of NtGTBP1. We generated RNAi-mediated NtGTBP1 suppressed transgenic tobacco plants. The knock-down plants had significantly smaller leaves and shorter stems than wild type plants and these phenotypes were gradually intensified during plant growth. Telomeres of NtGTBP1-RNAi plants were lengthened during plant growth, matched to increasing severity of abnormal phenotypes. This suggests that NtGTBP1 participates in telomere length homeostasis. Extrachromosomal telomeric DNA circles, which are detected in alternative lengthening of telomeres (ALT)-positive cancer cells, are found in NtGTBP1-RNAi plants by 2-dimensional pulse-field gel electrophoresis. Because NtGTBP1 blocks stand invasion of single stranded telomere repeats to the double stranded repeats, telomere lengthening in NtGTBP1-RNAi plants may result from the single stand invasion and replication between telomeres. Moreover, like other telomere related mutant plants, we observed abundant formation of anaphase bridges in NtGTBP1-RNAi pollen mother cells. These results propose that NtGTBP1 functions as an inhibitor of misplaced homologous recombination between telomeres in tobacco.

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CELL CYCLE-DEPENDENT PHOSPHORYLATION OF ORC2 DISSOCIATES ORIGIN RECOGNITION COMPLEX FROM CHROMATIN AND REPLICATION ORIGINS

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The origin recognition complex (ORC) bound to the replication origin leads to the assembly of the pre-replicative complex (pre-RC) for subsequent initiation of eukaryotic chromosome replication. We demonstrated that the cell cycle-dependent of human Orc2, one of the six subunits of ORC, during the S phase dissociates ORC from chromatin. Consistently, the phospho-mimetic Orc2 protein exhibits defects in the bindings to replication origins as well as chromatin, whereas the phospho-defective protein persisted in the bindings throughout the cell cycle. These results suggest that the phosphorylation of Orc2 inhibits not only dissociates ORC from replication origins, but also binding of ORC to newly replicated DNA. Since the function of ORC precedes other proteins in the initiation of chromosome replication, controlling ORC by the phosphorylation of Orc2 will contribute to the regulation of chromosome replication initiation.

GENOME-WIDE MAPPING OF ACTIVE REPLICATION ORIGINS USING DEEP SEQUENCING REVEALS THE EXISTENCE OF A DNA MOTIF TO DEFINE ORIGIN LOCATIONS

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Mechanisms implicated in replication origins selection in Metazoans are still elusive due to the absence of consensus DNA sequence to define their position, despite recent initiatives on 1% of the genome covered by the ENCODE regions. To extend this study genome-wide, we coupled the purification of short nascent strands (SNS) at replication origins to highly sensitive latest development of next-generation sequencing. It allowed us to map active replication origins genome-wide in four different human cell lines : Hela cells, IMR90 fibroblasts, human embryonic stem cells (hESC) and induced pluripotent stem cells (iPSC). We discovered using highly deep sequencing, an unprecedented high number of potential replication origin positions, heterogeneously distributed, conserved in different cell types, exhibiting a cell type specific variability in efficiency. Origin density is globally correlated to the G:C content, the replication timing but not to the chromosome size, confirming their non-random distribution. Moreover, a comparative analysis of inter-origin distances measured by DNA molecular combing indicates that only a subset of potential origins is activated each cell cycle, demonstrating a great flexibility in origin selection. Finally, we identified a major consensus DNA motif to predict their positions, which unravels the percieved importance of DNA structures in the regulation of replication initiation.

ANALYSIS OF DNA REPLICATION IN HUMAN CELLS UPON KNOCKDOWN OF LICENSING AND INITIATION FACTORS

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Chromosome replication occurs precisely once during the cell cycle. The regulation of licensing, activation and firing of the origins scattered along the genome is crucial for maintaining genomic stability. Incomplete replication, as well as re-replication leads to double strand breaks, a hallmark for cancer.
The aim of this work was to understand how the cells regulate early stages of DNA replication and respond to its alterations.
We treated two human cancer cell lines (MDA-MB-231 and HCT116) and one non transformed cell line (MCF10A) with siRNAs targeting either one component of the pre-replication complex (Cdc6), the origin activator Cdc7, and the pre-initiation complex component Cdc45, also known as “limiting factor” for replication initiation.
We monitored the cellular responses applying the following methods:
1) Alteration of cell cycle profile and BrdU incorporation by FACS analysis
2) G1/S transition and the activation of DNA damage by western blot, immunofluorescence and Comet assay
3) Alteration of DNA replication pattern by molecular combing (single DNA molecule analyses).
We find striking differences in the way the DNA replication machinery acts after depleting the different replication factors. In particular, non-transformed cells appear to detect the misregulation of licensing/initiation and arrest in G1 before engaging S-phase. On the contrary, cancer cells initiate S-phase and do not seem to detect the alterations until much later, and die 5 days later.
Our studies also revealed a distinct response of the cancer cells to the block of origin licensing (siCdc6), activation (siCdc7) or firing (siCdc45) steps. In all the cases, replication patterns were grossly altered, and a mutual regulation of fork progression and inter origin distances was observed. In particular, when Cdc45 was silenced, we observe replication fork slowdown and overall reduction in the DNA synthesis but, in parallel, also activation of dormant origins within replication clusters consisting on average of 3-6 origins 35 kb far apart from each others.
These observations shed light on the mechanisms that regulate the early stages of DNA replication and the different ways human cells are able to respond to the alterations of this essential step of their cycle.

INVESTIGATION OF SIGNALING PATHWAYS INVOLVED IN DEGRADATION OF DNA POLYMERASE Δ SUBUNITS IN RESPONSE TO DNA DAMAGE

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DNA polymerase δ (Pol δ) has been intensively studied as a key enzyme in eukaryotic DNA replication. However, much less is known of how its activity may be modulated in response to different cellular states such as during proliferation or DNA damage. As yet, the only mechanism uncovered for the modulation of mammalian Pol δ is the alteration of its quaternary structure in response to DNA damage by the degradation of the p12 subunit. This converts the Pol δ heterotetramer to a trimer, Pol δ3, that lacks the p12 subunit. Our laboratory reported that p12 is rapidly degraded in response to UV, the alkylating agent methyl methanesulfonate (MMS), and replication stress induced by hydroxyurea and aphidicolin (1). In addition, analysis of Pol δ3 has shown that it exhibits altered properties – increased capacity for proofreading, and reduced tendency for bypass of DNA lesions – that are consonant with a function in the DNA damage response (2,3). However, an understanding of signaling processes involved in initiating the degradation of p12 and the generality of this response to different types of DNA damage is still incompletely understood. Here, we report preliminary findings in regard to the range of DNA damage that elicits p12 degradation and the signaling pathways that are required for this process to occur.

One of the major sources of DNA damage is oxidative stress, whether endogenous or exogenous. We found that p12 is degraded during oxidative stress elicited by H_2O_2. Other alkylating agents such MNNG were also able to lead to degradation of p12. We had previously shown that UV-C (280 nm) efficiently elicited the degradation of p12 in an ATR dependent but ATM independent manner. We tested UV at longer wavelengths, and found that UVB and UVA also elicited the p12 response.

The other major form of DNA damage in addition to base modification is double stranded DNA breaks, which can be generated by exposure to IR and by chemical agents including topoisomerase inhibitors and neocarzinostatin. We found that IR was able to elicit p12 degradation at high levels of exposure, in an ATM independent process, but not at lower levels. Neocarzinostatin, a radiomimetic agent which generates increased oxidative stress, also was able to induce p12 degradation in an ATM independent manner. From these results we propose that p12 degradation is triggered by ATR, but not ATM. The degradation observed with neocarzinostatin and IR is most likely triggered by activation of ATR, or possibly by the p38MAPK signaling pathway.

MOLECULAR SNAPSHOTS OF THE PROTEIN MACHINES AT EUKARYOTIC DNA REPLICATION ORIGIN

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The initiation of DNA replication in eukaryotic cell is a multi-step process involving several protein complexes. In S. cerevisiae, the first step is the ATP-dependent binding of ORC, a six-protein Origin Recognition Complex, to the origins of DNA replication. Three additional replication factors are recruited: first Cdc6, then Cdt1, and finally another six-protein helicase core complex MCM2-7. Sequential ATP hydrolysis by Cdc6 and ORC loads MCM2-7 onto DNA as a head-to-head double-hexamer. Genetic and biochemical studies have shown that these fourteen proteins are necessary and sufficient for Pre-RC (Pre-Replication Complex) assembly and helicase loading.

We previously reported the overall architecture of ORC and ORC-Cdc6 based on negative stain TEM analyses. We have now obtained molecular snapshots of several purified protein complexes by using cryo-EM and single particle 3D image reconstruction. These include the S. cerevisiae ORC bound onto the ARS1 origin DNA, ORC in complex with the replication initiation factor Cdc6, the Pre-RC complex composed of ORC-Cdc6-DNA-Cdt1-MCM2-7, and the MCM2-7 double hexamer that is loaded onto the dsDNA. In our presentation, we will describe the structures of these complexes, and the potential insights into the molecular mechanism of eukaryotic DNA replication initiation.

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YEAST FACT COMPLEX PREVENTS TURNOVER OF NUCLEOSOMAL H3-H4

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Nucleosome, the fundamental repeating unit of chromatin, consists of two H2A-H2B dimers and one $(H3\text{-}H4)_2$ tetramer. Once assembled into nucleosomes, $(H3\text{-}H4)_2$ tetramers are relatively free from turnover compared to H2A-H2B dimers. This stability is hypothesized to be important for the maintenance of chromatin integrity and propagating the epigenetic memory. However, factors involved in preventing the histone H3-H4 turnover remain largely unknown. Yeast FACT complex, containing two essential subunits Spt16 and Pob3, functions in both DNA replication and gene transcription. Here we show that in Saccharomyces cerevisiae compromising the function of either Spt16 or Pob3 results in a dramatic increase of newly synthesized H3 marked by acetylation of histone H3 lysine 56 (H3K56Ac) on chromatin. Moreover, the accumulation of H3K56Ac on chromatin is independent of the cell cycle. Remarkably, we observed a global loss of nucleosome positioning upon inactivation of Spt16. Interestingly, the alterations in nucleosome positioning were mainly detected at gene bodies, but not at the +1/-1 nucleosomes surrounding transcriptional starting sites. Finally, we found that the deposition of H3K56Ac onto chromatin upon Spt16 inactivation is partially mediated by histone chaperone Rtt106. Therefore, we propose that FACT plays an important role in stabilizing the H3-H4 in a nucleosome and maintains chromatin integrity.

R LOOP-MEDIATED GENOMIC INSTABILITY IS CAUSED BY IMPAIRMENT OF REPLICATION FORK PROGRESSION

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Transcriptional R loops are anomalous RNA:DNA hybrids that have been detected in organisms from bacteria to man. These structures have been shown in eukaryotes to result in DNA damage and rearrangements; however, the mechanism(s) underlying these effects has (have) remained largely unknown.

In this study, we first present evidence that transcription through a fragment of the mouse Immunoglobulin (Ig) gene Sg3 region, in its physiological orientation, results in R loop-mediated DNA rearrangement and recombination in E. coli, indicating that transcriptional R-loop formation is a conserved threat to genome integrity throughout evolution. More importantly, we then show, by analysis of the pattern of replication intermediates, that R-loop formation impedes replication fork progression. Furthermore, we find that the impact of R loops on genome stability is in fact dependent on the presence of active replication, both in E. coli and in SRSF1-depleted HeLa cells. Our findings thus provide a direct demonstration that R-loop formation impairs DNA replication, and that this is responsible for the deleterious effects of R loops on genome stability, from bacteria to man.

It has been known for some time that G-rich regions in nascent transcripts favor R-loop formation due to the exceptional stability of rG:dC base pairs. Although stable R loops have only been detected under physiological conditions in switch regions of Ig genes, which indeed produce G-rich transcripts, there are of course numerous G-rich motifs with various densities and lengths spread throughout human genome. The findings that cotranscriptional R-loop formation poses a significant barrier to DNA replication and genome stability highlight the importance of understanding in more detail the molecular determinants and pathological consequences of transcriptional R-loop formation, including its possible role in human disease.

AND-1 IS REQUIRED FOR THE STABILITY OF HISTONE ACETYLTRANSFERASE GCN5

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Histone acetyltransferases (HATs) play a central role in the modification of chromatin and gene transcription as well as in the pathogenesis of a broad set of diseases including cancers. Gcn5 is the first identified transcription-related histone acetyltransferase (HAT) that has been implicated in the regulation of diverse cellular functions. However, how Gcn5 proteins are regulated remains largely unknown. Here we show that And-1, a component of replisome, has the remarkable capability to regulate the stability of Gcn5 proteins and thereby histone H3 acetylation. We find that And-1 forms a complex with both histone H3 and Gcn5. Downregulation of And-1 results in Gcn5 degradation, leading to the reduction of H3K9 and H3K56 acetylation. And-1 overexpression stabilizes Gcn5 through protein-protein interactions in vivo. Furthermore, And-1 expression is increased in multiple cancer cells in a manner correlating with increased Gcn5 and H3K9Ac and H3K56Ac. Thus, our data reveal not only a functional link between Gcn5 and And-1 that is essential for Gcn5 protein stability and histone H3 acetylation, but also a potential role of And-1 in cancer. We will present the mechanistic insight into how And-1 regulates the stability of Gcn5 proteins in cancer cells.

HIGH THROUGHPUT, HIGH-RESOLUTION MAPPING AND CHARACTERIZATION OF AUTONOMOUSLY REPLICATING SEQUENCES IN DIVERSE BUDDING YEASTS

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DNA replication initiates at loci termed origins of replication. In yeast, replication origins are short A/T-rich sequence elements (ARSs) that can initiate autonomous replication of plasmids. ARSs have been most well studied in S. cerevisiae where a motif called the ACS is necessary but not sufficient for ARS function. Because chromosomal origins are redundant in eukaryotes, we proposed that over evolutionary time, sequences and locations of ARS have diverged. Since origin function in other budding yeasts remains largely uncharacterized, we have developed tools for rapidly isolating and characterizing large numbers of ARSs and have applied our methodology to a number of budding yeast species (spanning ~500 million years of evolution) including S. cerevisiae, S. bayanus, S. castellii, S. kluyveri, K. lactis, and P. pastoris.

Combining the classic ARS assay with Ilumina sequencing we have developed several methods that allow us to quickly and comprehensively map ARSs (ARS-seq), to determine their ACS (miniARS-seq, mutARS-seq), and to identify ACS-accessory sequences (flankARS-seq). By applying these new assays to S. cerevisiae we have refined the ACS motif, confirmed more than 60 new ARSs, and have performed a genome wide screen for sequences that can function as B-elements.

Applying our high-throuhput assays to other budding yeast species has uncovered a striking diversity of ARS sequences and genomic distributions. S. bayanus shares ARS sequences essentially identical to S. cerevisiae but has a higher density of functional ARSs in its genome. S. castellii and S. kluyveri have permissive mechanisms for ARS selection using degenerate A/T rich sequences resembling the S. cerevisiae ACS. K. lactis has significantly fewer ARSs/kb in the genome and uses an ACS motif dissimilar from all other species. Industrial yeast P. pastoris has approximately the same ARS distribution as S. cerevisiae, but a GC-rich motif.

In addition to mapping ARSs in their native genomes, using cross-species screening and manual validation we have identified foreign DNA sequences that have ARS function in each of the host species. Some species (S. cerevisiae and S. bayanus) can use each others' ARSs interchangeably. On the other hand, K. lactis ARSs do not function in other species (and vice versa) suggesting the trans-factors that recognize origins have also diverged. Some species show ARS selection promiscuity: while over half of S. cerevisiae ARSs function in P. pastoris, PpARSs do not function in S. cerevisiae. Our findings bring us closer to a complete understanding of the molecular determinants of ARS function and provide interesting insight on the evolution of replication origins and their distribution in yeast genomes.

ASYMMETRY IN THE ENZYMATIC ACTIVITIES INVOLVED IN POST-REPLICATIVE DNA REPAIR

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The DNA mismatch repair (MMR) system repairs replication errors, with an efficiency that depends on the composition of the mismatch and the surrounding sequence context. MMR efficiency may also depend on how and where the replication error was made; e.g., lagging strand replication errors appear to be more efficiently repaired when made by DNA polymerase α near the 5′ end of Okazaki fragments as compared to more 'internal' errors made by pol δ. To further understand the relationship between MMR efficiencies and the asymmetry of the replication machinery, we are investigating the nucleolytic activities involved in MMR in budding yeast. This is done using mutator strains for pols α, δ, and ε, in order to infer which polymerase generates a particular replication error. These strains are being used to study the involvement of three nucleases that have previously been implicated in MMR, Exonuclease 1, a 5′-3′ exonuclease, Rad27, a nuclease with 5′-3′ exonuclease and endonuclease activity that functions in Okazaki fragment maturation, and the MutLα heterodimer, whose Pms1 subunit has an endonuclease activity that is essential for MMR. Work in progress will be presented, including an initial result demonstrating that deleting Exo1 increases the spontaneous mutation rate of a pol α mutator strain to a significantly greater extent than does deleting Exo1 in pol δ or pol ε mutator strains. Thus, like DNA replication, MMR enzymology is also functionally asymmetic.

CRYSTAL STRUCTURE OF HUMAN MONO-UBIQUITINATED PCNA: IMPLICATIONS FOR THE MODULATION OF FEN1 ACTIVITY AND OKAZAKI FRAGMENT MATURATION AS A CONSEQUENCE OF DNA DAMAGE.

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The mono-ubiquitination of PCNA coordinates the DNA damage response, a process of DNA damage avoidance that utilizes TLS polymerases to bypass bulky lesions that block DNA replication. PCNA ubiquitination initiates the switch from the replication polymerase (Pol δ) to the translesion polymerases. In order to understand how mono-ubiquitination of PCNA might affect its transactions with its protein partners, particularly those in DNA replication, we have determined the three dimensional structure of mono-ubiquitinated human PCNA. We solved the three-dimensional structure of ub-PCNA and refined it to a nominal resolution of 2.9 Å. There is one trimeric ring in each asymmetric unit with two ubiquitin molecules modeled in. The overall structure displays overall high temperature factors (B factor) that implies structural high flexibility. The ubiquitin molecules are radially extended away from the trimeric ring of PCNA with a bend towards the back face of the ring. In fact, there is no other contact between ubiquitin and PCNA aside from the isopeptide linkage. Therefore, mono-ubiquitination of PCNA would not be expected to obstruct the binding of proteins through the PIP box binding site or the interdomain connecting loop (IDCL) regions. This minimal interaction between PCNA and ubiquitin could also imply that ubiquitin can recruit interacting proteins even when other proteins are bound through the PIP box interactions. On the other hand, the binding of molecules adopting conformations to the side of the trimeric ring could be sterically hindered because of the radial extension of the ubiquitin. The Fen1-PCNA complex (1UL1) has revealed three conformations of Fen1 and these interconversions are essential for its activity. When we superimposed the structure of ub-PCNA onto the Fen-1/PCNA structure, we observed that the ubiquitin molecules interfere with one of the conformations of Fen1. We therefore initiated studies on the effects of ub-PCNA on Okazaki fragment processing by the combined actions of Pol δ /Fen1/PCNA on model primer/templates. Consistent with our structural analysis, we demonstrate that ub-PCNA does not stimulate Fen1 cleavage of the flap in our model system for Okazaki fragment maturation, in contrast to unmodified PCNA.

In conclusion, our studies demonstrate that the mono-ubiquitination of PCNA is not only essential for the recruitment of TLS polymerases but may also result in the stalling of lagging strand synthesis and thus replication fork progression.

VISUALIZATION OF EUKARYOTIC DNA REPLICATION USING PHADE, A NOVEL SINGLE-MOLECULE IMAGING APPROACH

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We introduce a novel, single-molecule imaging approach, PhADE (PhotoActivation, Diffusion, and Excitation) that is compatible with micromolar concentrations of fluorescent protein. A protein of interest is labeled with a photoactivatable fluorophore and is allowed to bind to a surface-immobilized substrate. After local photoactivation, rapid diffusion of unbound molecules out of the detection volume eliminates background fluorescence, whereupon the substrate-bound protein is excited for imaging. We used PhADE to dynamically visualize replication of individual DNA molecules in Xenopus egg extracts. PhADE imaging of the Okazaki-fragment processing enzyme Flap endonuclease 1 (Fen1) allowed dynamic tracking of the initiation and growth of replication bubbles over time revealing an unexpectedly high initiation density. Furthermore, imaging of individual Fen1 molecules on replicating DNA suggests that Fen1 is recruited via cooperative interactions with PCNA and DNA. Given its ability to image single molecules at physiological protein concentrations, PhADE represents a powerful tool to elucidate the dynamics of complex biochemical reactions.

CHARACTERIZATION OF HUMAN ORC6 PROTEIN

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The origin recognition complex (ORC) plays a central role in origin selection and pre-RC assembly in eukaryotic cells. Orc6 protein is the smallest and least conserved of all ORC subunits. The functions and regulation of Orc6 protein vary significantly in different organisms. In S. cerevisiae, Orc6 is not important for ORC-DNA association, but it is required for cell viability and the maintenance of pre-RC. In Drosophila, Orc6 is a DNA binding protein and is essential for ORC-dependent DNA binding and DNA replication. In Xenopus and human Orc6 is less tightly associated with other ORC subunits, and some of the published data suggest that Orc6 may not be important for DNA binding and replication.
In this study, we characterized the functions of HsOrc6 in DNA binding and DNA replication using recombinant HsOrc6 and HsORC proteins. In vitro DNA binding assays showed that HsORC complex containing HsOrc6 demonstrates higher DNA binding affinity than the complex without HsOrc6. We also found that HsOrc6 enhanced the salt resistance of HsORC-DNA interaction. Interestingly, in vivo depletion of HsOrc6 significantly changed the phosphorylation state of HsMcm2 protein. The phosphorylation states of HsCdc45 and HsCdc7 were also decreased in HsOrc6 depleted cells, leading us to examine whether a connection between HsOrc6 and HsCdc7 exists. A weak but direct interaction between these two proteins was identified by co-immunoprecipetation in both cellular extract and purified proteins. Moreover, HsOrc6 enhanced HsCdc7/Dbf4 kinase activity in in virto assays, strongly suggesting that HsOrc6 is a regulator of the important S phase kinase HsCdc7.

SEARCHING FOR SPECIFICITY: CHROMATIN SIGNATURES THAT DEFINE REPLICATION ORIGINS IN METAZOA.

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Metazoan origins of replication, unlike those of yeast and bacteria, lack a consensus sequence that can be recognized by the Origin Recognition Complex (ORC). Despite this apparent lack of sequence specificity, ORC still binds, and replication initiates, at specific chromosomal locations. Instead of sequence specificity, metazoan origin selection may be regulated by the local chromatin structure and environment. Chromatin structure is extremely versatile and may confer both the specificity and flexibility required to coordinate origin selection with developmental and tissue specific transcription programs. To better understand the chromosomal features that direct ORC localization, we identified approximatey 5000 ORC binding sites throughout the Drosophila genome and analyzed these sites in the context of almost a thousand genomic datasets produced by the modENCODE (model organism encyclopedia of DNA elements) consortium. We did not identify a simple consensus sequence for ORC binding but rather found that primary sequence, chromatin modfications and chromatin associated proteins exhibit an additive effect on our ability to predict and classify ORC binding sites. We have selected several chromatin associated proteins (e.g. GAGA factor (GAF)) and chromatin marks (e.g. H3K36 methylation), that have shown a strong positive or negative correlation with ORC binding sites and/or replication timing for further analysis. We are surveying the direct impact of these factors on the Drosophila replication program by using RNAi to knockdown the relevant chromatin associated proteins or chromatin modifying enzymes and generating replication timing profiles by high-throughput sequencing. Our preliminary data indicate that the replication program is quite robust and exhibits little change in response to RNAi depletion of specific factors. Together these data suggest that there is likely redundancy in the chromatin factors that regulate origin selection and activation.

COMPARING REPLICATION DYNAMICS, GENOMIC INSTABILITY, AND CHROMATIN STRUCTURE IN NORMAL AND CANCER CELLS

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Previous studies suggest that chromatin structure influence replication dynamics, and that these parameters are deregulated in cancer cells. Furthermore, common fragile sites (CFSs), which represent highly unstable regions of the genome, manifest DNA replication stress and genomic instability in the very early steps of cancer development in mammalian cells. To advance our understanding of the role of chromatin structure, DNA replication, and genomic instability in the pathogenesis of human tumors, we are comparing origin usage and chromatin structure within CFS and non-fragile regions (NCFSs) in non-malignant cells and cancer cells. For this purpose, we chose to compare a non-malignant EBV-transformed mature B cell line (control) and two malignant, mature B cell lines derived from multiple myeloma, the NTKO parental line, over-expressing the histone methylase, MMSET (Multiple Myeloma SET domain), and TKO, an MMSET knock-out cell line derived from NTKO. The overexpression of MMSET in the NTKO cell line leads to a global increase in H3K36 methylation and global decrease in H3K27 methylation, suggesting a more open structural state of the chromatin.
We mapped origins of replication, using a microarray-based assay (Lucas et al., EMBO Rep. 8: 770, 2007), within six CFSs, FRA3B, FRA16D, FRA7G, FRAXB, FRA2G, and FRA7H, and their surrounding NCFSs in the control cell line. Under basal conditions, we found that origin usage within CFSs is comparable to that of flanking NCFSs, with the exception of the two most highly-expressed CFSs, FRA3B and FRA16D. Following replication stress (low doses of aphidicolin, APH), there is a decrease in origin usage for FRA3B and FRA16D. In contrast, increasing the chromatin acetylation during replication stress increased the origin usage for these CFSs. We also showed that APH delays the replication timing of FRA3B and FRA16D, and that increasing histone acetylation alleviates this delay. Overall, these results indicate a link between the origin replication defect detected in APH-treated cells, the level of histone acetylation, and the level of instability observed at some CFSs. We have now expanded our microarray design and are analyzing the origin mapping results obtained for the control, TKO, and NTKO cell lines under basal conditions. We are also evaluating the effect of MMSET overexpression, by comparing CFS breakage levels in the three cell lines. We will present the results of our expanded origin mapping study and the correlation of these results with chromatin structure.

