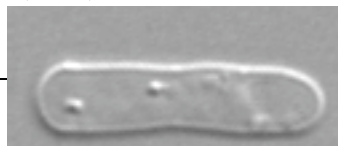


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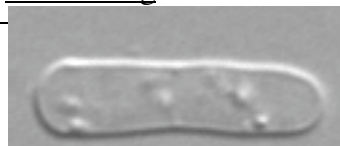


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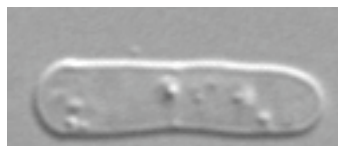
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**btn1, the *Schizosaccharomyces pombe* homologue of the human Batten disease gene CLN3, regulates vacuole homeostasis**

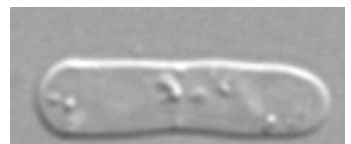
Sandra Codlin<sup>1</sup>, Y Gachet<sup>2</sup>, JS Hyams<sup>3</sup> and SE Mole<sup>1</sup>

<sup>1</sup> MRC Laboratory for Molecular Cell Biology, University College London,

<sup>2</sup> Institut d'Exploration Fonctionnelle des Génomes, Université Paul Sabatier,

<sup>3</sup> Institute of Molecular Biosciences, Massey University

We have cloned the *Schizosaccharomyces pombe* homologue of the human Batten disease gene, CLN3. Mutations in CLN3 cause a severe neurodegenerative disease in children leading to premature death. CLN3, encodes a predicted transmembrane protein and Btn1p shares 30% identity and 48% similarity to its human counterpart. Cells deleted for *btn1* are viable, but have defects in vacuolar homeostasis. *btn1Δ* cells have enlarged and more alkaline vacuoles and are sensitive to the chloramphenicol base ANP. Overexpression of an N-terminally fused GFP-Btn1p construct complements these vacuolar defects. Vacuolar defects of *btn1Δ* cells are also rescued by heterologous expression of GFP-CLN3, proving that Btn1p and CLN3 are functional homologues. In fission yeast, both Btn1p and CLN3 traffic to the vacuole membrane via FM4-64 staining pre-vacuolar compartments, suggesting an endocytic trafficking route for Btn1p. Localisation of Btn1p to the vacuole membrane is dependent on the Ras GTPase Ypt7p, with Btn1p being held in prevacuole compartments in *ypt7Δ*. Cells deleted for both *ypt7* and *btn1* show synthetic lethality at 36°C. In addition, vacuoles in these cells are larger than those of cells deleted for *ypt7* alone. *btn1* must therefore have a functional role in the prevacuolar compartments which also impacts on vacuolar homeostasis. Btn1p is able to localise to vacuoles in cells deleted for *vma1* (a subunit of the vacuolar ATPase). Cells deleted for both *vma1* and *btn1* grow slowly and show synthetic lethality at 30°C. This demonstrates that *btn1* also plays an essential role at the vacuole. The disease severity of expressed Btn1p containing Batten disease-causing mutations (G187A, E295K and V330F) correlated with their effect on vacuolar pH, suggesting that elevated lysosomal pH contributes to the disease process. Cells deleted for *btn1* also exhibit temperature sensitive growth defects resulting in accumulation of neutral lipids and cell death. Fission yeast is therefore a good model system to understand the molecular basis of Batten disease.



**Mining G protein structure-function via genetic analysis in  
*Schizosaccharomyces pombe*.**

E. Douglas Ivey, Fran Taglia, Fan Yang, Matt Ziparo and Charles S.  
Hoffman

Boston College Biology Department

The *S. pombe* glucose response pathway provides a genetically pliable system to isolate and characterize mutations that activate the Gpa2  $G\alpha$  subunit required for adenylate cyclase activation in response to glucose detection. The expression of constitutively activated gpa2\* alleles suppresses the loss of both the Git3 glucose receptor and the Git5-Git11  $G\beta\gamma$  dimer. We have isolated thirteen gpa2\* alleles that alter twelve different residues of Gpa2. This collection of activated gpa2\* alleles represents the largest individual collection of activated  $G\alpha$  subunits and is, in fact, larger than all of the combined activating mutations present in the  $G\alpha$  literature. The majority of identified changes affect residues conserved across  $G\alpha$  subunit families. However, they have not been previously characterized as residues critical for  $G\alpha$  regulation. The activating potential of gpa2\* alleles identified in our primary screen has been reconfirmed by gene replacement into the endogenous gpa2+ locus. Functional analysis of reporter gene activity places the gpa2\* alleles into two fundamental groups: strong or moderate defect in response to glucose starvation. Biochemical and physical properties, for example GTP hydrolysis and GDP exchange rates and GTP-induced conformational changes of purified Gpa2\* protein preparations are used to connect the observed in vivo effects of these mutations with their actual molecular defect. In one case, a mutation affecting a residue distantly located from the guanine nucleotide-binding pocket results in both a GTP hydrolysis defect and an increased GDP- GTP exchange rate. This study provides a wealth of structure-function data that will extend our understanding of G protein-mediated signaling and provides a valuable resource to scientists across multiple disciplines.