MISMATCH REPAIR OF LEADING AND LAGGING STRAND DNA REPLICATION ERRORS

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Mismatch repair (MMR) corrects mismatches made in *S. cerevisiae* by variants of DNA polymerases α and δ. We now describe a similar analysis for errors made by the M644G mutator variant of DNA polymerase ε. Pol ε M644G preferentially incorporates rNMPs into the nascent leading strand but not into the nascent lagging strand, providing physical evidence to strongly support previous genetic studies implying that Pol ε is the major leading strand replicase in *S. cerevisiae*. Comparison of the mutation rates of MMR-proficient *pol2-M644G* strains with the rates of MMR-deficient *pol2-M644G msh2*Δ strains indicates that the vast majority of leading strand single base mismatches are corrected by MMR, indels moreso than transitions moreso than transversions. The MMR efficiency is high and similar for errors made by proofreading-proficient Pols ε and δ. Errors made by the less accurate and proofreading-deficient Pol α, all located near to the 5′ ends of Okazaki fragments, are corrected with even greater efficiency. Collectively, MMR correction factors suggest that on average, natural single base mismatches made during leading and lagging strand replication are repaired about equally well. Nonetheless, MMR efficiency varies from site to site, even for the same type of mismatch, with the extreme case of a T-T mismatch made by Pol ε that largely escapes MMR. This mismatch is flanked by a triplet repeat sequence that, when interrupted, partially restores MMR *in vivo*. This identifies a base pair in the yeast genome that has an up to 10,000 fold greater risk of mutation due to a triplet repeat contextual inefficiency of the wild type MMR machinery.

KNOCK-OUT MOUSE MODELS OF MCM8 AND MCM9 REVEAL A MCM8/MCM9 COMPLEX ESSENTIAL FOR FERTILITY AND GENOME STABILITY

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MCM8 and MCM9 belong to the MCM2-9 family of helicases that are involved in DNA replication. Whereas the first six family members, the MCM2-7 proteins, are well characterized as the main replicative helicase during S-phase, the functions of the more recently discovered MCM8 and MCM9 proteins are much less understood.
Using the in vitro Xenopus egg extract system, we previously showed that MCM8 and MCM9 were involved in DNA replication. We have now created mouse models for both proteins to elucidate their functions in vivo and ex vivo more comprehensively. The phenotypes of MCM8- and MCM9-deficient mice show intriguing links between DNA replication, DNA repair and recombination.
Both MCM8- and MCM9 knock-out mice are viable, but sterile. Their gonads show severe proliferation defects and do not produce functional oocytes or spermatozoa due to recombination defects (although MCM9-deficient males can produce a low number of functional spermatozoa).
Both MCM8- and MCM9-deficient MEF cells also have severe proliferation defects. Knock-out cells are defective in replication fork maintenance, dormant origin activation and are sensitive even to mild fork blocking conditions. In the absence of exogenous impairment, MCM8-and MCM9-deficient cells also show a genetic instability unraveled by a high number of micronuclei, chromosomes breaks and a tendency of the cells to immortalize and transform. The similar phenotypes of both knock-outs is emphasized by the finding that MCM8 and MCM9 form a complex in vivo and that both proteins are coregulated and stabilize each other.
Our studies reveal a crucial role of MCM8 and MCM9 in the regulation of homologous recombination, replication fork maintenance and genome stability.

IDAS BINDING TO GEMININ REGULATES TIMELY REPLICATION

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Geminin is a central cell cycle regulator in metazoa, which safe-guards once per cell cycle replication by directly binding and inhibiting the DNA replication licensing factor Cdt1.

We have identified Idas as a previously uncharacterized human protein which possesses a coiled-coil domain homologous to the Geminin coiled-coil. Idas is found in vertebrates and its closest paralogue is GemC1, recenty shown to interact with TopBP1 and to be required for Cdc45 chromatin loading and S-phase onset. We show that Idas directly binds to Geminin through coiled-coil mediated interactions. Idas binding to Geminin inhibits Geminin-Cdt1 interactions both *in vitro* and in cells. The structure of a binary complex between Geminin and Idas suggests how the substitution of a Geminin molecule in the native Geminin homoduplex prevents Cdt1 binding. Indeed, Idas binding to Geminin relieves Geminin-mediated licensing inhibition, as shown in the Xenopus *in vitro* licensing system. Idas over-expression and depletion experiments in cultured human cells support a role for Idas in regulating timely replication through antagonistic interactions with Geminin. Our data suggest that competing interactions between Idas, Geminin and Cdt1 may provide a switch during DNA replication.

Our analysis highlights the presence of a Geminin superfamily in vertebrates, whose balanced interactions regulate timely replication.

GEMININ REGULATES SELF-RENEWAL AND DIFFERENTIATION DECISIONS OF NEURAL PROGENITOR CELLS IN THE BRAIN

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Cortical neural progenitor cells have the ability to generate the complex array of neurons comprising the adult cortex while maintaining their undifferentiated state. There are three distinct populations of cortical neural progenitors arising in an orderly fashion. Neuroepithelial cells (NE) comprise the earliest cortical progenitor cell population which undergoes symmetric divisions prior to the onset of neurogenesis, while they switch to mostly asymmetric divisions at the onset of neurogenesis in order to give rise to the earliest-born neurons of the cerebral cortex and to the second class of cortical progenitor cell population, the radial glia cells (RGCs). NE and RGCs occupy the apical layer of the neuroepithelium forming the ventricular zone. The third class of cortical progenitors, named basal progenitors, are produced by the asymmetric divisions of radial glia cells and reside in a distinct cell layer called subventricular zone. Radial glia and basal progenitor cells are responsible for the generation of the majority of cortical neurons. Neural progenitor cells should therefore regulate decisions for self-renewing and differentiating divisions in order to give rise to the appropriate cell number that will comprise the cortex.
It has been suggested that Geminin regulates decisions between proliferation and differentiation, through interactions with cell cycle regulators, chromatin remodeling complexes and transcriptional factors. At early stages of nervous system development, Geminin has been suggested to promote neural cell fate acquisition, while at later stages it promotes neuronal differentiation.
To elucidate the mechanisms regulating self-renewal and differentiation of cortical progenitors, we have generated mice lacking Geminin expression in the developing cortex. Our data show that in the absence of Geminin a delay in the generation of radial glia cells is observed. Moreover, early cortical progenitor cells show a preference towards self-renewing divisions and present increased S phase length. These altered features of early cortical progenitors resulted in the impaired production of early born cortical neurons. Moreover, Geminin overexpression in cortical progenitor cells reduces cortical progenitor cell numbers by promoting cell cycle exit and neuronal differentiation. Our data suggest that Geminin regulates cortical progenitor cells decision between self-renewal and differentiation.

P73 REGULATES AN ONCOGENIC NETWORK OF DNA REPLICATION GENES

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The tumor suppressor p53 is often mutated in cancer, while the related family members p63 and p73 are not. Instead, p73 is often overexpressed and this correlates with increased tumor aggressiveness and therapy resistance leading to an inferior patient outcome. While non-tumor cells predominantly express the p53-like tumor suppressive isoform of p73 (TAp73), cancer cells up-regulate a shorter inhibitory isoform (ΔNp73), which impairs p53 and TAp73 function and in addition exerts oncogenic activity. To develop more efficient cancer therapies it is therefore of great interest to identify the mechanisms underlying ΔNp73's oncogenic functions.

ChIP sequencing has revealed that ΔNp73 and p53 have many target genes in common and it could be confirmed that ΔNp73 functions as a global repressor of the p53 response. In addition, ΔNp73 directly binds and activates a transcriptional network of genes that is clinically correlated with poor patient survival and resistance to S phase targeting drugs. This target gene network is strongly enriched in DNA replication genes and, consistently, ΔNp73 overexpression increases chromatin-bound levels of several DNA replication factors. Vice versa, knockdown of endogenous p73 impairs tumor cell proliferation and renders cells hypersensitive to S phase targeting drugs. Importantly, p73-mediated expression of replication genes appears essential for the activation of dormant replication origins in response to replication stress. These results suggest that high-level expression of p73 in cancer renders tumor cells more tolerant to replication stress induced by oncogenes or chemotherapy. Together this provides an intriguing explanation why p73 is frequently found overexpressed in cancer patients.

AN *IN VITRO* SYSTEM TO STUDY REPLISOME-SPECIFIC UBIQUITYLATION OF THE MCM2-7 HELICASE

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A variety of E3 ubiquitin ligases play an important role in preserving genome integrity during chromosome replication in eukaryotic cells, but for several of these enzymes the substrates remain to be identified. By isolating replisome material from replication forks in yeast cell extracts, under conditions that inhibit the activity of ubiquitin proteases, we have found that a specific subunit of the MCM2-7 helicase is modified by ubiquitylation. The modification is very efficient within the replisome, but the bulk population of the MCM2-7 helicase away from the replisome remains unmodified. Moreover, other replisome subunits do not appear to be modified in the same way. We have found that the ubiquitylation reaction can occur in the cell extract, providing an in vitro system with which to study the underlying mechanism. We have now identified the responsible E3 and E2 enzymes, and determined the basis for the replisome-specificity of the reaction. Currently we are mapping the sites of modification so that we can generate mutations and examine the physiological consequences for the regulation of chromosome replication. Current progress with this work will be presented.

THE SPATIO-TEMPORAL REPLICATION PROGRAM CHANGES AT THE MID-BLASTULA TRANSITION IN *XENOPUS LAEVIS* EMBRYOS

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Eukaryotic DNA replication requires a strict control in space and time during S phase in order to maintain genome stability. In higher eukaryotes the DNA replication starts from several thousand replication origins, organized in replication clusters, which are activated at different time during the S phase. The spatio-temporal replication program changes during development but underlying mechanism are only poorly understood. In *Xenopus*, early embryonic development generally starts with rapid cell divisions driven by maternally derived products and exhibits little zygotic transcription. Since there is no growth during these early rapid stages, the ratio of nuclei to cytoplasm (nuclear-cytoplasmic ratio N/C) increases progressively. At the mid-blastula transition (MBT), the gap phases of the cell cycle are introduced, S phase becomes longer and widespread zygotic transcription initiates. Several lines of evidence suggested that titration of a maternally deposited replication factor by nuclear components triggers the MBT. Using the *Xenopus in vitro* replication system, we have shown that origins are asynchronously activated throughout S phase. Increasing the N/C ratio *in vitro* mainly changes the temporal replication program in sperm nuclei replicating in egg extracts and thereby increasing S phase length. Now, we have analysed the changes of the replication program on whole genome sequences in *Xenopus* embryos at the MBT by molecular combing of DNA. Our results suggest that the length of S phase increases in *Xenopus* embryos at the MBT by increasing both the mean origin distances and by modulating the temporal program.

DYNAMICS OF THE ORIGIN RECOGNITION COMPLEX IN VERTEBRATES

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DNA replication initiates at specific locations along the genome at sites termed origins of replication. How exactly these origins are defined is not yet well understood in higher eukaryotes. Temporal and spatial programs select replication start sites and control their timing of activation during the S phase. An increase or a decrease in the number of origins may generate genetic instability. ORC (Origin recognition complex) binding is the first step in the formation of the pre-RC (pre-replication complex). Pre-RC assembly occurs in G1 and is required prior to origin activation. The key point is to determine if all these assembled pre-RCs will be activated during the following S phase. One hypothesis sustains that only a fraction of the pre-RCs are used during a normal S phase. The excess of pre-RCs may be useful in case of replicative stress to prevent genomic damages linked to an incomplete replication.

Our goal is the establishment of a genomic map of ORC binding sites and its comparison with the genomic sites of replication initiation in order to ask whether an excess of pre-RC over origins is assembled in G1. We used the outstanding capacity of the avian DT40 cell line to perform homologous recombination to tag the endogenous version of these proteins. This allows us to overcome the problem of over-expressing ORC sub-units and the poor ChIP efficiency of antibodies against ORC subunits. Both the Orc1 and Orc2 subunits were tagged in different cell lines and the different clones grow normally and show no cell cycle perturbation. Chromatin immunoprecipitation (ChIP) experiments have been performed with an anti-tag antibody on asynchronous chicken cells and have shown small (3 to 5 fold) but highly reproducible enrichments of the Orc1 and Orc2 proteins at already known chicken replication origins and more precisely at origins, which are activated very early in S phase.

Our purpose now is to perform ChIP experiments at different time points during the cell cycle with chicken cells synchronised by elutriation. The elutriation technique is based on the separation of cells by their size by centrifugation. This will allow us not only to accurately characterize, with stronger enrichments, ORC binding sites but also to follow during the cell cycle the dynamic of ORC interaction with known replication origins. After all we will answer to the question of the existence of an excess of bound pre-RCs on the genome that constitutes a fail-safe mechanism in case of replicative stress.

THE STRUCTURE OF SACCHAROMYCES CEREVISIAE DBF4 MOTIF N REVEALS A UNIQUE FOLD NECESSARY FOR THE INTERACTION WITH RAD53

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Dbf4 is a conserved eukaryotic protein that functions as the regulatory subunit of the DDK (Dbf4-dependent kinase) complex. DDK plays essential roles in DNA replication initiation and checkpoint activation. During the replication checkpoint, *Saccharomyces cerevisiae* Dbf4 is phosphorylated in a Rad53-dependent manner and this, in turn, inhibits initiation of replication at late origins. We have determined the minimal region of Dbf4 required for the interaction with the checkpoint kinase Rad53 and solved its crystal structure. The core of this fragment of Dbf4 folds as a BRCT domain, but it includes an additional N-terminal α helix that is critical for the interaction with Rad53. The presence of this helix is predicted in lower eukaryotes, though the sequence conservation of this region is low. Our structure also reveals that previously characterized Dbf4 mutants with checkpoint phenotypes destabilize the domain, indicating that its structural integrity is essential for the interaction with Rad53. Collectively, these results allow us to propose a model for the association between Dbf4 and Rad53.

IMPORTANT FUNCTION FOR THE DEAD-BOX PROTEIN DDX5 IN TRANSCRIPTIONAL REGULATION OF DNA REPLICATION AND BREAST 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 against cancer, a disease typified by uncontrolled cell proliferation. To identify new proteins required for DNA replication in human cells we performed a screen for genes that are essential for stable maintenance of an episomal plasmid in a human colorectal tumor cell line. Stable maintenance of this plasmid in cells requires it to be replicated by host cell DNA replication proteins. We confirm this by demonstrating that RNAi knockdown of 14 known replication factors impairs plasmid stability. Results from the screen revealed an essential role for the DEAD-box protein DDX5 in both plasmid stability and cell proliferation prompting us to investigate whether it functions in DNA replication. Indeed, we found that DDX5 interacts with the E2F1 transcription factor at E2F-regulated promoters and is required for expression of DNA replication genes. This is consistent with our observations that DDX5 depletion impairs G1-to-S phase progression, BrdU incorporation, and the loading of DNA replication factors onto chromatin. These results indicate that DDX5 functions in the transcription of E2F-regulated genes and thus contributes toward the regulation of DNA replication. Consistent with this positive role for DDX5 in cell proliferation we have found that the DDX5 locus is frequently amplified and overexpressed in breast cancer. We identified breast cancer cell lines with or without amplification of the DDX5 gene and found that proliferation of breast cancer cell lines with DDX5 amplification are much more sensitive to its depletion than breast cancer cell lines and a breast epithelial cell line lacking amplification of DDX5. Moreover, DDX5 is frequently co-amplified in breast cancer along with the oncogene ERBB2 and combining DDX5 depletion with herceptin mediated ERBB2 inhibition in a DDX5/ERBB2 co-amplified breast cancer cell line results in greater inhibition of cell proliferation than either DDX5 knockdown or herceptin treatment alone. These results demonstrate a novel activity for DDX5 in regulating DNA replication and suggest DDX5 as a potential target of therapy in breast cancer treatment.

IDENTIFYING NEW PHYSIOLOGICAL PARTNERS OF BRCA1

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The detailed molecular mechanism(s) of how BRCA1 exerts its breast and ovarian tumor suppressive function remain a mystery. BRCA1 heterozygous women develop basal like breast cancer (BLC), a disease for which only a few targeted therapies (i.e. PARP1 inhibitors and cisplatinum) are available and the prognosis is poor. Though BRCA1 is not mutated in sporadic breast cancer, pathologically indistinguishable BLCs constitute 15% of sporadic breast cancers. Even at the level of gene expression analysis BRCA1-negative and sporadic BLC tumors cluster together (Sorlie et al. 2001 and 2003). However, the molecular pathways responsible for the similarities to BRCA1-mutant tumors are not known.

BRCA1 is required for cell survival, making analysis of cellular functions by conventional knockdown/out techniques difficult. Therefore we reasoned that a detailed proteomics analysis to identify novel BRCA1 associated proteins could yield (a) valuable new insight into BRCA1 physiological functions (b) new tools to study individual BRCA1-dependent pathways and their respective contributions to BRCA1 tumor suppressor activity and (c) the identities of promising targets for pharmaceutical intervention in BLC.

Historically such an analysis has been difficult to perform since ectopic expression of BRCA1 expression is not well tolerated by cells. Even short-term ectopic expression has not been easy to achieve, since BRCA1 is too large to package into most viral vectors. To circumvent this technical problem, we established stable mouse embryonic stem cell clones using a bacterial artifical chromosome (BAC) of the entire *Brca1* locus recombineered with epitope tags fused to the last exon of the gene. Using this unique resource, we purified the mouse BRCA1 complex using sequential HA and FLAG tag immunoprecipitations followed by mass spectrometric analysis. We identified many well-characterized BRCA1 partner proteins in addition to many proteins involved in pathways heretofore not associated with BRCA1.

We are in the process of verifying candidate proteins for their abilities to interact physically and functionally with BRCA1. In addition, we are using our arsenal of BRCA1 reagents to test whether BRCA1 might participate in cellular pathways suggested by the identities of the candidate proteins.

Sorlie, T., et. al. 2001. PNAS U S A 98:10869-74.
Sorlie, T., et. al. 2003. PNAS U S A 100:8418-23.

FURTHER EVIDENCE FOR SPIRAL ASSEMBLY BY THE SV40 LARGE T ANTIGEN ORIGIN BINDING DOMAINS AT THE ORIGIN OF REPLICATION

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Polyomavirus origins of replication, while highly conserved, also contain distinct features. They contain multiple G(G/A)GGC sequences and the arrangement and spacing of these sequences is critical for correct function. The viral protein termed large T-antigen (T-ag) binds with high affinity to these sequences through its central origin-binding domain (obd). We use Simian Virus 40 (SV40) as our model system to study the structures that occur during 'assembly' on these sites. SV40 T-ag assembly on the core origin (which contains four GAGGCs and is termed site II) culminates in the formation of two hexameric rings of T-ag; the exact structural details of this assembly process are unknown. We previously showed that in SV40, the T-ag obds can form a left-handed spiral in the crystal having six molecules per turn and have postulated that in the context of full length T-ag hexamers, one turn of this spiral may occur after origin recognition.
The nearby SV40 regulatory region termed site I contains a direct repeat of two GAGGC sequences separated by 7 bp. To further our understanding of T ag's interactions with site I we have solved a 3.5 Å co-structure of the T-ag obd bound to site I. The orientation of the T-ag obds on site I suggested a molecular model whereby the bound T-ag obds nucleate a single hexamer of T-ag which positions the helicase domains over the EP region of the origin of replication. EMSA experiments have confirmed that a single hexamer forms on site I.
The spacer length between the GAGGC pairs differs between site I (seven bps) and site II (one bp). A long-standing question in the field of polyoma virus replication is how do pairs of GAGGCs, separated by different spacer lengths, support hexamer formation? Our T-ag obd-site I crystal structure provides an intriguing link between GAGGC spacing and hexamer formation. Owing to the helical nature of DNA, the obds bound to the GAGGC -$(X)_n$-GAGGC sequences are on the same face for site I, but on the opposite face for site II. Surprisingly, our modeling studies indicate that T-ag bound to either of these two GAGGC arrangements may transition into a hexamer provided it utilizes different subunits within the spiral. For the obds bound to site I, the DNA contacts are predicted to be between spiral subunits 1 and 6; for site II, the contacts are predicted to be with spiral subunits 1 and 4. This correlation extends to other polyomavirus origins (e.g., Merkel). Finally, these and related data allow us to present a detailed model of how T -ag assembles on the origin and then transitions to the replication forks.

ROLE FOR CASEIN KINASE 1 IN THE PHOSPHORYLATION OF CLASPIN ON CRITICAL RESIDUES NECESSARY FOR THE ACTIVATION OF CHK1

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In eukaryotic cells, duplication of the genome is an elegantly orchestrated process that requires not only the DNA replication machinery itself, but also checkpoint proteins that monitor the fidelity of the process. The checkpoint kinases ATR and Chk1 play crucial roles in the response to replication stress. In particular, the mediator protein Claspin is critical for the activation of Chk1 during checkpoint responses to stalled replication forks. This function involves the Chk1-activating domain (CKAD) of Claspin, which undergoes phosphorylation on multiple conserved sites. These phosphorylations promote binding of Chk1 to Claspin and ensuing activation of Chk1 by ATR. However, despite the importance of this regulation, the kinase responsible for these phosphorylations has remained unknown. By using a multifaceted approach, including kinome-wide RNAi screens in *Drosophila* cells and biochemical assays in cultured human cells, we have found that the *Drosophila* Gish and human casein kinase 1 gamma 1 (CK1γ1) carry out this function. CK1γ1 phosphorylates the CKAD of Claspin efficiently *in vitro* and in cells. Furthermore, this function is mediated by the collective activity of multiple isoforms of CK1γ1. Depletion of CK1γ1 from human cells by siRNA results in dramatically diminished phosphorylation of Claspin. Consequently, the siRNA-treated cells display impaired activation of Chk1 and resultant checkpoint defects. These results indicate that CK1γ1 is a novel component of checkpoint responses that controls the interaction of a key checkpoint effector kinase with its cognate mediator protein.

GENOME WIDE ANALYSIS OF REPLICATION INITIATION IN A HUMAN LYMPHOBLASTOID CELL LINE

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Origins of replication were isolated from asynchronously growing populations of the human B_lymphocyte derived cell line GM06990 by way of trapping fragments in agarose that were in the act of initiating at the time the DNA was isolated. The information obtained from the subsequent sequencing of this material was used to generate an origin map of the human genome. In a previous study (Genome Res 21: 377) we determine the distribution of origins in the one percent of the genome that was a part of the ENCODE pilot project and we found a significant association between origins and transcriptionally active genes in this cell line. Because of these findings we also generated a gene transcription map of GM06990 by sequencing cDNA synthesized from poly-A selected RNA isolated under the same conditions that were used to generate the origin map. We are currently comparing the distributions of origins with those of transcription the results of which will be presented. In addition, the origin and gene expression maps will be compared to genome wide distributions of various structural and functional properties as well as a number of factors all of which were generated by other labs. These include chromatin conformation (Hi-C), replication timing from FACS sorted asynchronously growing cells, CTCF and several histone modifications. Available results from these cross comparisons will be presented.

STRUCTURE AND MUTAGENESIS STUDIES OF THE C-TERMINAL REGION OF CDT1 ENABLE THE IDENTIFICATION OF KEY RESIDUES FOR BINDING TO MCM PROTEINS

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In eukaryotes, DNA replication is fired once in a single cell cycle before cell division starts to maintain stability of the genome. This event is tightly controlled by a series of proteins. Cdt1 is one of the licensing factors and is involved in recruiting replicative DNA helicase Mcm2–7 proteins into the pre-replicative complex together with Cdc6. In Cdt1, the C-terminal region serves as a binding site for Mcm2–7 proteins, although the details of these interactions remain largely unknown. Here, we report the structure of the region and the key residues for binding to Mcm proteins. We determined the solution structure of the C-terminal fragment, residues 450–557, of mouse Cdt1 by NMR. The structure consists of a winged-helix domain and shows unexpected similarity to those of the C-terminal domain of Cdc6 and the central fragment of Cdt1, thereby implying functional and evolutional relationships. By performing in vitro binding and budding yeast viability experiments, we showed that 45 residues located in the N-terminal direction of the structural region are equally crucial for recognizing Mcm proteins. Our data suggest the possibility that winged-helix domain plays a role as a common module to interact with replicative factors in pre-replicative complex. Furthermore, we have characterized binding domain of Mcm6 to Cdt1. Finally, we found C-terminal domain of Mcm6 is responsible for the association with Cdt1. Interestingly, it has recently been reported by G. Zhu and colleagues that the structure of C-terminal domain of human Mcm6 exhibits a typical winged-helix fold. Whereas Mcm4/6/7 complex can associate with Cdt1 in the presence of 450 mM NaCl, C-terminal fragment of Mcm6 does not associate with Cdt1. We are now trying to examine the requirement of stable interaction of Mcm6 for Cdt1 binding just like C-terminal fragment of Cdt1.

SWE1-DEPENDENT AND -INDEPENDENT PATHWAYS CONTROLLING HYDROXYUREA RESISTANCE IN SACCHAROMYCES CEREVISIAE

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Hydroxyurea (HU) slows down or stalls DNA replication fork and activates the replication checkpoint. In budding yeast Saccharomyces cerevisiae the replication fork component proteins Tof1, Csm3, Ctf4 and Mrc1 as well as its regulators such as Dcc1, Ctf18 and Ctf8 confer HU resistance. In addition, approximately 300 genes from various pathways have also been identified that confer HU resistance suggesting involvement of multiple mechanisms in the process.

HU exposure also leads to accumulation of the cell cycle regulator Swe1. We have sought to investigate the possible role of Swe1 in HU sensitivity and HU-dependent morphological aberrations in various HU-sensitive yeast strains. The results show that HU resistance can be Swe1-dependent or Swe1-independent. Several replication fork- and replication checkpoint-associated, non-essential genes such as *TOF1*, *CSM3*, *CTF4*, *DCC1* and *CHL1* were found to confer HU resistance in a Swe1-independent manner. Morphological aberrations observed in strains lacking some of these genes also occurred in a Swe1-independent manner.

Interestingly, absence of a sphingolipid gene *ISC1*, which is involved in bioactive ceramide generation, causes HU sensitivity and abnormal morphology in a Swe1-dependent manner. HU-induced morphological aberrations in the isc1Δ cells also involved the checkpoint proteins Rad9 and Rad53, which are known to genetically interact with Swe1 to control cell morphology.

COMPARATIVE GENOMICS OF CHROMOSOME REPLICATION

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Precise, complete and timely replication of eukaryotic genomes is achieved through the use of multiple start sites, called replication origins. These are best understood in the budding yeast *S. cerevisiae*. Powerful comparative genomic approaches are possible in budding yeasts due to the evolutionary range of sequenced genomes available and their tractability to genetic approaches. We have compared the location and activity of replication origins between strains of *S. cerevisiae* and between the closely related species of the *sensu stricto* group to identify mechanisms involved in regulating replication origin function. Previously we identified functional sequence elements at replication origins based upon their phylogenetic sequence conservation. Measuring replication origin activity in different strains and species has allowed us to discover differences from which we can learn about the mechanisms regulating origin function. First, we identify single nucleotide polymorphisms responsible for the presence or absence of origins in *S. cerevisiae* strains. Second, despite only limited conservation of origin location we discover significant conservation in the temporal order of genome replication between *sensu stricto* species. Finally we measure the replication dynamics of an *S. cerevisiae* / *S. bayanus* hybrid and find that there are both local (cis-) and global (trans-) regulators of replication origin activity.

INHIBITION OF HUMAN BK POLYOMAVIRUS DNA REPLICATION BY SMALL NON-CODING CELLULAR RNAS

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Small non-coding RNAs regulate a variety of cellular processes including genomic imprinting, chromatin remodeling, replication, transcription, and translation (1, 2). Here we report that small non-coding cellular RNAs called srRNAs (small replication-regulating RNAs) are present in non-infected mouse cells and specifically inhibit DNA replication of the human polyomavirus BK (BKV) in vitro and in vivo. srRNAs isolated from FM3A murine mammary tumor cells were enriched by DNA replication assay-guided biochemically fractionation techniques and hybridization to the BKV Non-Coding Control Region (NCCR). The obtained cDNAs were transcribed in vitro and added to BKV and SV40 DNA replication systems specifically inhibiting BKV but not SV40 DNA replication in vitro. Selective mutagenesis of the cDNA sequences and their putative targets suggests that the inhibition of BKV DNA replication is mediated by srRNAs binding to the viral NCCR, hindering early steps in the initiation of DNA replication. Ectopic expression of srRNAs in human kidney cells inhibited BKV DNA replication in vivo. These biochemical and molecular biological analyses in vitro and in vivo suggest that the inhibition of replication is mediated by hybridization of srRNAs to sequences on opposite strands close to or within the BKV core-origin so as to form a replication-inactive initiation complex. Additional srRNAs were synthesized guided by the hypothesized mechanism to inhibit SV40 but not BKV DNA replication. As predicted these new srRNAs specifically inhibited SV40 DNA replication in vitro. The presence of the obtained srRNAs in non-infected mouse cells was verified by RT-PCR and sequencing of the PCR products. Our results using viral DNA replication in crude cell extracts and with purified proteins as well as in vivo point to novel mechanisms for regulating DNA replication by small non-coding cellular RNAs.

1. Bernstein, E., and C. D. Allis (2005) Genes Dev 19:1635-1655.
2. Fabian, M. R., N. Sonenberg, and W. Filipowicz.(2010) Annu Rev Biochem 79:351-379.

SUBUNITS OF THE ORIGIN RECOGNITION COMPLEX ARE ESSENTIAL FOR STABLE KINETOCHORE ATTACHMENT TO SPINDLES DURING MITOSIS IN HUMAN CELLS AND C. ELEGANS

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Accurate inheritance of genetic information requires precise coordination of DNA replication and chromosome segregation before cell division. Subunits of the Origin Recognition Complex that is required for the initiation of DNA replication are involved in chromosome segregation during mitosis. We show that hOrc1 is loaded onto chromatin as cells prepare to enter mitosis and that hOrc1 is the first ORC subunit loaded onto chromatin and is inherited into the daughter cells. hOrc2 and other ORC subunits are loaded as cells exit mitosis or in G1 phase. hOrc2 and hOrc3 come off chromatin as cells pass though S phase, but they co-localize with CENP-A at centromeres and from a phosphorylation-dependent complex containing the kinetochore proteins BubR1, CENP-E and Plk1 only during mitosis. In the absence of hOrc2 or hOrc3, spindles attach to the condensed chromosomes and the spindle assembly checkpoint is satisfied despite lagging chromosomes. During the metaphase to anaphase transition stable spindle attachment is lost and sister chromatids remain attached to each other, however chromosomes assume an abnormally condensed structure. Orc2 also localizes to the holocentric centromeres in C. elegans and its depletion causes defects in chromosome structure and segregation. We suggest an ancient link between origins of DNA replication and processes that ensure correct chromosome segregation.

COORDINATED DEGRADATION OF REPLISOME COMPONENTS ENSURE GLOBAL GENOME STABILITY UPON REPLICATION STRESS

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The replisome complex is essential for highly processive DNA replication, and its stabilization during S-phase of cycling cells is vital to preserve genomic integrity. Conversely, it would also be advantageous for the cell to abrogate replisome functions when DNA replication is adversely perturbed. However, such mechanisms remain elusive. Here we describe a genome-protecting response that is triggered when cells experience unstable replication forks in fission yeast. We found that replicative DNA polymerases and helicases, the major components of the replisome, are in concert degraded in response to replication stress caused by inactivation of $Swi1^{Timeless}$, a subunit of the replication fork protection complex. In sharp contrast, ORC and PCNA, which are essential for initiation of DNA replication, were stably maintained. Consistently, we observed that replisome progression, but not origin initiation, is delayed in the absence of $Swi1^{Timeless}$. We demonstrate that degradation of DNA polymerases and helicases is dependent on the ubiquitin-proteasome system, in which the SCF^{Pof3} ubiquitin ligase complex is required. Remarkably, forced accumulation of replisome components leads to catastrophic DNA replication and mitosis. We propose that the cell elicits a program to degrade replisome components to ensure global preservation of genomic integrity upon replication stress.

REGULATION OF DNA REPLICATION TIMING ON A HUMAN CHROMOSOME BY CELL TYPE SPECIFIC DNA BINDING PROTEIN SATB1

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Replication timing of metazoan DNA during S-phase is determined by many factors including chromosome structures, nuclear positioning, patterns of histone modifications, and transcriptional activity. Recent reports indicate developmental and cell type-specific regulation of replication timing domains, but the molecular basis of this regulation is still largely unclear.
We have been working on the human 5q locus (5q23/31), which contains clusters of cytokine genes, whose transcription is regulated in cell-type specific manner. We first examined replication timing by quantification of the replicated DNA in fractionated S-phase cells of Jurkat (T cells expressing cytokines) and HL-60 (non-T cells). We found that locations of early and late replicating domains on the 5q23/31 3.5-Mb segment were conserved, but the transition region between early and late were offset by 200-kb. Genome analyses of the 3.5-Mb segment revealed several clusters of potential binding sites for Special AT-rich sequence binding protein 1 (SATB1) in the vicinity of the transition region.
Next, we examined whether replication timing would be altered by SATB1. We generated HeLaS3 cells expressing SATB1 and showed that the transition region switched between ”Jurkat-type (SATB1(+), late)” and “HeLa-type (SATB1(-), early)” depending on SATB1 expression by FISH analyses.
Finally, we analyzed and compared replication timing pattern across the continuous 40-Mb segment spanning 5q23/31 in SATB1(+) and (-) HeLaS3 by microarrays. We found that the overall replication timing domains of these two cells are similar, but segments inefficiently incorporating BrdU were detected in SATB1-expressing cells. We showed that the replication fork indeed moves more slowly in the timing transition region. We also detected a significant SATB1 binding site within the transition region by ChIP assay. On the basis of these results, we speculate that SATB1 may regulate the replication fork propagation in the timing transition region which is generally a long origin-less segment where the fork moves unidirectionally.

ROLES OF THE HUMAN 9-1-1/TOPBP1 INTERACTION FOR CELLULAR DNA DAMAGE RESPONSES

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The checkpoint clamp Rad9-Hus1-Rad1 (9-1-1) is loaded onto damaged DNA by Rad17-RFC clamp loader. It also interacts with the ATR-activator, TopBP1 and plays a key role in the ATR-dependent checkpoint pathway. Recently, we demonstrated that phosphorylation of the C-terminal tail of Rad9 by Casein Kinase 2 (CK2) at Ser-341 and Ser-387 promotes the 9-1-1/TopBP1 binding, and is essential for DNA damage responses. However, their interaction, as well as the levels of the phosphorylation of Rad9 at the CK2 sites, was unchanged irrespective of DNA damaging treatments.
To understand roles of the interaction for cellular DNA damage responses, we have explored relation between the 9-1-1/TopBP1 interaction and their subcellular localization. Chromatin fractionation with HeLa cells revealed that TopBP1 was highly enriched in a chromatin fraction without DNA damage, while only a small portion of Rad9 localized on chromatin and the amount increased upon DNA damage. Interestingly, this damage-induced association was reduced in TopBP1 knocked-down cells. However, a phospho-deficient mutant of Rad9 that does not bind TopBP1 was able to exhibit DNA damage-induced chromatin association. This indicated that TopBP1 is involved in damage-induced chromatin localization of Rad9, but their direct association was not required. Furthermore, we observed that both Rad9 and TopBP1 localized to UV-irradiation sites by a local UV irradiation technique. Differently from previous models, their interdependency for the localization of Rad9 or TopBP1 to the damaged sites was limited. Therefore, TopBP1/Rad9 interaction will play an important role to mediate DNA damage signals through unknown mechanism after their assembly at DNA damage sites.

HUMAN SIRT1 REGULATES DNA-BINDING OF THE MCM10 DNA REPLICATION FACTOR VIA DEACETYLATION

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The eukaryotic DNA replication initiation factor, Mcm10, is essential for both replisome assembly and function. Human Mcm10 has two DNA-binding domains, the conserved internal domain (ID) and the C-terminal domain (CTD), which is specific to metazoans. SIRT1 is a NAD-dependent deacetylase that belongs to the sirtuin family. It is conserved from yeast to human, and participates in cellular controls of metabolism, longevity, gene expression and genomic stability.
We now report that human Mcm10 is an acetylated protein regulated by SIRT1, which binds and deacetylates Mcm10 both in vivo and in vitro. Moreover, the two DNA-binding domains of Mcm10 are modulated in distinct fashion by acetylation/deacetylation, which suggest a complex mechanism of regulation. Further analysis demonstrated that Mcm10 deacetylated by SIRT1 is required for both proper fork initiation and progression. This novel regulatory mechanism shows that like transcription and DNA repair factors, Mcm10 is also functionally regulated by acetylation-deacetylation. Moreover, our study suggests that SIRT1 may mediate a crosstalk between DNA replication and cellular circuits controlling metabolism and longevity.

STRUCTURAL AND FUNCTIONAL INSIGHTS INTO THE DNA REPLICATION FACTOR CDC45 REVEAL AN EVOLUTIONARY RELATIONSHIP TO THE DHH FAMILY OF PHOSPHOESTERASES.

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Cdc45 is an essential protein conserved in all eukaryotes and is involved both in the recruitment of the factors necessary for the initiation of DNA replication, as well as the subsequent progression of the replication fork. Together with GINS, Cdc45 has been shown to act as essential co-factor of the MCM2-7 complex that is directly responsible for the helicase activity at the replication fork. Despite its importance, no detailed information is available on either the structure or the biochemistry of the protein. Intriguingly, whereas homologues of both GINS and MCM proteins have been described in Archaea, no counterpart for Cdc45 is known.
We report a bioinformatic analysis that shows a weak but significant relationship among eukaryotic Cdc45 proteins and a large family of phosphoesterases that has been described as DHH family. This includes inorganic pyrophosphatases, polyphosphatases and ssDNA exonucleases (such as the RecJ enzymes) that catalyse the hydrolysis of a phosphodiester bond via a mechanism involving two Mn^{2+} ions. Only a subset of the amino acids that coordinate the metals are conserved in Cdc45 and no metal ion has been observed. We report biochemical and structural data on the recombinant human Cdc45 protein, consistent with the proposed DHH family affiliation. Like the RecJ exonucleases, the human Cdc45 protein is able to bind single-stranded, but not double-stranded DNA. Small angle X-ray scattering data are consistent with a model compatible with the crystallographic structure of the RecJ/DHH family members. The position of two putative insertions in the RecJ/DHH core can also be inferred.

RAD5 PLAYS A MAJOR ROLE IN TOLERANCE TO MMS-INDUCED DNA DAMAGE DURING CHROMOSOME REPLICATION

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The *RAD6/RAD18* pathway of DNA damage tolerance, necessary for cell viability in the presence of DNA polymerase-blocking lesions, is composed of two branches: translesion synthesis (TLS), an error-prone mechanism for most types of DNA damage, and an error-free process of damage avoidance that very likely involves template switching. TLS is mediated by DNA polymerases with low fidelity that replicate over and past DNA lesions, and in *Saccharomyces cerevisiae* is carried out by Polζ (Rev3/Rev7), Rev1 and Polη (Rad30). The error-free mechanism avoids lesions by using the newly synthesized, undamaged strand of the sister chromatid as the template for bypass replication. In budding yeast, this process is mediated by the Mms2-Ubc13 ubiquitin-conjugating enzyme in conjunction with the E3-ligase Rad5, which has also a helicase activity.

We analysed in *S. cerevisiae* the contribution of each branch of this pathway to cell survival during chromosome replication in the presence of DNA damage caused by the alkylating agent methyl methanesulfonate (MMS). We found that Rad5-deficient cells are much more sensitive to MMS during S phase than cells lacking all the TLS polymerases, showing loss of viability and defects in cell cycle progression even at concentrations of MMS at which the latter were unaffected. We studied the movement of DNA replication forks along an individual replicon and found that chromosome replication is highly defective in the absence of Rad5 when cells are treated with MMS, in contrast to *tls* mutants, which show only minor defects. These data indicate that Rad5 is mainly responsible for cell survival during S phase when cells are exposed to DNA methylation by MMS, allowing completion of chromosome replication under these conditions. Moreover, most of the problems derived from the absence of Rad5 are not due to its ubiquitin ligase function for PCNA polyubiquitylation, as Ubc13-deficient cells have only modest defects in tolerance to MMS during DNA replication in comparison to *rad5* mutants. Work in progress will be presented.

ANALYSIS OF THE CHROMATIN BINDING OF FORKHEAD TRANSCRIPTION FACTORS, FKH1 AND FKH2, IN REGULATING REPLICATION ORIGIN CLUSTERING AND INITIATION TIMING IN YEAST

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Eukaryotic chromosomes are organized in three-dimensions to facilitate the accurate duplication and regulated expression of the genome. DNA sequences located distally on the same chromosome or on different chromosomes coalesce into functionally specialized clusters (or "factories") of transcription or replication. The aggregation of factors required for DNA replication, for instance, together with the replication origins that serve as regulators and initial substrates presumably maximizes the efficiency and precise regulation of the replication process; however, little is known concerning the assembly and regulation of these factories. The differential efficiency and timing of replication origins throughout the genome may reflect their preferential assembly into replication factories. We have identified yeast Forkhead transcription factors, Fkh1 and Fkh2, as global determinants of replication origin efficiency and timing. In the absence of Fkh1 and Fkh2, most early origins fail to initiate promptly and efficiently. We further show that these origins fail to cluster in G1-phase with other origins destined to fire early in S-phase, suggesting that Fkh1 and Fkh2 are responsible for the selective recruitment of origins to emergent replication factories. To investigate possible mechanisms by which Forkhead proteins mediate origin clustering, we are examining Fkh1 and/or Fkh2 binding at Fkh-regulated origins, and genome-wide. We find that Forkhead proteins bind very extensively throughout the genome and correlate with the positions of Fkh-regulated origins. We are also examining origin clustering by chromosome conformation capture and live-cell imaging approaches. We will present our current results, and discuss their significance for the differential regulation of replication origin function and the three-dimensional organization of the replication process.

MCM8 AND MCM9 PROMOTE DNA HOMOLOGOUS RECOMBINATION AND DNA RE-REPLICATION

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The minichromosome maintenance proteins MCM8 and MCM9 are two MCM family members that were recently found to play a role in DNA replication elongation and pre-replicative complex (Pre-RC) formation, respectively. We found that MCM8 and MCM9 proteins interact with each other, but surprisingly do not stably interact with other DNA replication proteins. Additionally, the MCM8 protein is required for the stability of MCM9 protein, further demonstrating that these two helicases form a stable complex in mammalian cells. Depletion of MCM8 from mammalian cells by RNA interference only partially increased the population of cells in the G1 phase of the cell cycle, but surprisingly, did not significantly impact S-phase progression. Instead, we found that MCM8 and MCM9 are both required for aberrant re-replication induced by inactivation of the CRL4-Cdt2 E3 ubiquitin ligase complex. Finally, we show that MCM8/9 play a significant role in homologous recombination but not in non-homologous end jointing (NHEJ). Together, these findings support the hypothesis that these two metazoan-specific helicases may have evolved to promote genome stability, potentially by promoting homologous recombination repair under conditions that induce aberrant DNA replication.

REPLICATION-INDEPENDENT HISTONE H3 LYSINE 56 ACETYLATION IS RAPIDLY REESTABLISHED DURING S PHASE IN CULTURED ARABIDOPSIS AND RICE CELLS

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Acetylation of histone H3 on lysine 56 (H3K56ac) functions in DNA replication, DNA repair and gene activation. Many genes in both Arabidopsis and rice cells display abundant replication-independent H3K56ac at the transcription start site (TSS) of genes. However, in our study of the replication programs of Arabidopsis and rice cells, we also noted regions of H3K56ac in asynchronous cells that were inconsistent with either gene activation or DNA repair. Intriguingly, these regions tended to replicate early in S phase, suggesting that DNA replication origins may be associated with these regions. In yeast, DNA replication origins display increased H3K56ac during S phase. To dissect replication-independent and dependent H3K56ac in plant cells, we used a combination of EdU labeling, fluorescence activated cell-sorting and immunoprecipitations to isolate DNA associated with H3K56ac during G1 and S phase in Arabidopsis and rice cells, which was then analyzed on tiling microarrays to select portions of the Arabidopsis and rice genome. Surprisingly, in both Arabidopsis and rice, we detected few differences between the patterns of H3K56ac in G1 and S phase cells. However, in rice cells, we observed a quantitative increase in replication-independent H3K56ac at gene TSS, especially in the earlier replicating regions. This result in consistent with the increased copy number of early replicating DNA in S phase, and further suggests that patterns of replication-independent H3K56ac are rapidly reestablished by the removal or redistribution of replication-dependent H3K56ac.

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STRUCTURAL INSIGHTS INTO THE INITIATION OF DNA SYNTHESIS IN EUKARYOTIC REPLICATION.

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DNA polymerases need a primer to begin the synthesis of new DNA during replication. Nature has evolved specialised polymerases, termed primases, that are able to synthesise short RNA primers de novo. Because of the antiparallel nature of the DNA double helix, replication must start at least once on the leading strand and multiple times on the lagging strand. Thus, the enzymatic activity of the primase is constantly required at the replication fork. Our understanding of the molecular mechanisms by which primases synthesise the RNA primer is not well understood. Here we discuss our recent efforts to understand structure and function of the DNA Pol alpha/primase complex, responsible for initiation of DNA synthesis in eukaryotic replication.

THE ROLE OF THE CDC7 KINASE IN DNA REPLICATION AND IN THE DNA DAMAGE RESPONSE

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Initiation of DNA replication from licensed origins requires the action of two S-phase promoting kinases, the cyclin-dependent kinase (CDK) and Cdc7 kinase. Cdc7 is a serine/threonine kinase that phosphorylates chromatin-bound Mcm2-7 proteins. This is thought to be required for subsequent activation of the CMG helicase complex. However, little is known about how Cdc7 is regulated. In this study, we present results from the characterisation of a small molecule inhibitor, PHA-767491 (PHA), in the cell-free system derived from *Xenopus* eggs and how we use PHA as a molecular tool to dissect Cdc7 function in the cell cycle. We find that PHA inhibits DNA replication and hyper-phosphorylation of chromatin-bound Mcm4. We present evidence that the inhibition of DNA replication occurs largely through blocking an essential function of the Cdc7 kinase. We identify protein phosphatase I as a novel modulator of Mcm4 hyper-phosphorylation, acting in concert with Cdc7. Taking advantage of the rapid effect onset characteristic to chemical inhibitors, we conducted time-of-addition experiments. This revealed that Cdc7 appears to act early in S-phase to phosphorylate Mcm4 throughout the genome and execute its function to initiate origin firing. Once this is completed, Cdc7 no longer appears to be the rate-limiting step in driving the replication-timing programme in this *in vitro* system. With this work, we provide more details about how Cdc7 functions at the molecular level.

SACCHAROMYCES CEREVISIAE CENTROMERES ADVANCE THE ACTIVATION TIME OF NEARBY ORIGINS OF DNA REPLICATION

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Eukaryotic chromosomes are replicated from multiple origins that initiate synthesis at different times throughout S phase. In S. cerevisiae, the time of activation is not an intrinsic property of individual origins, but is influenced by the surrounding chromosomal neighborhood. For example, the late replication of *S. cerevisiae* telomeres is due to the ability of telomeres to delay the activation time of origins residing up to 30 kb away.

All centromeres in *S. cerevisiae* replicate early. Increasing evidence suggests that early centromere replication is a conserved feature of the eukaryotic replication landscape. Surprisingly, little is known about the biological significance or the mechanism of early centromere replication. We entertained two simple hypotheses: (1) centromeres ensure their early replication by promoting early activation of nearby origins; (2) centromeres have simply migrated over evolutionary time to reside in early replicating regions. Either hypothesis assumes that there is a selective advantage that early replication of centromeres provides the cell. To distinguish between these two possibilities a variation of the Meselson/Stahl density transfer assay was employed to determine replication times of centromeres and their flanking origins in haploid strains harboring centromeres residing in either their endogenous (wild type) position or translocated to a late replicating genomic region (rearranged). We show that the centromere in a rearranged strain promotes its own early replication by advancing the time of activation of its nearby origin, and this change in origin firing time is dependent on centromere function. Furthermore, we find that when a centromere has been removed from its endogenous position the origins in the formerly pericentric region replicate later than in wild type cells. Genome wide analysis indicates that centromeres advance the activation time of origins that are up to 11.5 kb away. Thus, centromeres function in cis to regulate the time of DNA replication initiation.

DNTP POOLS DETERMINE FORK PROGRESSION AND ORIGIN USAGE UNDER REPLICATION STRESS

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Hydroxyurea (HU) impedes replication fork progression by inhibiting ribonucleotide reductase (RNR), a key enzyme catalyzing a rate-limiting step of deoxyribonucleoside triphosphate (dNTP) production. Intriguingly, basal dNTP pools are preserved in the presence of HU, allowing *S. cerevisiae* cells to complete S phase at a very slow pace. However, the regulation of S-phase progression under low dNTP conditions remains poorly understood. Here, we show that HU-treated cells undergo a sharp transition from a fast- to slow-replication mode after activation of ~40% of the replication origins. Upregulation of RNR activity increases fork rate and delays this transition, allowing more initiation before checkpoint activation in HU. Conversely, faster elongation advances this transition and induces a premature repression of origins. Interestingly, mutants exposed to spontaneous replication stress show increased dNTP pools and HU-resistant DNA synthesis. We propose that dNTP levels regulate S-phase progression and that cells adapt to chronic replication stress by upregulating RNR activity.

A DOMINANT ROLE FOR TRANSLESION DNA POLYMERASE IV IN RECOMBINATION DRIVES EVOLUTION

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Homologous recombination (HR) repairs double-strand breaks (DSBs) by utilizing DNA polymerase (pol) activity to copy sequence information from a homologous donor DNA. This mode of repair is considered error-free which implies that only high-fidelity pols act at recombination intermediates called D-loops. Error-prone Y-family translesion pols of *Eschericia coli* (*E. coli)*, however, function with RecA, are highly upregulated during the SOS response, and promote mutations following DSBs, which suggests a possible role for these enzymes in HR. Here, we investigate pol activity at D-loops using the *E. coli* paradigm. We find that translesion pol IV (DinB) is highly proficient in D-loop extension even in the absence of the β-clamp. In contrast, translesion pol V ($UmuD'_2C$) lacks this activity and replicative pol III requires β and the τ-subunit of the clamp-loader for extending D-loops. Notably, pol IV extends D-loops more efficiently than pol III and is preferentially recruited to recombination intermediates under SOS response conditions. Unexpectedly, we find that the proofreading activity of Pol III is stimulated at recombination intermediates which inhibits its ability to extend D-loops. Finally, we show that pol IV extends D-loops containing heterologous sequences and promotes mutations at recombination intermediates. These findings demonstrate a dominant role for pol IV in initiating DNA synthesis at recombination intermediates and elucidate the mechanism by which pol IV promotes mutations that drive evolution under conditions of stress.

THE N-TERMINAL REGION OF HUMAN RECQL4 HELICASE POSSESSES MULTIPLE DNA DNA BINDING DOMAINS

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The RecQL4 helicase, a member of the RecQ family of DNA helicases, is involved in the maintenance of genome integrity and in DNA replication. Mutations in the human RecQL4 gene cause the Rothmund-Thomson, RAPADILINO and Baller-Gerold syndromes. These diseases are characterised by increased cancer risk and symptoms of premature aging such as early development of cataracts and loss of hair. The N-terminus of RecQL4 shows homology to the yeast Sld2 protein, which is an essential replication initiation factor. There is strong evidence that the N-terminus of human RecQL4 also has a fundamental function in DNA replication.
The well-conserved N-terminus of human RecQL4 carries an overall helical fold similar to homeodomains despite lack of significant sequence homology.
Using a screen for the identification of folded protein entities, we identified additional, potential domain boundaries in the N-terminal region of human RecQL4 and performed a biochemical and biophysical analysis of the corresponding recombinant fragments. DNA binding studies and the increasing annealing activity indicate the presence of multiple DNA binding sites in the N-terminus of human RecQL4. The analysis of the fragments facilitate the understanding of the roles of the N-terminal RecQL4 in DNA metabolism and hence in DNA replication in more detail.

DYNAMIC LOADING AND REDISTRIBUTION OF THE MCM2-7 COMPLEX THROUGH THE CELL CYCLE

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In higher eukaryotes there are many more Mcm2-7 complexes loaded onto chromatin than are required to complete an unperturbed S-phase. These excess Mcm2-7 complexes are thought to be important for the cell to recover from replicative stress. We have investigated the genome-wide distribution of the Mcm2-7 complex in Drosophila tissue culture cells. We have found that the Mcm2-7 complex is loaded in limited quantities at ORC binding sites in early G1, but by the G1/S transition there is significantly more Mcm2-7 complex associated with the chromatin. Furthermore, these excess Mcm2-7 complexes have redistributed throughout the chromosome and are no longer limited to sequences proximal to ORC. These redistributed helicases are functional and track with replication fork progression during S-phase. The redistribution of the Mcm2-7 complexes is dependent on cyclin E activity. In the absence of cyclin E, the cells arrest in G1 and we find a limited amount of Mcm2-7 complexes on the chromatin by chromatin fractionation. Inhibition of CDK activity by over expression of dacapo, a p27 homolog, also results in a G1 arrest; however, there are significantly more Mcm2-7 complexes associated with the chromatin. Together these results suggest that there is limited pre-RC assembly at ORC binding sites in the absence of cyclin E and that cyclin E, independent of CDK activity, is important for loading the full complement of Mcm2-7 complexes and the redistribution of the helicases throughout the chromosome. The loading of excess Mcm2-7 complexes and the redistribution of the complex throughout the genome during late G1 may, in part, explain the broad distribution of origin activity observed in metazoan genomes.

CELL CYCLE REGULATED, DYNAMIC ASSOCIATION OF ORCA/LRWD1 WITH PRERC COMPONENTS

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In eukaryotes, initiation of DNA replication requires the assembly of a multiprotein pre-replicative complex at the origins. We have recently reported that a WD-repeat-containing protein, ORC-associated (ORCA) or LRWD1 binds to ORC and play crucial roles in stabilizing ORC to chromatin. We now demonstrate that ORCA directly interacts with Orc2 subunit of the human ORC. Depletion of ORCA at specific stages of cell cycle reveals that ORCA is required for the entry into S-phase. Further, besides ORC, ORCA associates with several other pre-RC components, including Cdt1 and its inhibitor Geminin and forms independent complexes. ORCA associates with Orc1 and Cdt1 predominantly in G1, whereas it associates with Geminin during rest of the cell cycle. The WD-domain of ORCA is responsible for binding to ORC, Cdt1 and Geminin. Overexpression of Geminin, results in the loss of interaction between ORCA and Cdt1 suggesting that the increased levels of Geminin titrate ORCA away from Cdt1. Based on our results, we propose that at the end of G1 phase when the levels of Geminin begin to elevate, ORCA binding to Cdt1 is lost resulting in the disassembly of preRC. All the above results suggest a crucial role for ORCA in preRC assembly and cell cycle entry.

MUTATIONAL ANALYSIS OF MCM4

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MCM proteins are essential in the assembly and maintenance of a replication fork. Previous studies from our lab have shown that the mcm4 subunit in *Schizosaccharomyces pombe* is particularly important for replication fork stability, when replication is arrested by inhibitor such as hydroxyurea (HU), and suggest that Mcm4 is a substrate of the Cds1 checkpoint kinase. Interestingly, activation of the checkpoint rescues mcm4ts. To further study Mcm4 functions in the Cds1-dependent maintenance of the replication fork, we constructed a series of point mutations and truncation mutations.Complementation experiments have shown that these point mutation and truncations mutations can complement the mcm4ts mutation and the mcm4 null strain.The plasmid that contained the point mutations which disrupts all four potential phosphorylation sites has been integrated into a Mcm4 null strain and is currently being characterized by studying its response under different forms of drugs and more specifically focusing on how it responds to hydroxyurea under different conditions to determine its effects on replication fork stability.

GENOME-WIDE MAPPING OF *CAENORHABDITIS ELEGANS* ORIGINS OF DNA REPLICATION

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Genome integrity requires faithful chromosome duplication that starts at replication origins. To elucidate how and where origins are defined on chromatin in an *in-vivo* system is an important task that will help us to understand the complexity of genome organization and regulation.
In this study we mapped the replication origins of *C.elegans* in two different embryonic developmental stages. First, origins were mapped genome-wide in embryos from a proliferative stage (form first cleavage to the end of founder cells generation), and second, form embryos in a differentiating stage (from gastrulation to hatching). To do so, we have developed a technique of double synchronized large-scale liquid culture of *C.elegans* that allowed us to obtain a large quantity of embryos at the precise stage of interest. We have mapped *C.elegans* embryonic replication origins by high-throughput sequencing and also by microarray analysis of newly synthesized DNA. The results of these analyses will help us to understand the conservation of replication origins features and their organization throughout evolution.

FIDELITY OF RESTARTED REPLICATION FORKS

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DNA replication is a fundamental process, that needs to be completed prior to each cell division. However, lesions, protein barriers, secondary structures and other obstacles may interfere with this process and hinder the progression of the replication fork. In order to determine the accuracy of replication, we are using an assay which allows us to measure tandem repeat deletion rates of short sequences (41-251bp) in *Schizosaccharomyces pombe*. Furthermore, depending on the sequence content, secondary structures can form under certain conditions. These may interfere with replication and require specialised proteins or mechanisms for their replication. We are using G-rich sequences, prone to form G-quadruplexes, in combination with tandem repeats to investigate effects of such structures on replication fidelity.

DNA-protein complexes can also interfere with DNA replication. Natural replication fork barriers exist in cells in order to coordinate the direction of replication, which can be beneficial, for example, in highly transcribed loci like the rDNA. At least two types of unidirectional replication fork barriers can be found in the genome of *S. pombe*. The rDNA barrier and the replication terminating sequence (RTS1) in the mating type locus. Applying these sequences on either site of a marker gene confronts the cell with a region in the genome that is blocked for replication.
Work published in our lab (Lambert et al. 2005, Mizuno et al. 2009) has shown that controlled stalling of replication forks at RTS1 sites under these conditions, can cause gross chromosomal rearrangements and cell death. The data suggests that homologous recombination is required to restart the replication fork at RTS1 to allow completion of DNA replication at cost of genome integrity. The resulting genomic rearrangements observed after fork stalling could be due to the fidelity and different nature of the restarted fork.

The combination of the RTS1 system with the replication fidelity assays using tandem repeats and structured DNA, allows us to investigate the fidelity of DNA replication after replication fork stalling and restart at RTS1.

References
Lambert et al. Cell. 2005 Jun 3;121(5):689-702
Mizuno et al. Genes Dev. 2009 Dec 15;23(24):2876-86

INACTIVATION OF THE ESSENTIAL FISSION YEAST MCM^{MCM-BP} COMPLEX LEADS TO CELL CYCLE ARREST, RAD22 FOCI FORMATION AND CHK1-DEPENDENT DNA DAMAGE CHECKPOINT ACTIVATION

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The MCM helicase plays a vital role in chromosomal DNA replication in eukaryotic cells by unwinding double-stranded DNA at the replication fork. MCM is a hexamer comprising six related subunits, Mcm2-Mcm7. The distantly-related MCM-BP protein was first identified in human cells as a protein able to form a complex with Mcm3-Mcm7 but not with Mcm2. Evidence from *Xenopus* suggests that the MCM^{MCM-BP} complex may have a role in unloading the canonical MCM complex from chromatin at the end of S-phase, while work in plants and human cells has pointed to a role for MCM^{MCM-BP} in establishing and/or maintaining chromosome cohesion. The fission yeast *S.pombe* encodes a putative MCM-BP orthologue protein called Mhi1/Mcb1. To test whether Mhi1/Mcb1 forms an MCM^{MCM-BP}-like complex, we purified the protein by tandem affinity chromatography from native protein extracts and analysed the composition of the purified fractions by SDS-PAGE and mass spectrometry. As predicted from studies of the human MCM^{MCM-BP} complex, Mhi1/Mcb1 was found to co-purify with Mcm3-Mcm7 and not with Mcm2. These interactions have been confirmed by co-immunoprecipitation of individually-tagged MCM proteins with Mhi1/Mcb1 and by bimolecular fluorescence complementation analysis with Mcm4 and Mhi1/Mcb1. Analysis of the size, stoichiometry and structure of the MCM$^{Mhi1/Mcb1}$ complex is underway. To examine the cellular function of the MCM$^{Mhi1/Mcb1}$ complex, we have generated a large number of temperature-sensitive *mhi1*/*mcb1* mutant alleles. Inactivation of MCM$^{Mhi1/Mcb1}$ function by temperature shift results in cells arresting with an apparent 2C DNA content due to activation of the Chk1-dependent DNA damage checkpoint and is accompanied by a marked accumulation of Rad22 (Rad52) repair foci suggesting that chromosomal DNA replication cannot be completed successfully in the absence of MCM$^{Mhi1/Mcb1}$ function. The results of these studies will be presented.

ARCHITECTURE OF THE REPLISOME COMPLEX IN BUDDING YEAST

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The process of DNA unwinding is tightly coupled to DNA synthesis at replication forks by means of a multi-protein assembly termed the 'replisome', which regulates the speed of fork progression and reduces exposure of single-strand DNA during replication. The molecular nature of the replisome is well defined in *Escherichia coli*, wherein the clamp loader complex provides the essential connection between the replicative helicase and the DNA polymerases on the leading and lagging strand templates. Our present understanding of the composition of the eukaryotic replisome is very limited, owing to the intrinsic complexity of the eukaryotic system and the fact that the eukaryotic replisome is built in situ at nascent forks and then disassembled after replication.
We have developed a procedure for isolating replisome material from replication forks in yeast cell extracts, under physiological conditions that preserve the association of replicative helicase and polymerases. In contrast to the *E. coli* paradigm, our findings indicate that the clamp loader complex does not provide the essential link between the helicase and polymerases within the eukaryotic replisome. We reported previously that a complex of GINS and Ctf4 links the MCM helicase to DNA polymerase alpha on the lagging strand template, and the Mrc1 checkpoint mediator has been proposed to be the link between MCM and the leading strand DNA polymerase epsilon. However, we find that Pol epsilon still associates with the replisome in the absence of Mrc1, leading us to analyse systematically the requirements for coupling helicase to leading strand polymerase within the eukaryotic replisome. Current progress with this work will be presented.

DNA POLYMERASES AND THE REPLICATION FORK IN THE EXPANSION OF $(GAA)_N$. $(TTC)_N$ REPEATS IN YEAST

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Expansions of trinucleotide repeats have been discovered to cause severe neurodegenerative diseases in humans. Large-scale expansions of (GAA)n•(TTC)n repeats in the first intron of the Frataxin gene in humans are responsible for Friedrich's ataxia – the most common hereditary ataxia. The fine molecular mechanisms behind these large-scale expansions of trinucleotide repeats remain unknown. Our lab has previously developed an experimental system to study the large-scale expansions of these repeats in budding yeast. In this system, (GAA)n•(TTC)n tracts expand significantly mimicking expansions observed in human pedigrees.

We have previously proposed that DNA replication across the repeat tract gets compromised leading to repeat instability. In the current study, we evaluate the role of replicative DNA polymerases in the expansion process. Mutations affecting the catalytic subunit of DNA polymerase-primase α (alpha), replicative DNA polymerases δ (delta) and ε (epsilon) were tested for their effects on the rate and scale of expansion of the (GAA)•(TTC)100 repeat. Mutations in Pol δ and Pol ε resulted in a great increase in the rate of expansion. In contrast, mutations in Pol α had only a modest effect on the expansion rate, but significantly increased the scale of expansions.

To further investigate the mechanism of how DNA polymerase progression is regulated at the repetitive tract, mutations in PCNA were tested in conjunction with the DNA polymerase mutants. Our preliminary data suggest that the polymerases could switch templates back and forth without perturbing the replisome resulting in repeat expansions.

ROLE OF ORCA/LRWD1 IN REPLICATION INITIATION

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Initiation of DNA replication requires the ordered assembly of a multiprotein pre-replicative complex at the origins of replication. We have previously demonstrated that ORC-associated, ORCA/LRWD1, plays a key role in stabilizing ORC binding to chromatin. The roles of ORCA in preRC assembly remained to be elucidated. We show that ORCA directly interacts with Orc2, Cdt1 and Geminin, and forms independent sub-complexes in a cell cycle dependent manner. Further, ORCA association to ORC, Cdt1 as well as Geminin is mediated via its WD domain, whereas Geminin associates with ORCA and Cdt1 through different regions. Interestingly, overexpression of Geminin results in loss of Cdt1 association with ORCA, suggesting that increased levels of Geminin titrate ORCA away from Cdt1. Implications of these results will be discussed.

CDT1 PROTEOLYSIS IS PROMOTED BY DUAL PIP DEGRONS AND IS MODULATED BY PCNA UBIQUITYLATION.

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Cdt1 is a conserved and essential DNA licensing factor. The protein is strictly regulated in eukaryotic cells by a number of mechanisms, which include $CRL4^{Cdt2}$-mediated ubiquitylation in S phase and after DNA damage. In Metazoa, this ubiquitylation of Cdt1 is triggered by the DNA binding of proliferating cell nuclear antigen (PCNA). Using bimolecular fluorescence complementation, we show that fission yeast Cdt1 interacts with PCNA *in vivo*. In addition, we demonstrate that DNA loading of PCNA is needed for Cdt1 proteolysis after DNA damage and in S phase. Activation of this pathway by ultraviolet (UV)-induced DNA damage requires upstream involvement of nucleotide excision repair or UVDE repair enzymes. Unexpectedly, two non-canonical PCNA-interacting peptide (PIP) motifs at amino acid positions 28 and 301, both with basic downstream residues, function redundantly in Cdt1 proteolysis. Finally, we show that poly-ubiquitylation of PCNA, which occurs after DNA damage, reduces Cdt1 proteolysis. This may provide a mechanism for fine-tuning the activity of the $CRL4^{Cdt2}$ pathway towards Cdt1, allowing Cdt1 proteolysis to be more efficient in S phase than after DNA damage.

THE N-TERMINAL SERINE/THREONINE-RICH DOMAIN OF MCM4 MODULATES ORIGIN ACTIVATION AND REPLICATION FORK PROGRESSION

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In *Saccharomyces cerevisiae*, origins of DNA replication are licensed in G1 by formation of a pre-replicative complex that includes the hexameric mini-chromosome maintenance (MCM) complex (composed of Mcm2-Mcm7). The MCM complex forms the core of the CMG helicase that unwinds the DNA duplex during replication. Activation of licensed origins depends on several kinases. The S phase CDKs activate factors essential for facilitating assembly of active helicase complexes, while DDK removes an intrinsic barrier within Mcm4, a subunit of the MCM complex, that gates origin activation. Checkpoint kinases also regulate origin firing.

The structurally disordered N-terminal serine/threonine-rich domain (NSD) of Mcm4 is a target of multiple kinase regulatory pathways. The proximal portion of the Mcm4 NSD (residues 74-174) contains the essential target of DDK and the distal portion is phosphorylated by additional kinases, such as Mec1 and CDKs. We have found that the distal portion of the Mcm4 NSD (residues 2-145) is required for robust checkpoint responses through hyper-phosphorylation of Rad53. To uncover alterations in patterns of DNA replication that are not readily identified by traditional approaches, we have adapted NextGen sequencing technologies to establish DNA replication profiles at the whole-genome scale. Analysis of DNA replication profiles in mcm4 mutants show that mutants lacking the proximal NSD fire late origins frequently in the presence of hydroxyurea. These mutants also exhibit altered replication fork progression. Single-molecule DNA fiber analysis have given similar results. Thus, the Mcm4 NSD participates in modulating both origin firing and replication fork progression. Together, our data suggests that the Mcm4 NSD, a unique feature of eukaryotic MCM helicase, plays an important role in integrating multiple signals to regulate origin activation and replication fork progression in response to the environment.

SHARED AND DISTINCT FUNCTIONS OF HUMAN RECQ HELICASES WRN AND BLM IN REPLICATION FORK PROGRESSION, RECOVERY, AND NASCENT STRAND PROCESSING AFTER REPLICATION STRESS

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Human WRN and BLM genes are members of the conserved RECQ helicase family and mutations in these genes are associated with two distinct genomic instability disorders -- Werner and Bloom syndromes in humans. WRN and BLM proteins are implicated in DNA replication, recombination, repair, telomere maintenance, and transcription. We were interested in delineating common and unique functions of these related RECQ helicases within the cellular replication stress response pathway. Using our microfluidics-assisted display of stretched DNA, we analyzed replication tracks in human fibroblasts depleted of either WRN, BLM, or both RECQ helicases, to show that WRN and BLM contribute additively to normal replication fork progression, and epistatically, in a RAD51-dependent pathway, to resumption of replication after short-term arrests by a replication-stalling drug hydroxyurea (HU). We also show that WRN but not BLM is required to support fork progression after HU. However, resumption of replication by forks appears not to be sufficient for timely completion of the cell cycle after HU arrest. While depletion of either BLM or WRN delayed fork recovery to an equal degree, BLM depletion led to a far more prolonged delay of cell division, than WRN depletion. We determined that breakage susceptibility of DNA at sites of fork stalling could not account for this differential behavior since it was not substantially elevated and was not different in BLM versus WRN-depleted cells. Similarly, activation and deactivation of the S phase checkpoint, as measured by the abundance of CHK1 S317P and S345P species, proceeded with similar kinetics in WRN or BLM-depleted cells and thus could not explain BLM-specific delay of cell division. Instead, BLM but not WRN depletion caused an elevation in extensive chromatin bridging in the first division after HU. Based on these findings, we propose a model by which small perturbations in recovery of replication forks during S phase can result in problems of chromatin segregation in mitosis. Finally, by examining the fate of DNA synthesized by recovering replication forks within the first hour after release from HU in normal or transformed human fibroblasts, we found that some of this DNA appears to be replaced or degraded within an hour of being synthesized, a phenomenon we refer to as nascent strand processing, NSP. We found that WRN and BLM differentially affect NSP, and we will discuss its genetic determinants and potential mechanisms.

IDENTIFICATION AND CHARACTERIZATION OF QUINOLONE-BASED INHIBITORS OF THE MCM2-7 COMPLEX

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The Mcm2-7 complex is a toroidal heterohexameric ATPase that forms the core of the eukaryotic replicative helicase. Genetic and biochemical evidence from our lab and others suggests that the six component ATPase active sites are functionally and structurally distinct. As most mutations in these active sites are lethal, the identification of specific small molecule inhibitors for the different Mcm2-7 ATPase active sites would be of both considerable benefit in the study of the Mcms and have potential utility in the development of new chemotherapeutic drugs. As a general proof of principle, we have identified the fluoroquinolone ciprofloxacin as a selective inhibitor of Mcm2-7. To identify molecules of greater affinity and specificity, we screened a small library of related molecules simultaneously against purified Mcm2-7, the DNA-unwinding competent Mcm467 subcomplex, and the related SV40 Large T-antigen (TAg). As hoped, additional compounds were isolated that were stronger inhibitors of Mcm2-7, some of which were more specific for Mcm2-7 than TAg. These molecules likely target one or more of the complex's six AAA+ ATPase active sites, since their inhibitory effects can be negated by increasing the ATP concentration of an in vitro helicase assay. In vivo, several of these compounds inhibit the growth of yeast and human cells at concentrations consistent with their ability to inhibit Mcm2-7 in vitro. At least one of these inhibitors blocks cell-cycle progression in drug-sensitive Saccharomyces cerevisiae strains similar to that observed for many Mcm conditional alleles, consistent with a role of these drugs in disrupting DNA replication.

EXO5 IS A NOVEL HUMAN SLIDING 5'-EXONUCLEASE ESSENTIAL FOR GENOME STABILITY

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We have previously characterized a 5'-exonuclease from S. cerevisiae that is essential for mitochondrial genome maintenance. However, no detectable nuclear phenotypes were associated with EXO5 deletions. We have now characterized the S. pombe, mouse, and human Exo5 homologs. Human Exo5 localizes to the nucleus in response to DNA damage, and is involved in UV- and interstrand crosslink DNA repair. Knockdown of Exo5 in human cells, or deletion of S. pombe Δexo5 leads to increased sensitivity to various DNA damaging agents including UV- irradiation, cis-platin, and mitomycin C. A S. pombe Δ pso2 Δexo5 double mutant is exquisitely sensitive to cis-platin. A biochemical analysis of Exo5 from human, mouse, and S. pombe indicates a conserved nucleolytic activity for Exo5 that is divergent from that in S. cerevisiae. Exo5 is a 5' single-strand specific exonuclease that has the propensity to slide along the single-stranded DNA prior to strand scission, hence the designation: Sliding 5'-exonuclease. These proteins also share four conserved cysteine residues that form a structural motif for an iron-sulfur complex. The likely presence of an iron-sulfur cluster was indicated by UV-VIS spectroscopy.

A NOVEL KINASE-INDEPENDENT FUNCTION OF CHK1 IN THE REPLICATION OF DAMAGED DNA

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Chk1 is broadly known as a central kinase involved in the cell cycle response to the accumulation of damaged DNA. The kinase activity of Chk1 is also essential for unperturbed DNA replication. The activation of Chk1 is totally dependent on its phosphorylation by ATR and in general, the negative regulation of ATR or Chk1 cause similar consequences in cells. Intriguingly, using DNA combing technology and specific siRNA-dependent downregulation, we have observed that Chk1 contribution to the maintenance of fork progression after an UV insult is more robust when compared to ATR. In line with this, we found that the kinase domain of Chk1 is not required for the maintenance of fork progression immediately after UV irradiation. Instead, a recently identified motif of Chk1 involved in its release from chromatin is necessary to maintain replication fork progression after UV-induced DNA damage. Moreover, this motif of Chk1 also promotes the organization of the specialized polymerase pol η into subnuclear focal structures. This is particularly interesting since pol η sustains DNA synthesis by using UV-damaged DNA as templates for replication. Together, our data put forward a potential kinase-independent contribution of Chk1 to the replication of damaged DNA.

AID BINDS COOPERATIVELY WITH UNG AND MSH2-MSH6 TO IG SWITCH REGIONS DEPENDENT UPON THE AID C TERMINUS

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Activation-induced cytidine deaminase (AID) is induced in B cells during an immune response and is essential for both class switch recombination (CSR) and somatic hypermutation (SHM) of antibody genes. The C terminal 10 amino acids of AID are required for CSR but not for SHM, although their role in CSR is unknown. Using retroviral transduction into mouse splenic B cells, we show that the C terminus is not required for S region DSBs, and therefore functions downstream of DSBs. Using chromatin immunoprecipitation, we show that AID binds cooperatively with UNG and the mismatch repair proteins Msh2-Msh6 to Ig Sμ and Sγ3 regions, and this depends on the C terminus and the deaminase activity of AID. We also show that mismatch repair does not contribute to the efficiency of CSR in the absence of the AID C terminus. Although it has been demonstrated that both UNG and Msh2-Msh6 are important for introduction of S region DSBs, our data suggest that the ability of AID to recruit these proteins is important for DSB resolution, perhaps by directing the S region DSBs toward accurate and efficient CSR via non-homologous end joining.

ANALYSIS OF DOUBLE HEXAMERIC HELICASE ASSEMBLY PROVIDES MECHANISTIC INSIGHT INTO LOCAL ORI MELTING AND TEMPLATE UNWINDING.

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Preparation of DNA templates for initiation of DNA replication requires the sequential utilization of activities that recognize the origin of DNA replication (ori), melt the DNA duplex, and unwind the DNA template. The biochemical details of these events are poorly understood in higher organisms but initiator proteins from a handful of eukaryotic viruses can perform these processes.

The papillomavirus E1 protein forms two stable complexes with ori. One, the double trimer (DT), is a direct precursor for the E1 double hexamer (DH), which has helicase activity and unwinds the ori DNA. By following the transition from DT to DH we can provide a detailed description of the changes in template structure that accompany template melting and helicase loading.

Binding of E1 to the ori utilizes the E1 DBD for site-specific binding to E1 binding sites (E1BS) and an element in the helicase domain, the β-hairpin, for attachment of the helicase domain to the flanking sequences. The attachment of the helicase domain results in permanganate reactivity at the attachment sites. In a process that requires ATP hydrolysis, the E1 DT complex untwists the DNA between the two attachment sites, generating permanganate reactivity extending across the E1 BS and resulting in local melting of ~30bp. This local melting is necessary for formation of the DH.

The untwisting can be interrupted by nicks in the ori template between the attachment sites for the E1 helicase domains, by mutations in the β−hairpin, and by numerous mutations in the interface between individual subunits in the E1 hexamer. One mutation, which falls in the central channel, arrests with untwisted DNA, and fails to form the DH helicase and fails to unwind the ori DNA. Based on our comprehensive analysis of E1 we propose a model where iterative linear movement of the β-hairpins is converted into rotation of the DNA duplex by the interaction of the β-hairpins with the helical grooves in the DNA.

IRON-SULFUR CLUSTERS AND ZN BINDING MOTIFS IN EUKARYOTIC B-FAMILY DNA POLYMERASES MEDIATE SUBUNIT INTERACTIONS AND PROCESSIVE REPLICATION

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Eukaryotic B-family DNA polymerases α, δ, ε, and ζ have a highly conserved C-terminal domain that contains two cysteine-rich motifs, CysA and CysB, each with four appropriately spaced cysteines for potentially coordinating metal ions. We show here that all four DNA polymerases contain at least one $[4Fe\text{-}4S]^{2+}$ iron sulfur cluster in the C-terminal domain. Extensive mutational analysis of Pol3, the catalytic subunit of Pol δ, indicates that this iron-sulfur cluster resides in the CysB motif of Pol3. Cys -> Ser mutations in CysB of Pol δ result in complete loss of iron-sulfur occupancy and in loss of subunit interactions. Based on sequence and structural conservation, we propose that the CysB motif of the other eukaryotic B-family DNA polymerases also contain a $[4Fe\text{-}4S]^{2+}$ cluster. The CysA cluster shows a classical Zn ribbon pattern ($CX_2C\text{-}CX_2C$). Cys -> Ser mutations in CysA of Pol3 do not result in loss of iron-sulfur occupancy nor in subunit-subunit interactions, however, the mutant Pol δ complex is defective in PCNA-dependent processive DNA replication. Thus, we have identified a novel PCNA-binding motif in Pol δ that specifically mediates replication processivity.

THE MUTS COMPLEX INHIBITS NUCLEOSOME ASSEMBLY AROUND MISMATCH SITES IN XENOPUS EGG EXTRACTS

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The mismatch repair (MMR) system corrects replication errors to protect genomic information from mutations. The MMR reaction initiates with recognition of replication errors such as base-base mismatches by the heterodimeric MutS complex. The MutSα complex, composed of Msh2 and Msh6, recognizes base-base mismatches and small insertion/deletion loops, and the MutSβ complex, composed of Msh2 and Msh3, recognizes large insertion/deletion loops. The MMR system then identifies the erroneous newly synthesized strand by searching signals that discriminate the strands. In eukaryotes, single strand breaks such as gaps or nicks function as the strand discrimination signals. The strand discrimination step likely involves translocation of the MutS complex along DNA strands. After recognition of the signals, the MutL endonuclease is activated to initiate removal of the erroneous bases on the newly synthesized strand. Importantly, the post-replicative MMR system operates after DNA synthesis, at the time when re-assembly of chromatin also takes place. However, how the MMR reaction occurs in the context of chromatin is not understood.
To elucidate how the MMR reaction deals with chromatin structure, we employed nucleoplasmic extracts (NPE) of Xenopus eggs. NPE is an undiluted extract of nuclei assembled in Xenopus egg extracts, and it efficiently recapitulates reactions in the nucleus such as chromatin assembly in vitro. We found that NPE promotes strand-specific repair of base-base mismatches and insertion/deletion loops when a gap is given as a strand discrimination signal. In the absence of a gap, both MutS and MutL bound to DNA in a mismatch- dependent manner, but removal of the mismatch bases was not initiated, suggesting that the MMR reaction arrests at the strand-discrimination step. Under this condition, interestingly, nucleosome assembly around mismatch sites was strongly inhibited. Immunodepletion experiments revealed that the inhibition of nucleosome assembly around base-base mismatches depends on Msh2 and Msh6, but not on the MutL complex. These results suggest that the MutS complex inhibits local nucleosome assembly around mismatch sites at an early step of the MMR reaction, likely to promote the strand-discrimination reaction. We propose that the MutS-dependent inhibition of chromatin assembly ensures efficient MMR reaction in the context of chromatin replication.

ORIGIN FIRING IS FINE-TUNED BY FORK SPEED INDEPENDENTLY OF CHK1 STATUS

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The amount of DNA polymerized in a given period of time relies on both the speed of replication forks and the density of active origins. In mammalian cells, depletion of checkpoint kinase 1 (Chk1) reduces fork movement and activates latent origins, showing that Chk1 regulates the replication dynamics in unchallenged cells. It was recently proposed that Chk1 represses the firing of some origins thereby reducing the consumption of replication factors, which maintains their concentration at a level permitting the forks to travel fast. An alternative hypothesis could be that Chk1 increases fork speed and, consequently, turns off some origins by a mechanism called compensation. In support to this hypothesis, Rad53p the functional homolog of Chk1 has been involved in the control of deoxyribonucleotide pools (dNTPs) in budding yeast. Whether Chk1 primarily controls origin firing or fork movement thus remained to be determined. We show here that addition of nucleotide precursors in the growth medium alleviates fork slowing in Chk1-depleted cells, suggesting that Chk1 also controls the availability of dNTPs for the replication machinery in mammalian cells. Strikingly, precursor addition also suppresses the consequences of Chk1 depletion on origin density. Thus, the spacing of initiation events depends solely on fork movement, not on a direct effect of Chk1 at latent origins.

PCNA BINDING BOX-LIKE SEQUENCES OF REV3 ARE REQUIRED FOR REV3/REV7 COMPLEX TO INTERACT NOT ONLY WITH PCNA BUT ALSO WITH REV1 IN FISSON YEAST

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DNA molecules in cells are consistently damaged by various genotoxic stresses. DNA damages not only inhibit the transcription but also the replication of DNA if these damages were not removed in time. These inhibitions may cause the cell death or carcinogenesis. Since replicative polymerases can not replicate damaged DNA, cells have evolved a specific mechanism (TLS; translesion synthesis) to bypass the damaged region in DNA replication. TLS is considered to be performed by binding of specialized polymerases to PCNA. In fission yeast, Eso1 (Polη), DinB, Rev1 and Polζ (a complex of Rev3 and Rev7) are identified as TLS specialized polymerases.

Among these TLS polymerases, Rev3 is thought to have unique role; since, Rev3 belongs to B family DNA polymerase and has relatively high processivity. Rev3 is relatively well characterized as an enzyme. However, the mechanism how Rev3 is recruited to damaged region is still unknown.

According to the preceding researches, Rev3 cooperatively works with Rev1 and Rev7 in TLS. We also identified the interactions among Rev1, Rev3 and Rev7 by immunoprecipitation. In addition, each protein was coprecipitated with PCNA. These results implied that Rev1, Rev3 and Rev7 interact with PCNA as a complex. To elucidate the role of Rev3 in these interactions, we initiated a mutational analysis of Rev3.

First we genetically characterized a deletion mutant of *rev3*, *rev3Δ*. *rev3Δ* was sensitive to cisplatin treatment which causes an intra-strand crosslink. Next we analyzed the biochemical property of Rev3Δ. Rev3Δ abolished the interaction of Rev7 to PCNA. On the other hand, Rev1Δ even increased the interaction of Rev7 to PCNA. From these results, we considered that Rev3 is able to bind to PCNA directly.

Taking these results into consideration, PCNA binding box (PIP box)-like sequences of Rev3 were intensively mutagenized. These mutants were sensitive to cisplatin, although the sensitivities were varied. We thoroughly characterized one of the mutants, *rev3FL1335-6AA*. As a genetic analysis, we found that this mutant exhibited a milder sensitivity to cisplatin than *rev3Δ* but the cisplatin sensitivity of this mutant was not lowered in *rev1Δ* background. As a biochemical analysis, we found that this mutation decreased the interaction of Rev7 with PCNA and completely abolished the interaction of Rev7 with Rev1 when immunoprecipitated. These results imply that PIP-like sequences of Rev3 are not only required for binding to PCNA but also to Rev1.

According to this study, we have concluded that Rev3 is able to work as a regulator of TLS besides as an effector of TLS.

A GENOME-WIDE VIEW OF PROTEIN DYNAMICS DURING REPLICATION STRESS IN S. CEREVISIAE

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In *S. cerevisiae* a number of proteins involved in the DNA damage response relocalize within the cell following exposure to DNA damage or DNA replication stress. We used the collection of *S. cerevisiae* strains in which ~4500 genes, representing 75% of the genome, have been fused to GFP to systematically screen for proteins whose abundance or localization changes in the presence of DNA replication stress caused by MMS or HU. Overall, we observe localization changes for 226 proteins and abundance changes for 395 proteins. Changes in protein abundance in response to MMS correlate only modestly with known transcriptional changes, indicating that many of these abundance changes are post-transcriptional. Groups of proteins that undergo similar localization changes are enriched for specific GO functions, protein-protein interactions, and genetic interactions, indicating that they represent functional replication stress response modules. For example, the group of proteins that localize to cytoplasmic foci upon replication stress is enriched for genes that function in mRNA processing, and in particular indicates a role for the LSM complex, a regulator of translation and transcript stability, in the replication stress response. Consistent with this assertion, Lsm1-GFP foci formation in response to HU is regulated by the checkpoint kinases Mec1 and Tel1. Strains lacking LSM1 are HU sensitive, and this HU sensitivity is suppressed by deletion of CRT1. Interestingly, this suppression is mediated through a decrease in histone mRNA abundance and points to a novel role for Crt1 in histone gene expression. We also identified a group of proteins that form nuclear foci in response to DNA replication stress. This group is highly enriched for proteins involved in DNA repair, and includes several unannotated ORFs. Among these, YDL156W is required for MMS resistance, highlighting the ability of our screening method to identify novel regulators of the DNA replication stress response. Together, our data indicate that analysis of protein dynamics following chemical perturbation is a powerful tool for elucidating DNA replication stress response pathways.

ATP-DEPENDENT CHROMATIN REMODELING FACTORS TUNE S PHASE CHECKPOINT ACTIVITY

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The S phase checkpoint response slows down replication in the presence of replication stress such that replication can resume normally once conditions are favorable. Both proper activation and deactivation of the checkpoint are crucial for genome stability. However, mechanisms of checkpoint deactivation have been largely unknown. We show that two highly conserved *Saccharomyces cerevisiae* ATP-dependent chromatin-remodeling factors, Isw2 and Ino80, function to attenuate and deactivate S phase checkpoint activity. Genetic interactions revealed that these chromatin remodeling factors and the Rad53 phosphatases function in parallel in the DNA replication stress response. Following a transient replication stress, a double mutant *isw2 nhp10* displays stronger and prolonged checkpoint activation without experiencing increased replication fork troubles. Isw2 and Ino80 are both enriched at stalled replication forks, and physically and specifically interact with single-stranded DNA binding protein (RPA). Based on these results, we propose that Isw2 and Ino80 are targeted to stalled replication forks via RPA and directly control the amplitude of S phase checkpoint activity and the subsequent deactivation process.

SCF-DEPENDENT REGULATION AND THE INTERACTION WITH CHECKPOINT PROTEINS OF REV1 ARE IMPORTANT FOR DAMAGE BYPASS IN FISSION YEAST.

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Translesion synthesis (TLS) is a specific mechanism to bypass the damaged region in DNA replication. In fission yeast, Eso1 (Polη), DinB, Rev1 and Polζ (a complex of Rev3 and Rev7) are identified as TLS polymerases.
One of these TLS polymerases, Rev1, exhibited an interesting phenotype. Namely the active site mutant showed a mild cisplatin sensitivity compared to a deletion mutant, *rev1Δ*. This result indicates that the existence of Rev1 itself is more important than its activity. To confirm this hypothesis, we first analyzed cell cycle oscillation of Rev1. We found that the amount of Rev1 dropped at G1/S boundary. SCF-dependent degradation is known to work at G1/S; therefore, we suspected that Rev1 is controlled by this mechanism. We examined Rev1 protein amount in deletion mutant of *pop1* or *pop2*, a subunit of SCF ubiquitin-ligase complexes. We observed increased amount of Rev1 in these mutants. We also tested in *mts2* temperature-sensitive mutants, which demolish the function of 26S proteasome. These mutants also up-regulated the amount of Rev1. Since ubiquitination is targeted to Lys reside, we created an internal deletion mutant, Rev1dK, which lacks Lys rich region. This mutation stabilized Rev1 protein. Despite the increment of protein amount, this mutant exhibited cisplatin sensitivity. These results apparently suggest that the destruction of Rev1 at G1/S is important for the function of Rev1.
Even though the potential importance of Rev1 down-regulation had been revealed, the mechanism remained unclear. A recent analysis on siRNA treatments for TLS polymerases suggests that TLS polymerases affect the activation of checkpoint. Thus, we suspected that Rev1 degradation is important for this pathway. First we examined Rev1 interaction with Rad9, a 9-1-1 checkpoint clump subunit, by immunoprecipitation. Not only wild type Rev1 but also Rev1dK interacted with Rad9. Next we analyzed Rev1 interaction with Rad26, a component of ATR kinase, by immunoprecipitation. Rev1 interacted with Rad26 after cisplatin treatment. Surprisingly, Rev1dK failed to interact with Rad26 after cisplatin treatment regardless of the increased amount of protein. Checkpoint-dependent DNA repair of the bypassed region is essential after TLS. Thus, it is plausible that the interaction between Rev1 and Rad26 contributes to link between TLS and repair. Failure to interact with Rad26 could be the primary reason for cisplatin sensitivity of Rev1dK.
Taking these results into consideration, we hereby propose an hypothesis that SCF-dependent degradation of Rev1 at G1/S ensures the cross talk between TLS and checkpoint.

EFFICIENT EXPRESSION AND PURIFICATION HUMAN CLASPIN FROM MAMMALIAN CELLS: DNA BINDING ACTIVITY AND NOVEL PROTEIN INTERACTIONS

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Purification of recombinant proteins of a large size often poses problems of instability or low expression in bacterial or insect cells. Here we established a method for a high level expression of large-sized recombinant proteins in mammalian cells and subsequent purification of the full-length proteins and complex of proteins. We, at first, applied this method to express and purify human Claspin which play important roles in DNA replication checkpoint responses as fork-stabilizing factors, and successfully purified them in functional forms in amount sufficient for enzymatic characterization. Purified Claspin behaves as a monomer and binds preferentially to fork-like DNA. Overexpression of tagged Claspin in mammalian cells facilitated the detection of its interacting factors. Claspin interacts with many factors involved in checkpoint regulation and replication fork machinery, including ATR, ATM, Chk1, Tim, MCM4, MCM10, Cdc45, DNA polymerases α, δ, ε and Cdc7 kinase. We will discuss the potential implication of these findings in architecture of replication fork. Purified Claspin inhibits DNA helicase activity of MCM4-6-7 complex on a synthetic Y-fork structures and facilitates the annealing of the single-stranded DNAs to generate a Y-fork structure. These activities of Claspin are now being examined in more detail. The current system for overproduction and purification of replication factors from mammalian cells is applicable to other proteins that are large and/or insoluble and have been difficult to deal with in other systems.

CIS REGULATORY ELEMENTS CONTROL SPATIAL AND TEMPORAL INITIATION OF REPLICATION

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Eukaryotic DNA replication is a highly regulated process that guarantees the faithful duplication of the genome in every cell cycle. However, mechanisms governing the spatio-temporal regulation of the replication initiation are still poorly understood in metazoa. In *Saccharomyces cerevisiae*, ORC recognizes a specific AT rich DNA consensus element the so-called ARS element. In order to pinpoint an enriched motif inside human replication origins, we analyzed the dataset of 283 origin segments recently mapped in HeLa cells in our laboratory (Cadoret *et al*, 2008). By using several methods aimed at identifying enriched motifs, we found in 80% of the origins a highly significant enrichment of a 22 bp GC rich sequence. Using the avian DT40 cell line we precisely deleted this motif in the well characterized chicken βA promoter/replicator. Origin activity is reduced by four folds after deletion showing the critical role of this motif in origin selection.

Moreover, the nuclear genomes of vertebrates show a highly organized program of DNA replication where GC-rich isochores are replicated early in S phase, while AT-rich isochores are late replicating. GC-rich regions are gene dense and are enriched for active transcription, suggesting a connection between gene regulation and replication timing. Insulator elements can organize independent domains of gene transcription and are suitable candidates for being key regulators of replication timing. We have tested the impact of inserting a strong replication origin flanked by the beta-globin HS4 insulator, on the replication timing of naturally late replicating regions. We find that the HS4 insulator has the capacity to impose a shift to earlier replication. This shift requires the presence of HS4 on both sides of the replication origin. Moreover, we find that the USF transcription factor binding site from the HS4 insulator is sufficient to control replication timing. Previously, it has been shown that the USF binding site, recognized by USF1 and USF2 transcription factors, is responsible for the recruitment of several active histone modifications at the HS4 insulator. Therefore the replication shift observed may be linked to the local chromatin structure. In conclusion, our data identify a consensus motif critical for origin activity in vertebrates and a combination of cis-elements that might constitute the basic unit of multi-replicon megabase-sized early domains of DNA replication.

MCM10 IS REQUIRED BEYOND ASSEMBLY OF THE CDC45-MCM-GINS HELICASE COMPLEX FOR ORIGIN UNWINDING DURING INITIATION OF CHROMOSOME REPLICATION

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The Mcm10 protein is essential for chromosome regulation but its roles remain poorly defined in all species. Previous studies with budding yeast showed that the effects of particular mutations of the MCM10 gene could be suppressed by specific mutations in MCM2-7 genes, indicating a common function. In contrast, however, other studies indicated that Mcm10 has other roles including the stabilisation of DNA Polymerase alpha. We have found that Mcm10 interacts specifically with the inactive form of the MCM2-7 helicase that is loaded at origins of DNA replication during the G1-phase of the cell cycle. Using a novel allele that allows very efficient inactivation of the Mcm10 protein, we show that Mcm10 is essential for unwinding of origins during the initiation step of chromosome replication, but is not needed for assembly of the Cdc45-MCM-GINS helicase complex. It thus appears that inactivating Mcm10 produces an inactive Cdc45-MCM-GINS helicase complex at origins, and these effects are distinct from those produced by inactivation of DNA polymerase alpha. Our findings suggest a novel role for Mcm10, in the activation of the Cdc45-MCM-GINS helicase during the initiation of chromosome replication.

A DUAL DOCKING MECHANISM LEADS TO NANOMOLAR INHIBITION OF MITOTIC CYCLIN-CDK1 BY CDC6

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Cyclin-dependent kinases (Cdks) prevent DNA rereplication during the cell cycle by restricting the replication origin firing licencing to the G1 phase. Cdc6 is a component of the prereplicative complex (preRC) whose synthesis at the end of mitosis is required for preRC assembly. Cdk1-dependent phosphorylation in S phase sends Cdc6 to SCF-mediated degradation. Here we report that Cdc6, while being an efficient substrate for the S-phase specific Clb5-Cdk1 and S/G2-specific Clb3-Cdk1, is a tight nanomolar inhibitor of the mitotic Clb2-Cdk1 complex. We present a unique inhibition mechanism involving dual docking by the phospho-adaptor subunit Cks1 and the hydropobic patch of the cyclin. Phosphorylation of site Thr-7 by Clb2-Cdk1 during the binding event was shown to be essential for tight inhibition via Cks1 subunit. Additionally, an unique leucine-rich motif in Cdc6 was found to be the second element of the inhibitory interaction via Clb2 hydrophobic patch, suggesting that the two docking interactions act synergistically to form a complex with an extremely slow off-rate. On the other hand, Clb5-Cdk1 and Clb3-Cdk1 utilize the hydrophobic patch and the Cks1 for cooperative phosphorylation of the N-terminal sites of phosphodegron by using R/KXL motifs as dockig sites, while binding of Cdc6 to the Clb2-Cdk1 complex shields these sites from Cdk1. To understand the functional implications of the described findings, we present here a systems model predicting that the tight inhibition of the Clb2-Cdk1 complex by Cdc6 is essential for preventing the premature origin firing at the newly forming pre-RCs during mitotic exit.

POSSIBLE ROLES OF RNA IN ORIGIN SELECTION THROUGH TWO DISTINCT MECHANISMS INVOLVING DIRECT INTERACTIONS OF RNA WITH DNA, A TRANSCRIPTION FACTOR AND HUMAN ORIGIN RECOGNITION COMPLEX

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It has been reported that several factors including transcription factors, chromatin structures and histone modifications are involved in selection of replication origins in metazoans. However, the precise mechanism for origin selection remains unclear. Recent studies by Lieberman and his colleagues have suggested that RNA mediates EBNA1-dependent recruitment of human ORC to the viral origin oriP. We thus examined whether purified recombinant human ORC would directly recognize RNA by gel mobility shift assay with a fluorescence-labeled RNA probe. We found that purified human ORC bound to GU-rich RNA. ATP enhanced the RNA-ORC complex formation, and non-hydrolysable ATP analogs exhibited modest effect. AC-rich RNA, GT-rich ssDNA or AT-rich dsDNA did not effectively compete for GU-rich RNA-ORC complex formation, indicating that ORC has sequence preference for its RNA binding and that the binding affinity to RNA is comparable to that to DNA. The protein analyses of the RNA-ORC complexes showed that ORC1 alone, the sub-complex as well as the entire ORC bound to RNA. Indeed, purified recombinant ORC1 was capable of binding to GU-rich RNA, and we found that the region adjacent to the AAA+ domain in ORC1 exhibited RNA binding activity.

We next examined whether RNA would affect DNA binding of ORC by analyzing ORC binding to circular plasmid DNA. PolyG or polyU RNA polymer effectively inhibited ORC binding to plasmid DNA. We found, however, that pre-incubation of plasmid DNA with polyG resulted in a marked increase in ORC binding to plasmid. Although the precise mechanism for the increase is unclear, these results imply the formation of an RNA-DNA complex suitable for ORC recruitment, possibly at a specific site.

We also investigated the activity of a cellular transcription/replication factor AlF-C, which binds to the origin that is located in the promoter region of rat aldorase B gene. We found that AlF-C also bound to RNA. Furthermore, polyG but not polyA stabilized AlF-C-ORC interaction in vitro, implying the possibility that G-rich RNA may mediate AlF-C-dependent ORC recruitment to the cellular origin. In conclusion, human ORC directly binds to RNA, and its RNA binding activity may provide two distinct mechanisms for ORC recruitment; one is by forming a RNA-DNA complex possibly at a specific site, the other is by interaction of RNA with DNA-binding proteins.

DNA ROAD BLOCKS & REPLICATION PLAYERS IN SYNTHESIS OF COMMON FRAGILE SITE SEQUENCES

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Common fragile sites (CFS) are specific genomic loci prone to formation of breaks and gaps that can be seen on metaphase chromosomes. Although they normally exist in individuals as stable chromosomal components, CFS instability is known to be induced by replication stress, and recent studies have uncovered several cellular proteins necessary for their stability. Due to their propensity for breakage, CFS are also frequently deleted or translocated in cancer. Sequence analyses of many CFS have determined that they contain repetitive DNA sequences with non-B DNA potential such as hairpins, triplex structures and bent DNA. FRA16D, located at 16q23.3, is one of the most commonly expressed and well characterized CFS. Previously, our lab showed that the replicative polymerases δ and α are significantly inhibited during synthesis of FRA16D sequences with non-B DNA structure potential (Shah et al., 2009). Additionally, loss of the specialized DNA polymerase η has been shown to cause increased instability at CFS (Rey et al., 2009). We hypothesize that the clustering of DNA elements with non-B DNA structure potential in CFS sequences inhibits replicative DNA polymerases, and that specialized DNA polymerases/replicative proteins are required to complete CFS replication. To test this hypothesis, templates were generated that correspond to regions of FRA16D with non-B DNA potential, including microsatellites, inverted repeats and regions of high DNA flexibility. Synthesis of these templates by polymerase δ and specialized polymerases κ and η was examined with and without associated replication factors using our established biochemical assay, which was modified to better reflect nuclear physiologic conditions. Synthesis quantification showed significant slowing of polymerase δ, particularly at predicted hairpins and mononucleotide repeats. Polymerase η showed similar patterns of slowing at microsatellites, but overall completion of template synthesis was significantly greater than with δ. Polymerase κ showed the least slowing overall, and the greatest ability to completely synthesize FRA16D templates. This suggests that polymerases κ and η contribute to replication at CFS in order to maintain stability in normal cells. Our findings of overall slowed synthesis at FRA16D sequences support a model whereby sequence elements with non-B DNA potential impede replication and contribute to CFS breakage under replication stress by preventing complete replication.

LICENSING OF DNA FOR REPLICATION IN MAMMALIAN ZYGOTES FOLLOWS A DIFFERENT PATHWAY THAN SOMATIC CELLS

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INTRODUCTION: The mammalian one-cell embryo is the only cell in which the maternal and paternal genomes undergo different processes in the same cell. After fertilization the maternal DNA completes meiosis and the paternal DNA is decondensed. Each genome is then undergoes DNA replication within separate pronuclei before eventually existing in the same nucleus in the two-cell stage. The two pronuclei develope asynchronously, with the maternal pronucleus forming slightly more rapidly than the paternal. We tested whether DNA replication licensing of the two pronuclei occurred simultaneously, or whether there are recognizable differences. In somatic mammalian cells, the origin recognition complex (ORC1-6) mediates DNA-protein interactions by recruiting Cdc6, Cdt1 and the minichromosomal maintenance (MCM 2-7) proteins. We examined DNA replication licensing in the one cell embryo and found it was significantly different from that of somatic cells, and that there were differences between the two pronuclei. METHODS: Mouse embryos were created by intracytoplasmic sperm injection (ICSI) to ensure timing of development. The loading of ORC2 and MCM7 were monitored by immunocytochemistry through the two-cell stage. RESULTS: Neither ORC2 nor MCM7 were detected in mature oocytes prior to fertilization. Surprisingly, the first licensing protein to bind chromatin in zygotes was MCM7, which first appeared on the maternal (and polar body) chromatin during anaphase II by 1 hr after fertilization. At this same time point, ORC2 was clearly localized between the separating chromosomes but was not detected on the chromosomes, themselves. Neither protein was visible in the paternal pronucleus. By 2 hrs after fertilization, both MCM7 and ORC2 were visible in the maternal pronucleus. MCM7 was visible on the paternal DNA by 2 hrs after fertilization, but ORC2 did not appear until 3 hrs after fertilization. In S-phase of both the first and second cell cycle, ORC2 disappeared from the pronuclei, but MCM7 remained. During the second cell-cycle ORC2 appeared on the spindles and formed a ring structure surrounding the chromatin during anaphase while MCM7 loaded onto each set of DNA. Both proteins were present on the nuclei after 1hr into G1 of the second cell-cycle. CONCLUSIONS: Licensing of the maternal and paternal pronuclei appears to follow independent pathways with each pronucleus. This suggests licensing is relatively independent of cytoplasmic control. Both pronuclei appear to be fully licensed by 3 hrs after fertilization. It appeared that MCM7 bound the chromatin of the maternal and paternal pronuclei before ORC2. Current models for DNA licensing in somatic cells suggest that ORC2 binding is required for MCM7 loading. Our data suggest that either this is not the case in zygotes. This research was supported by NIH grant HD060722.

MCM10 IS REQUIRED FOR ORIGIN UNWINDING IN *S.CEREVISIAE*

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Previous reports have shown that Mcm10 clearly plays important roles during chromosome replication in eukaryotic cells, however, we have not fully understood the essential role of it yet. By combining a recently developed auxin-inducible degron with the conventional *mcm10-1* ts mutation, we have successfully generated a tight conditional allele in which the expression of a degron fused Mcm10 can be controlled by addition of auxin. The resultant strain, *mcm10-1-aid*, showed a strong defect in the initiation of replication but not in the elongation process, suggesting that Mcm10 plays an essential role in the initiation. Consistent with these results, our quantitative ChIP analysis with Mcm10 showed that the ChIP signal increased at early firing origins in a CDK and Cdc45 dependent manner. Interestingly, we did not observe a strong Mcm10 ChIP signal away from the origins under our experimental condition, implying that Mcm10 might not have a major role for fork progression. We also noted that Polα was not destabilized in our strain.
Our results suggest that Mcm10 plays an essential role either at or after formation of the CMG (Cdc45-Mcm-GINS) complex. A ChIP assay with Cdc45 showed that the Cdc45 ChIP signal increased at origins when they fired and stayed at a high level even at later time points in the absence of Mcm10, whereas it dropped immediately after formation of replisomes in the control. Consistently, we could identify CMG by IP even in the absence of Mcm10, showing that CMG can be formed at origins without Mcm10. On the contrary, we found that RPA loading and the downstream events were not seen in the absence of Mcm10, suggesting that origin unwinding is defective. We therefore propose that the essential role of Mcm10 is to convert assembled CMG to a functional state in the initiation process.

RIBONUCLEOTIDE INCORPORATION INTO DNA DURING LEADING AND LAGGING STRAND DNA REPLICATION

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In reactions containing dNTP and rNTP concentrations present *in vivo*, *S. cerevisiae* DNA polymerases α, δ and ε incorporate rNTPs into DNA in amounts suggesting that rNMPs could be among the most common of all non-canonical nucleotides introduced into the nuclear genome. Pol ε, inferred to be the primary leading strand replicase, also incorporates rNTPs *in vivo*. The rNMPs are normally removed by RNase H2-dependent repair, but a defect in this repair leaves rNMPs in the genome that are subject to attack by topoisomerase 1, ultimately yielding short deletions in repetitive sequences. The deletions often occur in a strand-specific manner, consistent with rNMP incorporation by Pol ε during leading strand replication. We are now determining whether rNMPs are also incorporated in vivo by lagging strand polymerases α and δ, and if so, whether these rNMPs result in genome instability. Variant forms of these polymerases, L868M Pol α and L612M Pol δ, incorporate rNMPs into DNA *in vitro* about 10-fold more frequently than do wild type Pols α and δ. Despite this fact, and in contrast to a 'homologous' RNase H2-deficient pol ε-M644G mutant strain, loss of RNase H2 in polα-L868M and pol δ-L612M strains has little effect on S-phase progression, genotoxin-sensitivity, dNTP pools, or the rate of short deletions in repetitive sequences. Thus, the consequences of rNMPs in DNA appear to depend on whether they are introduced during leading or lagging strand replication. Experiments are in progress to understand this asymmetry.

IN VITRO STUDY OF THE INITIATION OF DNA REPLICATION IN BUDDING YEAST.

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Initiation of chromosomal DNA replication in eukaryote is a highly regulated process and requires a series of events including assembly of distinct protein complexes, unwinding ds-DNA and synthesis of primer RNA and DNA. In budding yeast, all factors involving the initiation seems to be identified and order of their assembly on replication origins is mostly revealed in vivo; Orc1-6, Cdc6, Mcm2-7 form pre-replicative complex (pre-RC) on replication origins in G1 phase, and Sld3, Sld7 and Cdc45 associate with early-firing origins in late G1 phase. When CDK is activated at G1/S boundary, Dpb11, Sld2, GINS and Polε form the pre-loading complex (pre-LC) and the pre-LC is recruited to origin. However, the connection between the events on origin and complex formation is still unclear.

We have purified all replication proteins participating in the initiation step of chromosomal DNA replication in budding yeast. Using these purified proteins as well as the crude extract of the yeast cells, we have tried to reconstitute each step of the initiation in chromosomal DNA replication.

HUMAN RIF1 PROTEIN, A KEY REGULATOR OF THE GENOME-WIDE DNA REPLICATION PROGRAM

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DNA replication is spatially and temporally regulated during cell cycle. Recent reports indicate that the site selection of replication origin firing and its timing during S phase are under developmental and cell type-specific regulation. The chromosomes appear to be organized into distinct replication timing domains, which could specify specific cell type or developmental stages, suggesting that this could be a novel epigenetic mark. However, mechanisms for this epigenetic regulation are totally unknown. Here, we show that Rif1, Rap1 interacting protein 1, is a critical determinant of replication timing program in human cells. Rif1 exclusively localizes in nuclei and tightly binds to chromatin during interphase. Depletion of Rif1 leads to stimulation of initiation events in early S phase and persistent S phase replication foci profiles all though the S phase. Consistent with this, genome-wide determination of replication timing shows dramatic alteration of replication timing domain structures including reversion of the timing, and consolidation and homogenization of replication timing domains in Rif1-depleted cells. Furthermore, replication fork rate and inter-origin distances increase, suggesting that the chromatin loops defining replicons may be enlarged in the absence of Rif1. Our results indicate that Rif1 determines the replication timing domain structures through regulating the chromatin organization within nuclei.

RNAI PROMOTES HETEROCHROMATIC SILENCING THROUGH REPLICATION-COUPLED RELEASE OF RNA POLII

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Heterochromatin comprises tightly compacted repetitive regions of eukaryotic chromosomes. The inheritance of heterochromatin through mitosis requires RNA interference (RNAi), which guides histone modification during the DNA replication phase of the cell cycle. Here, we show that the alternating arrangement of origins of replication and non-coding RNA in pericentromeric heterochromatin results in competition between transcription and replication. Co-transcriptional RNAi releases RNA polymerase II (PolII), allowing completion of DNA replication by the leading strand DNA polymerase, and associated histone modifying enzymes which spread heterochromatin with the replication fork. In the absence of RNAi, stalled forks are repaired by homologous recombination without histone modification. (* These authors contributed equally)

HUMAN FANCA IS A NUCLEIC ACIDS BINDING PROTEIN

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Fanconi anemia (FA) is an autosomal recessive or X-linked disorder characterized by bone marrow failure, developmental defects, predisposition to cancer, and hypersensitivity of crosslinking agents. Fanconi anemia complementation group A (FANCA) gene is one of the 15 disease-causing genes and has been found to be mutated in ~60% of all FA patients. Using the purified human WT-FANCA protein, we report that FANCA possesses intrinsic affinity to nucleic acids. FANCA binds to single-stranded DNA with affinity significantly higher than double-stranded DNA. FANCA also binds to single-stranded RNA with similar affinity to single-stranded DNA. FANCA requires a certain length of nucleic acids for optimal binding. Using DNA and RNA ladders, we determine the minimum numbers of nucleotides required for FANCA recognition are ~30 and ~40 for DNA and RNA respectively. A patient-derived FANCA truncation mutation, Q772X, has diminished affinity to both DNA and RNA. On the contrary, the complementing C-terminal fragment of Q772X, 772-1455, retains the differentiated DNA binding activity (ssDNA>dsDNA), indicating that the nucleic acids binding domain of FANCA is located at its C-terminus where most disease-causing mutations are found.

AND-1 IS ESSENTIAL FOR THE CENTROMERIC NUCLEOSOME ASSEMBLY AND CHROMOSOME CONGRESSION

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The coordination between DNA replication and chromosome segregation is critical for the genome stability. The centromere is an epigenetically designated chromatin domain that is essential for the accurate segregation of chromosomes during mitosis. The incorporation of CENP-A, a histone H3 variant, into chromatin is a fundamental step in defining the centromeric loci and the assembly sites for the kinetochore complex. Unlike canonical histones, new CENP-A is loaded at centromeres in early G1 phase via a complex process involving multiple proteins including histone chaperones. However, the mechanisms by which CENP-A is deposited onto centromeres remain largely unknown. Here we identify And-1 as a key deposition factor required for the assembly of CENP-A nucleosomes. And-1 depletion results in the accumulation of cells in early stages of mitosis with chromosome congression defects. Using immunofluorescence, we found that And-1 downregulation severely compromises the mitotic spindle checkpoint and kinetochore protein assembly at centromeres. To have a mechanistic insight into how And-1 regulates centromere structures, we examined the centromeric assembly of CENP-A by immunofluorescence assays. Our data showed that And-1 depletion significantly impairs the centromeric localization of CENP-A. Consistently, over-expression of And-1 promotes CENP-A chromatin association. Our studies establish an important role of And-1 in the regulation of centromere function and support the notion that there is an ancient link between DNA replication and chromosome segregation

MECHANISM OF INHERITANCE OF POLYCOMB GROUP PROTEINS THROUGH DNA REPLICATION IN VITRO.

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A major class of mechanisms for epigenetic inheritance of gene regulatory information is believed to involve heritable chromatin features. During genome duplication, chromatin based information faces two challenges: 1) the physical disruption of chromatin by passage of the replication fork; 2) the two-fold increase in DNA. Propagation of a chromatin mediated epigenetic state would thus require that information be transferred from parent to daughter, and that, ultimately, the parental structure be duplicated. Polycomb Group (PcG) proteins are believed to maintain epigenetic silencing through modifying chromatin structure. Previously we found that a PcG complex (PRC1) can be transferred from parent to daughter chromatin during DNA replication in vitro and thus could transfer silencing information. Here we dissect the mechanism of transfer. Posterior Sex Combs (PSC), a key subunit of PRC1, is sufficient for transfer through DNA replication. PSC, and PRC1, can bridge nucleosomes by a combination of nucleosome binding and self-association, activities which map to different regions of the protein. Both activities are required for stable chromatin binding and transfer through DNA replication, suggesting transfer could involve bridging of chromatin in front of and behind the DNA replication fork.

NUCLEOSOME-BINDING ACTIVITIES WITHIN THE EUKARYOTIC REPLISOME

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Little is known about how the eukaryotic replisome is able to pass through chromatin and facilitate the preservation of epigenetic histone modifications in the replicated sister chromatids. We showed previously that multiple factors assemble around the MCM helicase at replication forks, in addition to Cdc45 and GINS, including proteins with known roles in regulating fork progression, as well as the FACT complex that helps RNA polymerase pass through chromatin during transcription. Purification of this 'Replisome Progression Complex' from replication forks also revealed the presence of all four core-histones, raising the possibility that the replisome itself might be able to bind to nucleosomes that are released from DNA when the parental duplex is unwound at replication forks.
We have developed an assay for factors that can bind to nucleosomes when they are released from DNA, and found that this activity is shared by FACT and the MCM2-7 helicase, but not by conventional histone chaperones that assemble chromatin *de novo*. Moreover, we found that FACT and the MCM helicase can bind together to the same nucleosome to form a stable complex, and this feature appears to be conserved throughout evolution. Importantly, we show that the association of FACT with the Cdc45-MCM-GINS helicase, and with DNA polymerase alpha, is independent of its ability to bind nucleosomes. It thus appears that FACT and MCM are two replisome components that have the ability to hold nucleosomes released from the parental DNA, and that might potentially play a role in transferring nucleosomes onto the newly replicated DNA. We have mapped the nucleosome-binding activity of the MCM2-7 helicase and explored the *in vivo* consequences of blocking this activity.

PHOSPHORYLATION OF HISTONE H4S47 DIFFERENTIALLY REGULATES CAF-1- AND HIRA MEDIATED NUCLEOSOME ASSEMBLY

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Assembly of histone H3-H4 tetramers into nucleosomes is a critical step in maintaining genome integrity and epigenetic memory following DNA replication, gene transcription and DNA repair. In mammalian cells, canonical histone H3 (H3.1) and H3 variant H3.3 are assembled into nucleosomes via distinct mechanisms. H3.1, along with histone H4, is assembled into nucleosomes in a DNA replication coupled, CAF-1 dependent reaction, whereas H3.3-H4 tetramers are assembled into nucleosomes mainly by the histone chaperone HIRA. Here, we report that phosphorylation of histone H4 serine 47 (H4S47ph) differentially regulates CAF-1- and HIRA-mediated nucleosome assembly. H4S47 is phosphorylated by PAK2 prior to the assembly of H3-H4 into nucleosomes, and this modification, enriched in H3.3-containing nucleosomes, increases the binding of H3.3-H4 to HIRA and reduces binding of H3.1-H4 to CAF-1. Depletion of PAK2 results in decreased H3.3 occupancy and increased H3.1 occupancy at the candidate genes tested, whereas expression of a phospho-mimic H4 mutant, H4S47E, has an opposite effect. These results demonstrate that H4S47ph promotes the assembly of H3.3-H4 and inhibits the assembly of H3.1-H4 into nucleosomes. In addition, we have shown that depletion of PAK2 leads to a global change in gene expression, the degree of which correlates inversely with H3.3 occupancy at transcription starting sites. These studies reveal a novel mechanism by which two major nucleosome assembly pathways are differentially regulated by a kinase involved in cell signaling and suggest that this regulation is important for regulation of gene expression.

NEW HISTONE SUPPLY CONTROLS REPLICATION FORK SPEED

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Duplication of DNA sequence and its organization into chromatin takes place in S phase of the cell cycle. Restoration of chromatin structures on new DNA necessitates a considerable supply of canonical histones at replication sites. Early studies in mammalian cells proposed that supply of new histones is a requirement for DNA synthesis to ensure propagation of defined chromosomal structures (Sariban et al., 1985; Weintraub, 1972). Yet clear evidence of this basic link is still missing. Here we show that histone supply directly controls ongoing DNA replication in human cells. Shortage of canonical histones impedes fork progression, leaving initiation largely unaffected. Electron microscopy and nuclease digestion reveal that up to 6 kb of new DNA is found in a partially assembled immature chromatin state behind paused forks. Surprisingly, these forks do not expose ssDNA and remain stable. Consistently, this chromatin assembly defect remain invisible to conventional checkpoints with the advantage that histone biosynthesis is not further repressed by checkpoint signaling. We find that the sliding clamp, PCNA, accumulates in immature chromatin behind paused forks and that PCNA unloading is facilitated by nucleosome assembly in vitro. We thus propose that de novo nucleosome assembly is required for efficient PCNA recycling at active forks, providing a possible link between fork speed and chromatin maturation. Tight coordination of DNA replication and histone deposition is essential for rapid packaging of new DNA into chromatin, thus the regulatory mechanism described here is likely to have implications for both genetic and epigenetic integrity in dividing cells.

Sariban, E., Wu, R.S., Erickson, L.C., and Bonner, W.M. (1985). Interrelationships of protein and DNA syntheses during replication of mammalian cells. Mol Cell Biol 5, 1279-1286.
Weintraub, H. (1972). A possible role for histone in the synthesis of DNA. Nature 240, 449-453.

ATR-LIKE KINASE MEC1 REGULATES CHROMATIN ACCESSIBILITY AT DNA REPLICATION ORIGINS AND REPLICATION FORKS

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Faithful DNA replication is essential for the maintenance of genetic integrity. Because chromatin strongly inhibits protein-DNA interactions required for DNA replication, and DNA replication is associated with chromatin disassembly and assembly, chromatin and DNA replication are believed to strongly influence each other. However, it is not understood how chromatin structure influences DNA replication nor how DNA replication affects chromatin structure. Here, we have developed a novel approach to assess chromatin accessibility genome-wide. Using this approach, we demonstrate that chromatin accessibility increases around DNA replication forks. Additionally, we present the first evidence that chromatin at early replicating origins is more accessible than at late replicating origins. These results suggest a model in which both the origin firing and the progression of replication forks are facilitated by an increase in chromatin accessibility. Importantly, we find that Mec1, an ATR-like key regulatory kinase in the S phase checkpoint, is required for normal chromatin accessibility at both origins and DNA replication forks. Our results begin to unravel the complex relationship between DNA replication, chromatin structure and the S phase checkpoint, and uncover a previously unknown mechanism by which the key checkpoint kinase Mec1 functions.

DOUBLE-STRAND BREAK REPAIR INDEPENDENT ROLE FOR BRCA2 IN BLOCKING STALLED REPLICATION FORK DEGRADATION BY MRE11

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Breast cancer suppressor BRCA2 is critical for maintenance of genomic integrity and resistance to agents that damage DNA or collapse replication forks, presumably through homology-directed repair (HDR) of double-strand breaks. Using single-molecule DNA fiber analysis, we show here that nascent replication tracts created before fork stalling with hydroxyurea are degraded in the absence of BRCA2 but are stable in wild-type cells. BRCA2 mutational analysis reveals that a conserved C-terminal site, involved in stabilizing RAD51 filaments but not in loading RAD51 onto DNA, is essential for this fork protection but dispensable for HDR. RAD51 filament disruption in wild-type cells phenocopies BRCA2 deficiency. BRCA2 prevents chromosomal aberrations upon replication stalling, which are alleviated by inhibition of MRE11, the nuclease responsible for this novel fork instability. Thus, BRCA2 prevents rather than repairs nucleolytic lesions at stalled replication forks to maintain genomic integrity, and hence likely suppresses tumorigenesis through this novel replication-specific function.

COUPLING OF POLY(ADP-RIBOSE) POLYMERASE (PARP1) AND TYROSYL-DNA PHOSPHODIESTERASE (TDP1) FOR THE REPAIR OF TOPOISOMERASE I (TOP1)-MEDIATED DNA DAMAGE

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A common source of endogenous DNA double-strand breaks (DSBs) is by conversion of single-strand breaks during replication. Once such instance is the conversion of Top1 cleavage complexes (Top1cc) into DSBs. When a replication fork proceeds toward a stalled Top1cc, the extension of the leading strand is terminated with replication fork run-off, resulting in Top1-linked DSBs. Thus, the DNA damage response (DDR) pathways to Top1-induced DNA damage constitute a complex network of factors that carry out the detection and repair of the damage. Poly(ADP-ribose) polymerases catalyze the covalent attachment of poly(ADP-ribose) chains (PAR) to a variety of proteins including themselves and chromatin proteins (histones, XRCC1,Top1). ADP-ribosylation is a key post-translational modification implicated in DDR pathways. Negatively charged ADP-ribose polymers facilitate the recruitment of DDR and repair factors such as XRCC1 and cause PARP to dissociate from the DNA. PARP inhibitors are effective antitumor agents. They trap PARP on damaged DNA and enhance the activity of Top1 inhibitors. How PARP1 facilitates the repair of Top1-induced damage is not fully understood. One of the key repair enzymes for Top1 is Tdp1. Tdp1 hydrolyzes phosphodiester bonds at a DNA 3'-end linked to a tyrosyl moiety. This type of linkage is found in stalled Top1cc, and Tdp1 is implicated in the repair of such complexes. Here we show that PARP1 is activated in response to the Top1 poison camptothecin, with subsequent increase in PAR and Tdp1 levels in human cancer cells. Using pull-down experiments, we demonstrate that PARP1 directly interacts with Tdp1, enhances the catalytic activity of Tdp1, and poly(ADP-ribosylates) Tdp1. The PARP inhibitor, veliparib (ABT-888) abrogates the formation of repair complexes between XRCC1 and Tdp1 and the formation of CPT-induced XRCC1 nuclear foci. Consistent with the PARP inhibitor experiments, PARP1 knockdown cells are deficient in forming Tdp1-XRCC1 complexes and XRCC1 foci in response to camptothecin. Finally we show that ADP-ribosylation increases the half-life of Tdp1 after DNA damage, and induces the formation of repair complexes including XRCC1 and Ligase III. Increased DNA damage with PARP inhibitors is also evidenced from the rapid induction of the camptothecin-induced histone γH2AX, ATM activation and phosphorylation of Tdp1 at serine 81. Together, our findings demonstrate the importance of PARP for Tdp1 activation together with XRCC1 and provide a rationale for the sensitization of cancer cells to Top1-targeted drugs by PARP inhibitors.

A REPLICATION-BASED MODEL FOR GENERATING PALINDROMIC AMPLICONS

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Many models explaining the generation of palindromic amplicons propose double-stranded DNA breaks as the initiating event and repair by a variety of means, e.g., end-to-end fusions, break-induced replication, or non-allelic homologous recombination, as the process leading to amplification. However, amplicons that consist of interstitial segments in head-to-head/tail-to-tail orientation, with retention of distal chromosomal sequences, cannot be explained in any simple manner by such models. Such structures have been found in organisms as diverse as yeast and humans, including some patients with developmental and physical abnormalities who harbor interstitial triplications with an inverted central copy. Based on detailed examination of the molecular structure and junction sequences of one such amplicon in the budding yeast *Saccharomyces cerevisiae*, we derived a model that proposes a simple but underappreciated potential error in replication to explain the generation of an initial hairpin-capped linear intermediate.

The potential error we have identified is the ligation of the 3' end of leading strand to the 5' end of the lagging strand at a replication fork mediated by short, closely spaced inverted repeats. This ligation would generate a "closed" fork that is not capable of extension. In the process of removing the aborted nascent strands by displacement replication, an amplification intermediate would be produced that can generate tandem, inverted repeats by integration into the genome by homologous recombination. We think that this model is noteworthy because (1) the initiating event for rearrangement is not a double stranded break, (2) the strands that make up the displaced "closed" fork would be capable of autonomous replication, and therefore, the creation of the amplification intermediate and its integration back into the genome need not be temporally coupled, and (3) it provides an alternative way to create the initial "bridge" in the McClintock Bridge-Breakage-Fusion cycle. The model furthermore can explain the final structure and the pathway for forming this and other types of amplicons in both yeast and humans. We are in the process of testing specific predictions of the model. Because the model requires the presence of both an origin of replication and short, closely spaced, flanking inverted repeats, we call this model Origin-Dependent Inverted-Repeat Amplification (ODIRA).

DEVELOPMENTAL REGULATION OF ORC BINDING 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 the mechanisms that control proper gene copy number during development are poorly understood. Although Drosophila polytene cells contain multiple copies of the genome, there is differential DNA replication, that is, genomic regions of increased or decreased DNA copy number relative to overall genomic ploidy. We used genome-wide array based comparative genomic hybridization (aCGH) to profile differential DNA replication in isolated and purified larval salivary gland, fat body and midgut tissues as well as adult ovarian follicle cells of Drosophila. In the larval tissues we identify sites of euchromatic underreplication that are common to all three tissues and others that are tissue specific. In the follicle cells, we find six domains of gene amplification.

We have exploited examples of under-replication and gene amplification in differentiating Drosophila tissues to define parameters regulating metazoan ORC binding and replication fork progression. We have mapped ORC binding in the larval salivary gland and ovarian follicle cells, the first demonstrations of ORC binding in primary tissues. Under-replicated regions in the salivary gland are devoid of ORC in this tissue but not in cell culture. Approximately 30% of ORC binding sites are unique to the salivary gland. Strikingly, salivary gland-specific ORC binding sites are underrepresented in the promoters of active genes when compared to the distribution of ORC binding sites in Drosophila cell culture. In the ovarian follicle cells specific genomic regions undergo developmentally programmed gene amplification by re-replication based mechanism. ORC localizes in broad domains at all sites of amplification in these cells.

All sites of underreplication are dependent on the chromatin protein SUUR, a component of a newly identified repressed chromatin state. We find that SUUR does not block origin specification in salivary glands, suggesting it inhibits fork progression. Gene amplification in ovarian follicle cells provides another model to investigate fork elongation. SUUR represses fork progression in the follicle cells, highlighting the effect of chromatin configuration on fork elongation. Through genetic screens we have identified other regulators, isolating two mutants that result in enhanced fork movement. These developmental replication models provide powerful insights into metazoan DNA replication and perturbations that can occur in cancer cells.

THE MID-BLASTULA TRANSITION DEFINES THE ONSET OF Y RNA-DEPENDENT DNA REPLICATION IN *XENOPUS LAEVIS*

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Non-coding Y RNAs are essential for the initiation of chromosomal DNA replication in mammalian cells and cell extracts. We have recently shown that Y RNAs associate with unreplicated chromatin, interacting and co-localising with the origin recognition complex (ORC) and other initiation proteins. Following initiation, Y RNAs are displaced locally form replicated chromatin, consistent with a function as mammalian DNA replication licensing factors (Zhang AT, Langley AR et al, J Cell Sci 124, 2058-2069, 2011).
Here, we investigate Y RNA function in developing *Xenopus laevis* embryos and egg extracts. We show that embryos in which Y RNA function is directly inhibited by injection of antisense morpholino nucleotides (MOs) develop normally until the mid-blastula transition (MBT), but then fail to replicate their DNA and die before gastrulation. Consistent with this observation, Y RNA function is not required for DNA replication in Xenopus egg extracts, but is required for replication in a post-MBT cell line. In early embryos, Y RNAs do not bind chromatin before MBT. In contrast, they associate with interphase nuclei after MBT in an ORC-dependent manner. Y RNA-specific MOs inhibit the association of Y RNAs with ORC, Cdt1 and HMGA1a proteins, suggesting that these molecular associations are essential for Y RNA function in DNA replication. The MBT is thus a transition point between Y RNA-independent and Y RNA-dependent control of vertebrate DNA replication. Our data imply that in vertebrates, the non-coding Y RNAs function as a developmentally regulated layer of control over the evolutionarily conserved eukaryotic DNA replication machinery.

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TURNING ON AND OFF THE HELICASE FUNCTIONS OF THE MCM2-7: BIOCHEMICAL, STRUCTURAL AND GENETIC STUDIES

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We previously showed that the four GINS proteins and Cdc45 activate the helicase activity of the Mcm 2-7 complex (Ilves et al Mol Cell 2010). Further, we speculated that the controlled assembly of such Cdc45-MCM-GINS (CMG) replicative helicase complex defines a central switch for starting the S phase. Single-particle 3D-EM images of the Drosophila Mcm 2-7 complex showed that the complex is in equilibrium between two forms: an open lock -washer with a gap between Mcm 2 and 5, and a planar notched form. The Mcms adopt a planar conformation in CMG, with GINS and Cdc45 constraining and closing the Mcm ring while providing an additional channel on the side of the complex parallel to the long axis of the Mcm 2-7. The central channel in the Mcm ring of CMG is tightened when the complex binds ATP. These differences between Mcm 2-7 and the CMG provide a structural explanation for the activation of the Mcm motor (Costa et al 2011 NSMB). Cooperative interactions between Mcm subunits require tight contact, and mutational data of individual subunits as well as kinetic studies of ATPase activity validate the importance of such allosteric interactions. The CMG cannot bind duplex DNA but can bind forked or circular substrates. The results of mutation analysis of Mcm subunits suggest that the leading strand of the fork passes through the central channel of Mcm2-7 hexamer. Cdc45 shows sequence and structural homology to the prokaryote RecJ nuclease, and we will discuss that in the CMG Cdc45 can be cross-linked to single-stranded DNA. Our crosslinking data also indicate that when the CMG is bound to a forked substrate, duplex DNA does not pass into the central channel. These data lead us to propose that a single strand of DNA passes through the central channel and that the lagging strand might be guided by the DNA-binding domain of Cdc45.

We find that the DmChk2 (Lok) can modify the CMG subunits Mcm3, Mcm4 and Psf2. Such modification interferes with the helicase activity of the purified CMG. We have used deletion mutations and mass spectrometry to define the modified sites and used these data to probe the role of these modifications in potential S phase regulation. A null mutation in Mcm 3 has been created, which can be rescued by a wt Mcm3 transgene and a transgene encoding a form of Mcm3 that cannot be modified by Chk2. Larvae expressing the mutant Mcm3 transgene show decreased survival when irradiated as compared to larvae expressing the wt transgene. We posit that double stranded DNA breaks created by irradiation activate the Chk2 dependent damage response that leads to a slow down or halt of the replication forks.

COUPLING OF THE HUMAN CMG REPLICATIVE HELICASE WITH THE HUMAN POLYMERASE ε ON A ROLLING CIRCLE SUBSTRATE

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DNA replication requires the coupled action of a DNA helicase to unwind double stranded DNA and DNA polymerases that duplicate the template. In eukaryotes, substanstial data suggest that the replicative helicase is the CMG complex, consisting of the Cdc45 protein, the MCM(2-7) complex and the tetrameric GINS complex[1].
We purified the human CMG complex following co-expression of the 11 subunits in baculovirus-infected Sf9 cells. The purified CMG complex possesses 3'-5' helicase activity on both linear and circular DNA fork-like substrates. CMG helicase activity requires ssDNA at the 3'-end of substrates. The CMG helicase activity can be further stimulated by increasing the length of the 3'-tail as well as by the presence of dT within the tail.
In order to model and study DNA replication in vitro, we constructed a rolling circle substrate consisting of a 200-nucleotide minicircle and a hybridized DNA fragment, providing a 5'-tail and a 3'-annealed duplex region. Under the experimental conditions used, the human CMG complex unwinds rolling circle substrates efficiently if the double-stranded region is shorter than 40 nucleotides, while human DNA polymerase ε alone extends the 3'-hydroxyl end until completion of at least one round of the minicircle. The concomitant presence of the CMG complex and DNA polymerase ε, together with the processivity factors RFC and PCNA, produced fragments longer than 10 kilobases (representing more than 50 turns of the circle), suggesting that DNA polymerase ε increased the processivity to the CMG complex. These findings, the first to our knowledge, indicate that rolling circle assays can be carried out using a 3'-5' DNA helicase. The synthetic circle contains only 3 nucleotides on the leading strand and 3 nucleotides on the lagging strand. This bias will provide rapid mean to monitor the simultaneous synthesis of leading (dAMP incorporation) and lagging (dTTP incorporation) strand in vitro.

1. Moyer, S. E., P. W. Lewis, et al. (2006). "Isolation of the Cdc45/Mcm2-7/GINS (CMG) complex, a candidate for the eukaryotic DNA replication fork helicase." Proc Natl Acad Sci U S A 103(27): 10236-41.

CLAMP LOADERS ACTIVELY CLOSE SLIDING CLAMPS ON DNA

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Sliding clamps are ring-shaped protein complexes that encircle DNA and at the same time bind to DNA polymerases to increase the processivity of DNA synthesis. Clamp loaders assemble sliding clamps onto DNA in a multi-step reaction in which clamps are bound and opened, clamps are placed around DNA, and clamps and DNA are released to allow polymerase binding. This mechanical reaction requires ATP binding and hydrolysis by the clamp loader to modulate the interactions of the clamp loader with the clamp and DNA. ATP binding promotes clamp binding and opening as well as DNA binding, and ATP hydrolysis promotes release of the clamp on DNA. Given that sliding clamps exist as stable rings in solution, it has been assumed that clamps rapidly "snap shut" around DNA when released by clamp loaders. Our laboratory has developed separate clamp closing and clamp release assays to measure the relative timing of these two events and to determine whether clamps spontaneously close around DNA or are actively closed by clamp loaders. Our results show that clamp closing is faster than clamp release for both the *E. coli* and *S. cerevisiae* clamp loaders supporting a mechanism in which clamp loaders actively close clamps around DNA prior to releasing the clamps. This active clamp closing is triggered by DNA binding and requires ATP hydrolysis. In contrast, in the absence of DNA or in the presence of a nonhydrolyzable ATP analog, ATPγS, clamps passively dissociate from the clamp loader and the timing of clamp closing and release are the same. If clamps were released prior to being closed, this would leave open the possibility that the clamps could dissociate from DNA before closing, but active clamp closing by clamp loaders eliminates this possibility.

THE REPLISOME UTILIZES OPPOSING MECHANISMS TO COORDINATE CYCLING OF THE LAGGING STRAND POLYMERASE

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Semidiscontinuous replication requires the lagging strand polymerase to rapidly cycle among Okazaki fragments as the replisome moves along DNA. This process requires detachment from the processivity clamp to release the highly processive replicases. Two mechanisms have been proposed to trigger polymerase dissociation: “collision release” where collision with the 5’terminus of the previous fragment leads to polymerase dissociation, and “signal release”, where an extrinsic signal causes polymerase to release before completing an Okazaki fragment. Here we demonstrate that the E. coli replisome utilizes both release mechanisms during uninterrupted DNA synthesis. We find that signal release is not triggered by primer synthesis or clamp assembly, but instead correlates with long Okazaki fragments. Using single-molecule methods, we demonstrate that signal release occurs as a function of force applied to the replisome. These findings suggest a model whereby the replisome intermittently employs signaling release to relieve tension created by large lagging strand loops.

TESTING THE TOOL BELT MODEL FOR PCNA FUNCTION IN HUMAN CELLS

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PCNA is the sliding clamp at the centre of the replicative process. Many key replication enzymes interact with a region of PCNA known as the interdomain connecting loop (IDCL). As a homotrimeric ring, PCNA contains three identical protein binding surfaces. It has been suggested that this permits PCNA to bind three different enzymes simultaneously, and that this might enable PCNA to choreograph enzymatic transitions occurring at the replication fork. In such a model PCNA acts as a replication "tool belt" presenting enzymes successively to their sites of action.

In order to test this model in vivo, we constructed a monomeric, triple-PCNA-fusion protein fused to GFP. We demonstrated that this was able to interact normally with known PCNA partners and that it was properly localised to replication factories in the cell. We then mutated the individual IDCL motifs, singly or in combination, to generate triple-PCNA-fusions with 0,1,2 or 3 IDCL regions intact. These mutations had the expected effect on binding to Fen1 in in vitro pull down experiments. All the constructs were able to localise to sites of DNA replication in human cells, but none of the constructs had a dominant negative effect on cell viability or growth when expressed in the presence of endogenous PCNA. Using siRNA targeted against untranslated regions of the PCNA mRNA we then depleted the endogenous PCNA so that the cells became more reliant on the triple-PCNA-fusion constructs. We show that a single, unmutated, IDCL surface within a PCNA trimer is both necessary and sufficient for PCNA function during replication and the repair of UV light induced DNA damage. This suggests that PCNA is not required to act as a tool belt for protein handovers during these processes.

ROLE OF HUMAN DNA HELICASE B IN REPLICATION FORK SURVEILLANCE AND RECOVERY

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Coordination of chromosomal replication with DNA damage bypass and repair at individual replication forks is essential for genome maintenance, but understanding of these mechanisms remains incomplete. DNA helicase B (HELB), a 5' to 3' superfamily 1 helicase conserved among vertebrates, was previously implicated in chromosomal replication and DNA repair. Here we report that the human HELB ortholog (HDHB) associates with chromosomal origins of replication in early G1 cells and dissociates at G1/S, but is dispensable for bulk DNA replication. However, in S-phase cells exposed to DNA damaging agents, HDHB accumulates on chromatin and co-localizes with RPA. This accumulation depends on RPA, but not on checkpoint signaling, suggesting that RPA may recruit HDHB to stalled forks. Biochemical analysis and NMR spectroscopy reveal direct physical interactions of a conserved acidic motif in HDHB with the basic cleft of the N-terminal domain of the RPA 70-kDa subunit (RPA70N). Mutational analysis of the HDHB motif confirms its role in binding to RPA70N and recruiting HDHB to chromatin. HDHB-silencing significantly reduces replication restart in S-phase cells exposed to hydroxyurea and increases chromosomal instability in cells exposed to aphidicolin. We suggest a role of HDHB in replication fork surveillance and recovery from replication stress. Potential implications of the observations will be discussed.

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G-QUADRUPLEX UNWINDING IS AN EVOLUTIONARILY CONSERVED ACTIVITY OF PIF1 FAMILY HELICASES

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G-quadruplex (G4) DNA is a four-stranded DNA secondary structure formed by Hoogsteen G-G basepairing. G4 structures are hypothesized to have roles in diverse cellular functions. The *Saccharomyces cerevisiae* Pif1 helicase binds G4 motifs *in vivo*, and, in its absence, replication forks slow and chromosomes break near G4 motifs (Paeschke *et al*., 2011, Cell; 145(5):678-91). Spontaneous mutations that abrogate the ability of G4 structures to form eliminate Pif1 binding and relieve replication defects. These data suggest that G4 structures form *in vivo* and must be resolved to allow complete DNA replication. Particular attention has been given to the possible roles of RecQ family helicases in unwinding G4 DNA, as human and *S. cerevisiae* RecQs unwind G4 substrates *in vitro*. However, human and *S. cerevisiae* Pif1 family helicases also unwind G4 structures *in vitro*. Here, we used biochemical and genetic approaches to compare the effects of the *S. cerevisiae* RecQ helicase Sgs1 and Pif1 on G4 DNA. Detailed biochemical analyses of full-length Pif1 and Sgs1 revealed that Pif1 preferentially binds G-rich single-stranded DNA (ssDNA) with high affinity, while Sgs1 preferentially binds poly(dT) ssDNA. *In vitro*, both Pif1 and Sgs1 unwound G4 substrates, but Pif1 did so with a 30,000-fold higher apparent specificity than Sgs1. Remarkably, Pif1-like proteins from five evolutionarily diverse prokaryotes also displayed strong G4 unwinding activity. *In vivo*, we used a modified gross-chromosomal rearrangement (GCR) assay with and without a G4 motif and determined GCR rates in the absence of the Pif1, Rrm3 (Pif1 paralog), Sgs1, and Hrq1 (RecQ4 homolog) helicases. In the presence of G4 inserts, the GCR rate increased two-fold in the *pif1* strain but was unchanged in the other single mutants. Although GCR rates slightly increased in double mutants relative to singles, these increases were not G4-specific, except in the *pif1 rrm3* strain (2700-fold over background). Correspondingly, while Rrm3 did not bind G4 motifs in wild type cells by ChIP, binding was observed in cells lacking Pif1. In *pif1* and *pif1 rrm3* GCR survivors, G4 inserts were altered by mutation, deletion, or recombination such that they are no longer predicted to form G4 structures. Finally, expression of human Pif1 and prokaryotic Pif1 homologs in *S. cerevisiae* decreased the G4-specific GCR rate of the *pif1 rrm3* strain. Together, these data suggest that resolving G4 structures is an evolutionarily conserved function of the Pif1 helicase family.

DEFINING THE ssDNA-BINDING TRAJECTORY OF REPLICATION PROTEIN A (RPA): INSIGHTS FROM SMALL-ANGLE X-RAY AND NEUTRON SCATTERING.

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As the primary ssDNA-binding protein in eukaryotes, Replication Protein A (RPA) serves to protect and organize ssDNA during replication and other DNA transactions. RPA engages its substrates in a progression of three binding modes (encompassing ~10-, ~20-, and ~30-nucleotides respectively). These binding states function as pivot points, where other DNA processing proteins may enter to accelerate, hinder, or reverse progression through RPA's DNA-binding trajectory. We propose that accompanying transitions in the spatial organization of the 7 globular domains of RPA are key to promoting DNA transactions, in particular by manipulations of RPA affinity for ssDNA by stabilization of specific RPA architectures by DNA processing proteins.

Using small-angle x-ray and neutron scattering (SAXS/SANS) on the DNA-binding core of RPA (RPA-DBC) and other multi-domain fragments, we are defining the structural transitions as RPA proceeds through its three modes of DNA binding. These studies reveal a progressive compaction of the modular DNA-binding core upon engaging 10- and 20-nucleotide substrates. However, this compaction is reversed for the 30-nucleotide substrate, resulting in an extended conformation closer to that of the DNA-free protein. Comparison of this data to a range of structural models indicates residual excursions of the ssDNA template away from the binding site may occur, suggesting a means by which DNA processing proteins might access exposed ssDNA. Together, these results support a model for RPA DNA-binding where the DNA-binding core is initially compressed to accommodate emerging ssDNA substrates. Extension out to 30-nucleotides allows RPA to 'spread out' and assume an extended conformation more favorable to manipulation of interactions with ssDNA by other DNA processing proteins. This structurally dynamic 'pathway' for RPA DNA-binding provides a critical foundation for understanding the dynamic progression of DNA processing machinery.

TIPIN/TIM1 COMPLEX MAINTAINS THE STRUCTURAL INTEGRITY OF THE REPLICATION FORK

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The accurate and timely duplication of chromosomes is arguably the most fundamental process underlying cell reproduction. Once two adjacent origins fire, the two converging forks progress until they meet, ensuring complete replication of the DNA segment between them. Forks can stall, however, if they encounter DNA lesions or fork barriers. When this happens, the 'Replication Pausing Complex' stabilizes stalled forks, a checkpoint response is activated to halt cell cycle progression, allowing DNA repair and resumption of replication. Among the molecular players of this response are Tipin and Tim1, which are associated with the replication fork and are required for fork stability, the S-phase checkpoint and restarting stalled forks. However, their contribution in this process is unclear.

We established a cell free systems based on Xenopus laevis egg extract to study the role of Tipin-Tim1 during DNA replication. We identified And1, a factor involved in the loading of Pol alpha on DNA, as new Tipin interacting-protein. The simultaneous depletion of Tipin-Tim1/ And1 from egg extracts impaired DNA synthesis due to a defect in the loading of Pol alpha on DNA. This indicated that Tipin collaborates with And1 in ensuring the stable binding of Pol alpha to chromatin, an essential step in DNA replication.

To better understand this process we have set up experiments based on the use of electron microscopy EM to visualize replication intermediates. We found that in the absence of Tipin/Tim1 complex there is a significant increase in the number of abnormal replication intermediates with reversed forks. In these structures newly synthesized DNA strands anneal to form cruciform intermediates resembling a chicken foot.

Tim1 and Tipin travel with replication forks and may play a structural role within the replisome to ensure concerted unwinding and DNA polymerization. Our data suggest that Tipin/Tim1 protects stalled forks by preventing their conversion to reversed forks. It is possible that these structures result from the uncoupling of DNA unwinding and DNA polymerization due to the loss of polymerase binding to DNA. Inappropriate resolution of these structures might lead to the formation of abnormal DNA intermediates at the stalled forks, which can ultimately lead to genome instability by promoting inappropriate recombination events.

A NOVEL FUNCTION OF THE DNA REPLICATION CHECKPOINT IN MODULATING THE ARCHITECTURE OF REPLICATING CHROMOSOMES.

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The Mec1/ATR checkpoint monitors replication fork integrity to counteract mutations and chromosomal rearrangements. We investigated the checkpoint-dependent mechanisms controlling fork stability following replication stress. We found that ablation of THO/TREX-2 factors or inner basket nucleoporins, mediating mRNA export and gene gating, promotes viability and fork stability in checkpoint mutants.

Mlp1 and other nucleoporins required for gene gating are phosphorylated by checkpoint kinases. We found that gene gating, which physically tethers transcribed chromatin to the nuclear periphery to assist efficient expression, is counteracted by the checkpoint response. Checkpoint mutants retain transcribed genes at nuclear pores likely failing to release topological impediments that drive the reversal of incoming forks. Consistently, relaxation of replicon topological complexity by introduction of a double strand break between a fork and a transcribed unit prevents fork collapse. Mlp1 mutants mimicking constitutive checkpoint-dependent phosphorylation impair gene gating and alleviate checkpoint defects.

We propose that the replication checkpoint assists the progression and stability of replication forks by counteracting gene gating through phosphorylation of key nucleoporins, thus neutralizing topological tension accumulating at nuclear pore tethered genes.

MAP KINASES CONTROL THE STABILITY OF THE CDT1 REPLICATION LICENSING FACTOR DURING G2 AND M PHASE

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Precise and complete genome duplication presents a unique challenge during the cell division cycle. During G1 phase, origins are loaded with an inactive form of the MCM helicase, which licenses origins for subsequent initiation in S phase. Origin licensing requires the Cdt1 protein, and Cdt1 abundance and activity are tightly regulated such that Cdt1 must only participate in licensing during G1 phase. During S phase, Cdt1 is ubiquitinated and degraded as one of the many mechanisms to avoid re-licensing and re-replication. During G2 phase Cdt1 re-accumulates and is abundant from G2 phase through the subsequent G1.

We have discovered that human Cdt1 is a substrate of the stress-activated MAP kinases, p38 and JNK. Both p38 and JNK activities are high during a cellular stress response and during unperturbed G2 and M phases. MAP kinases phosphorylate five positions in Cdt1, and phosphorylation renders Cdt1 resistant to ubiquitin-mediated degradation during S phase. The mechanism of phosphorylation-mediated Cdt1 stabilization is to block Cdt1 binding to the Cul4 E3 ubiquitin ligase adaptor protein, Cdt2. Phospho-mimetic mutations recapitulate the stabilizing effects of Cdt1 phosphorylation, but also reduce the ability of Cdt1 to support MCM chromatin loading. Thus, Cdt1 re-accumulates in G2 phase in a form that is CRL4-resistant but has low origin licensing activity. We have further shown that failure to re-accumulate Cdt1 after S phase ends leads to profound defects in spindle-kinetochore attachments and a block to anaphase onset. Endogenous Cdt1 is localized to kinetochores via association with the Hec1 subunit of Ndc80 during prometaphase and metaphase, and this localization is essential for mitotic progression. We suggest that Cdt1 is stabilized in G2 phase by MAP kinase-dependent phosphorylation to prepare for an essential mitotic function that is distinct and separable from its role in replication licensing during the subsequent G1 phase.

SUBSTRATE RECOGNITION BY THE UBIQUITIN LIGASE CRL4^{CDT2} REQUIRES A DIRECT INTERACTION WITH PCNA

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The E3 ubiquitin ligase CRL4^{Cdt2} is emerging as an important cell cycle regulator that targets numerous proteins for destruction in S phase and after DNA damage, including Cdt1, p21 and Set8. CRL4^{Cdt2} substrates contain a 'PIP degron,' which consists of a canonical PCNA interaction motif (PIP box) and an adjacent basic amino acid. Substrates use their PIP box to form a binary complex with PCNA on chromatin and the basic residue to recruit CRL4^{Cdt2} for substrate ubiquitylation. Using Xenopus egg extracts, we identify an acidic residue in PCNA that is essential to support destruction of all CRL4^{Cdt2} substrates. This PCNA residue, which adjoins the basic amino acid of the bound PIP degron, is dispensable for substrate binding but essential for CRL4^{Cdt2} recruitment. In addition, this PCNA residue is also required for Cdt1 destruction in fission yeast. Our data indicate that the interaction of CRL4^{Cdt2} with substrates requires direct interaction with molecular determinants not only in the degron, but also on PCNA. The results illustrate a potentially general mechanism by which E3 ligases can couple ubiquitylation to the formation of protein-protein interactions.

MCK1P, A YEAST HOMOLOGUE OF GSK-3 KINASE, PROMOTES CDC6P DEGRADATION TO INHIBIT DNA RE-REPLICATION

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Cdc6p is an essential DNA replication factor and 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 takes place only once per cell cycle. After the initiation of DNA replication, pre-RC components such as Cdc6p are phosphorylated by Cyclin/CDK in order to inhibit DNA re-replication. It has been shown that N-terminal region of Cdc6p is phosphorylated by Cyclin Dependent Kinase (CDK) for its degradation. Here we show that Mck1p, a yeast homologue of GSK-3 kinase, promotes Cdc6p degradation. Cdc6p was stabilized in the *mck1* deletion cells during late S phase and mitosis, and overexpression of Mck1p promoted rapid Cdc6p degradation. Mck1p interacted with Cdc6p through C-terminal region. There is a GSK-3 consensus site in CDC6 at C-terminus 368-372 (TPTTS) which is overlapped with CDK consensus site. The protein binding between Cdc6p and Mck1p was abolished in cells which have mutation at CDC6 T368A.

We also obtained evidences that Mck1p inhibits DNA re-replication. The *mck1* deletion genetically interacted with ORC6 or ORC2 phosphorylation mutant. To study the genetic interaction between *MCK1* (a yeast homologue of GSK-3 kinase) and *ORC6* (origin binding protein), temperature sensitive mutant of mck1 was generated by PCR mutagenesis. The temperature sensitive mutant, *mck1-16*, in the combination of *ORC6-rxl* induced moderate DNA re-replication followed by DNA damage checkpoint activation at 37 degree. Combination of *mck1-16* with *MCM7-NLS*, *ORC6-ps* and *ORC2-ps* induced extensive DNA re-replication suggesting that mechanism on DNA re-replication inhibition by Mck1p is additive. However *mck1* deletion did not genetically interact with *CDC6* mutant such as *CDC6ΔNT*. This result confirms that Mck1p and Cdc6p act in the single pathway. Finally *CDC6T368A* cells which contain a mutation at GSK-3 consensus site caused lethality in the *ORC6-rxl* cells.

These results suggest that Mck1 kinase and CDK target Cdc6p for its degradation to inhibit DNA re-replication. We will discuss the molecular mechanism how Mck1p and CDK corporate to trigger complete degradation of Cdc6p.

DDK-DEPENDENT RECRUITMENT OF SLD3, SLD7 AND CDC45 SPECIFIES THE TIMING OF ORIGIN-FIRING IN BUDDING YEAST.

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Chromosomal DNA replication in eukaryotes initiates from multiple origins of replication. Because of the multiplicity, activations of replication origins are likely to be highly coordinated; origins fire at characteristic times with some origins firing on average earlier (early-firing origins) and others later (late-firing origins) in S phase in the budding yeast *Saccharomyces cerevisiae*. However, the molecular basis for such temporal regulation is largely unknown. Here, we show that origin association of low abundant replication proteins, Sld3, Sld7 and Cdc45, is a key determinant for temporal order of firing of replication origins. These proteins form a complex to associate with 'early'-firing origins in G1 phase in a manner that depends on Cdc7-Dbf4 kinase, also called Dbf4-dependent kinase (DDK), which is essential for the initiation of DNA replication. Increased dosage of Sld3, Sld7 and Cdc45 makes the late-firing origins fire earlier in S phase. In addition, the increased dosage of DDK also makes the late-firing origins fire earlier. Thus, the limited association between origins and Sld3-Sld7-Cdc45 specifies the timing of origin firing. Our results unveil not only the molecular basis of temporal regulation of origins, but also the novel level of the control of replication origins in the initiation of DNA replication.

REGULATION OF DNA REPLICATION DURING MEIOSIS IN FISSION YEAST

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Meiosis is a specialized form of cell division. There is no S phase between the two rounds of chromosome segregation (meiosis I and II), which is of fundamental importance for effecting a reduction of ploidy in spore or gamete formation. How DNA synthesis is prevented during this period in fission yeast is unclear. Analysis of levels of replication proteins related to licensing (including Orc4, Orc6, Cdc18, Cdt1, Mcm2, Mcm6), initiation/elongation (Hsk1, Dfp1, Sld2, Sld3, Cdc45, Psf2, Cut5, Cdc23), and regulation of dNTP levels (Spd1, Cdc22, Suc22) identified that Cdc18 and Cdt1 are absent following pre-meiotic S phase, whilst Dfp1 is down-regulated and Spd1 is up-regulated after meiosis I.

Forcing expression of Cdc18 and Cdt1 during the MI-MII interval leads to partial DNA replication after meiosis I (approx 10% of a full round), which is detectable by 5-ethynyl-2'-deoxyuridine incorporation. Spore viability is also reduced if Cdc18 and Cdt1 are present following meiosis I. Over-expression of Cdc18 and Cdt1 also causes Mcm2 rebind to chromatin after meiosis I. However if Cdc18 and Cdt1 are expressed at levels comparable to those in pre-meiotic S phase, Mcm2 chromatin binding is not seen, suggesting that licensing may be inhibited by additional factors. Additional inactivation of Spd1 or stabilization of Dfp1 after meiosis I when Cdc18 and Cdt1 are also expressed does not enhance re-replication. Cells can still proceed into meiosis II when an incomplete round of DNA replication is initiated in the MI-MII interval, implying that there is no DNA replication checkpoint regulating entry into MII in fission yeast.

We conclude that down regulation of Cdc18 and Cdt1 plays an important role in preventing a second round of DNA replication during meiosis in fission yeast. However, there may be other restrictions since limited replication is seen, which may include CDK levels, chromosome condensation or other specialized meiotic controls.

CONTROL OF DNA REPLICATION AND CENTROSOME DUPLICATION BY ORC AND CYCLIN-DEPENDENT PROTEIN KINASES

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In metazoans, equal segregation of genetic material is mainly dependent on normal duplication of both DNA and centrosomes. Like DNA replication, centrosomes are also licensed once per cell cycle to ensure that each daughter cell acquires identical content of replicated DNA. Both DNA replication and centrosome duplication are committed as cells progress through G1 phase and into S phase, but little is known about the cross-talk between the two processes. In the past few years, there is evidence accumulating of the presence of replication proteins at centrosomes. We have previously demonstrated that the human Orc1 protein is present at centrosomes, while its depletion leads to centrosome re-duplication. Furthermore, expression of exogenous Orc1 blocks DNA damage induced centrosome re-duplication. We have found that human Orc1 contains a domain that inhibits the kinase activities of both Cyclin E-CDK2 and Cyclin A-CDK2. We show that Orc1 interacts directly with Cyclin A-CDK2 in a Cy-motif (RxL) dependent manner and this mutant does not block CDK kinase activity, but inhibition of Cyclin E-CDK2 is by a different mechanism. Consistent with this observation, Orc1 inhibits Cyclin A-CDK2, but not Cyclin E-CDK2, phosphorylation of Cdc6, an in vivo substrate of CDKs. We also present evidence that human Orc1 harbors a separate centrosome-targeting domain that shows similarity to the PACT domain present in other centrosome associated proteins. We have named the human Orc1 centrosome-targeting region the OPACT domain. The Cyclin/CDK2 kinase inhibitory domain of Orc1, when tethered to the OPACT domain, localizes to centrosomes and blocks Cyclin E-CDK2-dependent centrosome re-duplication that is induced upon hydoxyurea (HU) treatment of cells. We will discuss the interplay between Orc1 and CDKs in controlling both the initiation of DNA replication and centrosome copy number.

THE PRE-RC ASSEMBLY COUPLES COHESIN ACETYLATION TO THE INITIATION OF DNA REPLICATION IN XENOPUS EGG EXTRACTS

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Cohesion of sister chromatids is crucial for precise segregation of chromosomes. Sister chromatids are held together by the ring-shaped cohesin complex, which is loaded onto chromosomes by the Scc2-Scc4 complex before DNA replication. Experiments in yeast have shown that cohesins have to be loaded onto chromatin before or during DNA replication in order for them to connect sister DNA strands. In addition, the cohesion establishment reaction requires acetylation of two conserved lysine residues in the Smc3 subunit of cohesin by the Eco1 acetyltransferase. Eco1 homologs are conserved from yeast to vertebrates, in the latter of which two Eco1 homologs, Eco1/Esco1 and Eco2/Esco2, are identified. Importantly, Eco1 must be active during S-phase to establish cohesion, and the Smc3 acetylation peaks at S-phase. Therefore, both cohesin loading and cohesin acetylation are tightly coupled to DNA replication. However, the molecular mechanism that links sister chromatid cohesion and DNA replication is not clearly understood.
The cell-free extract of Xenopus eggs is a vertebrate model system that recapitulates essential cell cycle events including DNA replication and cohesion in vitro. We have previously shown that, in this system, the pre-RC recruits Scc2-Scc4 and cohesin onto chromatin via pre-RC-binding of the Dbf4/Drf1-dependent kinase (DDK), which physically interacts with Scc2-Scc4. Here, we report that the pre-RC also recruits the Eco2 acetyltransferase onto chromatin in Xenopus egg extracts. We found that Eco2, but not Eco1, is essential for sister chromatid cohesion and the Smc3 acetylation in Xenopus egg extracts. The Smc3 acetylation requires Scc2-Scc4, indicating that cohesin loading is a prerequisite for this reaction. Interestingly, the Smc3 acetylation is independent of DNA synthesis in Xenopus egg extracts, suggesting that cohesins are acetylated after its pre-RC-dependent loading and before the initiation of DNA replication Consistently, chromatin association of Eco2 requires the pre-RC assembly but not DNA synthesis. Chromatin association of Eco2 is independent of DDK or Scc2-Scc4, and the N-terminal domain of Eco2 is responsible for its pre-RC-dependent loading. These findings demonstrate that the pre-RC converges chromatin loading of cohesin and Eco2 to link the cohesin acetylation reaction to the initiation step of DNA replication in Xenopus egg extracts.

A DNA REPLICATION CHECKPOINT FUNCTION FOR THE COHESION CLAMP LOADER

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Sister chromatid cohesion (SCC) requires several conserved proteins whose loss of function results in defects in cohesion at steps post-cohesin binding, and includes the PCNA clamp loader Ctf18-RFC whose function during S-phase remains ill defined. Using siRNA technology and immunofluorescence microscopy (IF), we examined Ctf18p-depleted HeLa cells (Ctf18- cells) for defects in chromosome segregation, a marker of impaired cohesion, and DNA replication. We found that Ctf18- cells manifest (1) chromosome congression defects, (2) mitotic spindle checkpoint activation and (3) precocious sister chromatid separation, all phenotypes that collectively support a requirement for Ctf18-RFC for cohesion establishment. Impaired S-phase progression within asynchronous cultures of RFC-depleted (Rfc1-), Ctf18-, and Rfc1- /Ctf18- cells was investigated by determining the early, mid, and late S-phase distribution of BrdU and PCNA double-stained cells by IF, which revealed that the clamp loaders play unique roles in temporally distinct stages of S-phase. Whereas a significant increase in the number of cells trapped in early S-phase was observed in Rfc1- cells, a significant increase in the late S-phase population was evident in the Ctf18p-deficient cell population. In contrast to Rfc1- cells, which display activation of both Chk1 and Chk2 kinases, Ctf18- cells fail to activate both, and IF analysis of cells double-stained for either BrdU and phospho-histone H3 Serine 10 (PH3), or PCNA and PH3 revealed that these cells initiate chromosome condensation before completion of DNA replication, and enter mitosis with unreplicated DNA. Most interestingly, Rfc1- /Ctf18- cells manifested Chk1, but not Chk2 activation, and IF analysis of cells double-stained for BrdU and PH3 or PCNA and PH3 indicated these cells undergo initiation of chromosome condensation during S-phase, but not pre-mature entry into mitosis. We postulate that Ctf18-RFC and Chk2 are components of a novel checkpoint that prevents initiation of chromosome condensation before completion of DNA replication. Because both RFC and Ctf18-RFC metabolize PCNA, we asked if the level of PCNA associated with replication foci was dependent on the function of each clamp loader by measuring the mean fluorescence intensity of PCNA staining within nuclei in each transfected cell population. Our analysis suggests that the level of chromatin associated-PCNA is established by antagonistic activities of Ctf18-RFC and RFC, with higher levels present in the absence of Ctf18-RFC and lower levels present in the absence of RFC. This observation supports the idea that Ctf18-RFC functions as a PCNA clamp un-loader.

THE SLX4 AND RTT107 SCAFFOLDS COUNTERACT RAD9 TO PREVENT ABERRANT DNA DAMAGE SIGNALING

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In response to genotoxic stress, a transient arrest in cell cycle progression mediated by the DNA damage checkpoint (DDC) positively contributes to genome maintenance. However, the activity of DDC kinases needs to be tightly regulated during DNA damage responses as aberrant DDC hyperactivation can lead to a persistent and detrimental cell cycle arrest. Despite the importance, the mechanisms for monitoring and preventing DDC hyperactivation are not fully understood. Here we show that the scaffolding proteins Slx4 and Rtt107 in *Saccharomyces cerevisiae* prevent DNA lesions generated during DNA replication from hyperactivating the Rad53 checkpoint kinase. A complex formed by Slx4 and Rtt107 counteracts the checkpoint adaptor Rad9 by physically interacting with Dpb11 and phospho-H2A, two positive regulators of Rad9-dependent Rad53 activation. Cells lacking Slx4 or Rtt107 exhibit Rad53 hyperactivation upon replication stress, and hypomorphic mutations in RAD53 and H2A that reduce DDC signaling were found to rescue the hypersensitivity of slx4Δ or rtt107Δ cells to replication stress. We propose that Slx4 and Rtt107 function as anti-checkpoint factors that ensure the balanced engagement of Rad9 at replication-induced lesions. Our findings reveal a previously unappreciated mechanism for monitoring and controlling DDC activation during DNA replication stress.

CHECKPOINT KINASE-1 PREVENTS CELL CYCLE EXIT LINKED TO TERMINAL CELL DIFFERENTIATION

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Trophoblast stem (TS) cells proliferate in the presence of fibroblast growth factor-4, but in its absence, they differentiate into polyploid trophoblast giant (TG) cells that remain viable but nonproliferative. Differentiation is coincident with expression of the CDK-specific inhibitors p21 and p57; of which p57 is essential for switching from mitotic cell cycles to endocycles. Here we show that, in the absence of induced DNA damage, checkpoint kinase-1 (CHK1), an enzyme essential for preventing mitosis in response to DNA damage, functions as a mitogen-dependent protein kinase that prevents premature differentiation of TS cells into TG cells by suppressing expression of p21 and p57, but not p27, the CDK-inhibitor that regulates mitotic cell cycles. CHK1 phosphorylates p21 and p57 proteins at specific sites, thereby targeting them for degradation by the 26S proteasome. TG cells lack CHK1, and reestablishing CHK1 activity in TG cells suppresses expression of p57 and restores mitosis. CHK1 can also play this role in non-TS cells. Thus, CHK1 is part of a ‘G2 restriction point’ that prevents premature cell cycle exit in cells programmed for terminal differentiation.

TIMING AND SPACING OF UBIQUITIN-DEPENDENT DNA DAMAGE BYPASS

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DNA damage tolerance, also called postreplication repair (PRR), is a pathway that enables cells to overcome replication problems and complete the duplication of their genomes in the presence of DNA lesions. In eukaryotes, the pathway is controlled by ubiquitylation of the replication clamp protein PCNA. Monoubiquitylation of PCNA activates translesion synthesis by specialized damage-tolerant DNA polymerases, whereas polyubiquitylation is required for an alternative, error-free pathway. Ubiquitin-dependent PRR substantially influences the accuracy of replication and the resistance towards genotoxic agents. We have designed an inducible system of damage bypass in budding yeast that has allowed us to analyze the effects of PRR in the course of the cell cycle and to separate its activity from bulk replicative DNA synthesis. We show that PRR can act not only during S phase, but similarly contributes to survival when activated at a time when replication of the genome is largely complete. We find that UV lesions are processed predominantly by translesion synthesis, with error-free damage avoidance acting as a back-up pathway. PRR tracts analyzed by fiber spreading are clearly distinct from replicative DNA synthesis, ocurring in small patches along the chomosome. Our approach can thus reveal the distribution of PRR tracts in a synchronised cell population and allows an in-depth mechanistic analysis of the factors required for damage processing during and after replication.

SOLUTION X-RAY SCATTERING REVEALS MULTIPLE MODES OF ASSOCIATION FOR COVALENTLY-BOUND UBIQUITIN ON PCNA

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PCNA ubiquitination in response to DNA damage leads to the recruitment of specialized translesion polymerases to the damage locus. This constitutes the initial step in translesion synthesis (TLS) – a critical pathway for cell survival and for maintenance of genome integrity. The recent crystal structure of ubiquitinated PCNA (Ub-PCNA) shed light on the mode of association between the two proteins but also revealed that paradoxically, the ubiquitin surface engaged in PCNA interactions was the same as the surface implicated in translesion polymerase binding. This finding implied a degree of flexibility inherent in the Ub-PCNA complex that would allow it to transition into a conformation competent to bind the TLS polymerase. To address the issue of conformational flexibility and determine the structure of Ub-PCNA in solution, we combined multi-scale computational modeling and small angle X-ray scattering (SAXS). This combined strategy revealed alternative positions for ubiquitin to reside on the surface of the PCNA homotrimer, distinct from the position identified in the crystal structure. A mutation originally identified in genetics screens (pol30-113) and known to interfere with TLS, is positioned directly beneath the bound ubiquitin in our models. These computationally-derived positions, in an ensemble with the crystallographic and flexible positions, provided the best fit to the solution-phase scattering, indicating that ubiquitin is dynamically associated with PCNA and capable of transitioning between a few preferred sites on the PCNA surface. The finding of new docking sites and the positional equilibrium of PCNA-Ub occurring in solution provide unexpected insight into previously unexplained biological observations.

EFFICIENT REPAIR OF UV-INDUCED DNA DAMAGE DURING REPLICATION DEPENDS ON DIRECT XPA-PCNA INTERACTION

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Proliferating cell nuclear antigen (PCNA) is essential for vital processes such as DNA replication, DNA repair, cell cycle regulation, chromatin remodeling, and epigenetic. Many proteins interact with PCNA through the PCNA interacting peptide (PIP) -box or the newly identified AlkB homolog 2 PCNA interacting motif (APIM) [1, 2]. The Nucleotide Excision Repair (NER) protein, Xeroderma Pigmentosum group A (XPA), with a central but somewhat elusive role contains a conserved APIM motif [1]. In this study, we show that XPA binds directly to PCNA via the APIM motif. Surprisingly, we detect this interaction between endogenous XPA and PCNA in numerous foci in untreated S-phase cells, suggesting a role for XPA also in replication associated repair. Importantly, the tolerance to UV in XPA $^{-/-}$ fibroblast cells can only be fully reconstituted when the overexpressed XPA has an intact APIM motif. Also, cells without functional XPA-PCNA interaction fail to progress through S phase and accumulate arrested replication forks after UV irradiation. Moreover, we show that overexpression of an APIM-peptide sensitizes cells to UV irradiation, and that this increase in sensitivity is dependent on XPA. Our findings present a novel functional APIM-mediated interaction between XPA and PCNA required for efficient response to UV-induced DNA damage during replication.

1. Gilljam, K.M., et al., Identification of a novel, widespread, and functionally important PCNA-binding motif. J Cell Biol, 2009. 186(5): p. 645-54.
2. Warbrick, E., The puzzle of PCNA's many partners. Bioessays, 2000. 22(11): p. 997-1006.

THE CHOICE OF RECRUITMENT MECHANISM INFLUENCES TRANSLESION POLYMERASE USAGE AND BYPASS OUTCOME ON CPD LESIONS

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In proliferating cells DNA replication will inevitably encounter and stall at unrepaired bulky single strand DNA lesions. The mechanisms available to bypass these lesions include the potentially mutagenic translesion synthesis (TLS) as well as error-free recombination-type processes. We investigated the mechanisms recruiting TLS polymerases via a genetic analysis in the chicken DT40 lymphoblastoma cell line. TLS polymerase recruitment to DNA has been reported to occur either by binding to monoubiquitylated PCNA, or by binding to PCNA through REV1. We transfected cell lines mutant in distinct TLS polymerases or recruitment mechanisms with an episomally replicating plasmid carrying defined synthetic replication stalling lesions, using UV photoproducts as examples. Sequencing of the recovered replication product revealed that PCNA monoubiquitylation and REV1 play distinct non-redundant roles in TLS polymerase recruitment to cyclobutyl pyrimidine dimer (CPD) lesions. Specifically, the frequency of TLS decreases both in the non-ubiquitylable PCNA-K164R point mutant and in the REV1 mutant, and similarities of phenotypes suggest that PCNA ubiquitylation is required to recruit pol η for error-free TLS, while REV1 is required to recruit pol ζ for mutagenic TLS. Importantly, the defect of PCNA ubiquitylation dependent TLS results in a massive increase in post-replicative gaps on the nascent strand opposite the lesion, as detected by PCR on the recovered lesion-containing plasmids. Data demonstrating correlation between the TLS polymerase used, the recruitment mechanism, and the accuracy of CPD lesion bypass will be presented.

NEW INSIGHTS INTO REPAIR-SPECIFIC ROLES OF REPLICATION PROTEIN A

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Replication Protein A (RPA), the major eukaryotic single-strand DNA (ssDNA) binding protein, is essential for replication, repair, recombination, and cell cycle progression. RPA is recruited to stretches of single-stranded DNA generated during DNA replication and repair. The RPA-coated ssDNA acts as a signal for assembly of the replication folk, assembly of repair complexes and checkpoint activation. Defects in RPA-associated cellular activities lead to genomic instability, a major factor in the pathogenesis of cancer and other diseases.

RPA is a heterotrimeric complex with the largest subunit, RPA1, containing the major ssDNA-binding activity. The high affinity DNA binding domain interacts with ssDNA via a series of polar residues and four conserved aromatic residues. Mutation of the aromatic residues does not dramatically decrease ssDNA-binding activity of RPA, but results in a cell cycle defect. We have used a combination of biochemical analysis *in vitro* and knockdown and replacement studies in HeLa cells to characterize the contribution of these aromatic residues to RPA function. The aromatic mutants were able to function in replication and checkpoint activation, but exhibited a defect in DNA repair. Biochemical characterization of these separation-of-function mutants revealed that mutation of the aromatic residues severely decreased binding to smaller ssDNA fragments. These studies suggest RPA is more reliant on the core DNA-binding domains of RPA1 when binding to the short exposed ssDNA regions that occur in DNA repair pathways. We conclude that DNA replication and DNA repair require different RPA-DNA interactions and that these differences may depend on the length of exposed ssDNA intermediates.

Current studies are analyzing the effects of these mutants on individual repair pathways. These studies will determine whether these aromatic residue mutants have defects in repair pathways that have short ssDNA intermediates (NER and BER), but function in recombinational repair which has long ssDNA intermediates. These studies are defining functions of RPA in DNA repair and are contributing to understanding how human cells maintain genome integrity.

FUNCTION AND CONTROL OF TRANSLESION DNA POLYMERASES

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specialized DNA polymerases is an important mechanism of DNA damage tolerance in all domains of life. In eukaryotes, the major mutagenic branch of TLS is carried out by Rev and DNA pol zeta (Rev3/Rev7) and is controlled in part through interactions between the Rev1, Rev3, and Rev7 proteins. The development of cancer drug resistance is a persistent clinical problem limiting the successful treatment of disseminated malignancies. However, the molecular mechanisms by which initially chemoresponsive tumors develop therapeutic resistance remain poorly understood. To test whether resistance to front-line therapy results from mutations introduced by TLS over lesions introduced by DNA damaging chemotherapeutic agents, we utilized a tractable model of B cell lymphoma. As expected, suppression of Rev1inhibits both cisplatin- and cyclophosphamide-induced mutagenesis in cultured lymphoma cells. By performing repeated cycles of tumor engraftment and treatment, we showed that Rev1 plays a critical role in the development of acquired cyclophosphamide resistance. Thus, chemotherapy not only selects for drug-resistant tumor population but also directly promotes the TLS-mediated acquisition of resistance-causing mutations. Platinum-based chemotherapeutic drugs are front-line therapies for the treatment of non-small cell lung cancer. However, intrinsic drug resistance limits the clinical efficacy of these agents. We used a preclinical model of lung adenocarcinoma to investigate whether the effect of impairment of TLS on the response of aggressive late-stage lung cancers to cisplatin. We found that suppression of Rev3 led to a significant extension in overall survival of treated mice. Taken together, our results indicate the potential for inhibiting Rev1/3/7-dependent TLS as an adjuvant treatment during chemotherapy. Both bacterial DinB (DNA pol IV) and mammalian DNA pol kappa display a preferential ability to insert dC opposite N^2-furfuryl-dG. Our efforts to understand the basis of the lethality of DinB overproduction have led to new insights that have implications for the action of bactericidal antibiotics.

BEYOND TRANSLESION SYNTHESIS: POLYMERASE K FIDELITY AS A DETERMINANT OF REPETITIVE DNA STABILITY

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Microsatellite DNA synthesis represents a significant component of genome replication that must occur each cell cycle, yet little is known regarding the fidelity mechanisms operating genome-wide to maintain repetitive sequence stability. We recently published that yeast polymerases pol δ and pol ε holoenzymes do not possess high fidelity for microsatellite DNA synthesis (Abdulovic et al. 2011). In search of other replication factors involved, we examined the in vitro fidelity of human DNA polymerases δ holoenzyme (Pol δ4), κ (Pol κ), and η (Pol η) within [GT] and [TC] alleles. We observed that the human Polδ4 error frequency within both alleles was high, ~3 x 10^{-3}. Surprisingly, we observed that Pol κ is unusually accurate for unit-based indel events at the dinucleotide microsatellites. Thus, the relative hierarchy for polymerase accuracy within microsatellites follows the order: Pol κ ≥ Pol δ4 > Pol η. In contrast, the hierarchy we measured for indel errors within the HSV-tk gene target was: Polδ4 >> Pol κ > Pol η. The magnitude of fidelity difference was greatest between Pols κ and δ4 with the [GT] template. Mechanistically, the error rate for polymerase strand slippage errors is correlated with the degree of polymerase pausing within the repeat. We measured a significantly higher termination probability for Pol δ4 than for Pol κ within the [GT] repeat. We further examined whether Pol κ could compete with Pol δ4 during synthesis by following the differential termination signatures in dual polymerase reactions. We observed that microsatellite-specific termination decreased when Pol κ was added to the reaction, relative to the Pol δ4 only reaction. Pol κ effectively competed with either a moving or stalled Pol δ4 for primer-extension synthesis, even when Pol δ4 was present in a 10-fold molar excess over Pol κ. Our study demonstrates that Pols δ4 and κ can act cooperatively to reduce polymerase pausing at specific DNA sequences, and have complementary error rates that may enhance the fidelity of genome-wide DNA replication. Our study conceptually extends the requirement of Y family polymerases to maintain genome stability beyond accurate replication of DNA lesions to include accurate synthesis of repetitive elements, and challenge the ideology that pol κ is error-prone. We propose that multiple DNA polymerases with complementary biochemical properties function cooperatively to maintain repetitive sequence stability.

NEW PARTNERS OF DNA POLYMERASE ZETA SUGGEST A NOVEL MECHANISM OF POLYMERASE SWITCH

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All eukaryotes possess four B-family DNA polymerases: Pol α, Pol δ, Pol ε and Pol ζ. All are multi-subunit complexes of catalytic and accessory subunits. Pol α functions in initiation and early elongation steps of replication. Pol α is tightly associated with primase and extends RNA primers laid by primase. Pol δ plays a central role in DNA replication and DNA repair in eukaryotic cells. There is good evidence that Pol ε functions in the initiation of replication and in leading strand synthesis in the vicinity of the origins, while its role in bulk replication is less clear. Pol ζ is involved in translesion DNA synthesis. When replicative polymerases encounter replication-blocking lesions, there is a switch to translesion synthesis by damage bypass polymerases, including Pol ζ. Pol ζ performs some types of translesion synthesis and, more importantly, is ultimately involved in the extension of non-canonical primer/template combinations for mutation fixation. Pol ζ is responsible for nearly all mutations induced by DNA damaging agents and by replication stress. These properties put Pol ζ in a central position in the cellular machinery regulating outcomes of DNA damage and in the etiology of various diseases, including cancer.

We explore the structural features of multi-subunit complexes of B-family DNA polymerases and analyze how the inter-subunit interactions and conformational changes regulate the function of these polymerases. In particular, we have found that the iron cluster-binding C-terminal domain of the Pol ζ catalytic subunit recruits novel subunits. The disruption of Pol ζ interaction with these subunits by amino acid changes in the catalytic subunit abrogates Pol ζ function in UV mutagenesis. Based on this finding, we propose a novel model for the recruitment of Pol ζ to sites of translesion synthesis at replication-blocking lesions.

MCM8 AND MCM9 FORM A NOVEL COMPLEX INVOLVED IN RESISTANCE TO DNA CROSSLINKING REAGENTS

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Mcm8 and Mcm9, both of which are well conserved in higher eukaryotic cells, are proposed to have evolved from the ancestor of Mcm2-7 before the last common ancestor of the eukaryotes emerged (Liu et al, Curr Biol, 2009). Previous reports have shown that Mcm8 and Mcm9 are independently involved in chromosome replication. We report that Mcm8 and Mcm9 form a novel complex that is distinctive from Mcm2-7 in chicken DT40 cells. Interestingly, we have successfully generated knockout cell lines of *MCM8* and *MCM9*, indicating that both Mcm8 and Mcm9 do not play an essential role in chromosome replication of mitotic cells. Consistent with our identification of the Mcm8-9 complex, the expression level of Mcm9 is much reduced in cells without Mcm8. Both $MCM8^{KO}$ and $MCM9^{KO}$ cells are slightly defective in growth with higher population in G2-M phases, suggesting that the cells are defective in a process required for maintaining the genomic integrity. These mutants have a similar phenotype with weak sensitivities to IR, UV and MMS. However, reminiscent to the Fanconi anemia (FA) cells, the cells without Mcm8 or Mcm9 are hyper sensitive to DNA crosslinking reagents such as Mitomycin C (MMC) and Cisplatin. Moreover, these cells display an increased rate of chromosomal aberration when treated with MMC. Our current data suggest that the Mcm8-9 complex plays a role either in a process downstream of FANCD2 or in an independent pathway required for resistance against DNA crosslink.

THE *SACCHAROMYCES CEREVISIAE* HFANCM HOMOLOG, SCMPH1, FUNCTIONS IN A CONSERVED INTERSTRAND CROSSLINK REPAIR PATHWAY MEDIATED BY SCRAD5

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Interstrand crosslinks (ICLs) are toxic DNA lesions caused by bifunctional DNA damaging agents like nitrogen mustards. These lesions covalently link complementary DNA strands and block all forms of DNA metabolism, like DNA replication and transcription, which require strand separation. Accordingly, ICLs must be removed to allow cell survival. Several pathways have evolved that can faithfully repair the ICLs and thus maintain genomic integrity. Although several putative ICL repair pathways have been identified, the precise mechanism of each ICL repair process remains enigmatic. One repair pathway, the Fanconi Anemia (FA) pathway, is named for the human disease caused by defects in one of the fifteen identified pathway members which appear to function in ICL repair. In this study, we have used a genetics approach to characterize an evolutionarily conserved yeast ICL repair pathway comprised of the yeast homologs (Mph1, Chl1, Mhf1, Mhf2) of four FA proteins (FANCM, FANCJ, MHF1, MHF2). This pathway is epistatic with the recombination-based DNA damage bypass pathway mediated by Rad5 and distinct from the ICL repair pathways mediated by Rad18 and Pso2. In addition, consistent with FANCM's role in stabilizing ICL-stalled replication forks, we present evidence that Mph1 prevents ICL-stalled replication forks from collapsing into double strand breaks (DSBs). Furthermore, these studies reveal that the FA pathway is functionally conserved in the yeast *S. cerevisiae* and validate the yeast model for future studies that will further elucidate the mechanism of the FA pathway.

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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, east wing, lower level
PIN#: **Press 61300 (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 ($9.00 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