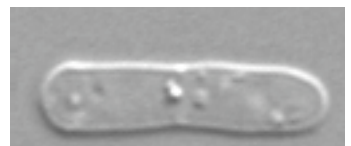
## **Cdc15p undergoes complex changes in its phosphorylation pattern during mitotic progression**

Rachel H. Roberts<sup>1</sup>, Curtis A. Thorne<sup>1</sup>, Robert H. Carnahan<sup>1</sup>, Ian McLeod<sup>2</sup>, John R. Yates III<sup>2</sup>, Bryan A. Ballif<sup>3</sup>, Steven P. Gygi<sup>3</sup>, Jun-Song Chen<sup>1</sup> and Kathleen L. Gould<sup>1,4</sup>

<sup>1</sup>Dept. of Cell and Developmental Biology, Vanderbilt University, <sup>2</sup>Dept of Cell Biology, The Scripps Institute, <sup>3</sup>Dept of Cell Biology, Harvard Medical School, <sup>4</sup>Howard Hughes Medical Institute

During cytokinesis in eukaryotic cells, a vast array of proteins must arrive at the medial region of the cell in a coordinated and well-regulated manner to ensure proper assembly of the contractile actin ring. In *S. pombe*, the PCH-family founding member Cdc15p arrives early in contractile actin ring assembly and is essential for medial recruitment of the formin Cdc12p and the Arp2/3 complex activator Myo1p. We have hypothesized that Cdc15p plays a central and regulated role in acting as a scaffold to recruit actin nucleators in order to promote contractile actin ring formation.

Though we have begun to understand the role of Cdc15p in contractile ring formation, the mode by which Cdc15p is regulated is unknown. Cdc15p is a known phosphoprotein that becomes dephosphorylated upon onset of mitosis and phosphorylated once again coincident with cytokinesis. We show that this dephosphorylation correlates with the ability of Cdc15p to bind Cdc12p and with localization of Cdc15p to the medial ring. In order to address the role of Cdc15p phosphorylation in its function, we have purified Cdc15p in maximally and minimally phosphorylated states and mass spectrometry has been used to identify Cdc15p phosphorylation sites. Three independent analyses have revealed between 36 and 62 phosphorylated serines and threonines, some of which may be cell cycle regulated. Further examination of these potential phosphorylation sites may solidify phosphorylation of Cdc15p as a key regulatory mechanism in contractile ring assembly, stability, and disassembly.



## **Investigating the regulation of proteasome nuclear transport and assembly by int6**

Zhe Sha and Eric Chang

The Breast Center, Baylor College of Medicine

The mammalian int6 gene has been implicated in breast tumorigenesis, but its molecular functions are largely unknown. We have characterized the int6 ortholog in fission yeast *Schizosaccharomyces pombe* and concluded that one of its conserved functions is to influence mitosis control and chromosome segregation via regulating the localization and assembly of the 26S proteasome. The overall objective of my project is to better understand how int6 regulates proteasome localization and assembly. My first aim centers on the fact that many proteasome subunits and int6 itself contain a PCI domain, the latter of which is truncated in mouse breast tumors. I have characterized a gene encoding a novel PCI proteasome subunit, Rpn7, which was isolated as a high copy suppressor to rescue mutant growth defects in yeast int6 mutants. I showed that Rpn7 is essential for normal mitosis and chromosome segregation. Furthermore, during the analyses of pair-wise proteasome and Int6 binding, I found that PCI subunits could preferentially interact with one another. For example, both Rpn5 and Rpn7 bind Rpn9 and Int6 interacts with this complex via binding to Rpn5. To further ascertain the function of the PCI domain, I performed protein sequence analyses and mutagenesis and identified a conserved leucine residue in Rpn7, which is critical for its localization and binding with Rpn9. The importance of this residue has been further verified in Int6. These data support the hypothesis that PCI domain can facilitate protein-protein interaction that is pivotal for the assembly of the proteasome. In yeast, proteasome is predominantly assembled in the inner layer of the nuclear membrane. I examined whether int6 acts through a particular importin alpha or beta subunit to control proteasome nuclear localization. My data showed that deletion of int6 is synthetically lethal with the deletion of kap123, encoding an importin beta, but not with other importin beta, such as kap104 and kap122. Furthermore, Rpn7 is mislocalized in kap123 null mutant but not in any other importin beta or importin alpha mutants. Thus, it is possible that Int6 may interact with Kap123 with substantial specificity to mediate proteasome nuclear localization.

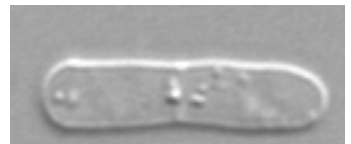
### **Hob3p, an ortholog of human BIN3, is a Cdc42p adaptor that regulates cell division**

Pedro M. Coll, Raul A. Izquierdo, Sergio Rincon and Pilar Perez

Instituto de Microbiología Bioquímica, Consejo Superior de Investigaciones Científicas / Departamento de Microbiología y Genética, Universidad de Salamanca

*cdc42+* is an essential gene required for maintenance of cell morphology and polarization of the actin cytoskeleton. Cdc42p is specifically activated by Scd1p and Gef1p, two guanine-nucleotide exchange factors (GEFs). Scd1p mainly regulates apical growth and Gef1p affects cytokinesis. In a two hybrid screening using *gef1+* as bait, Hob3p was identified as a Gef1p binding partner. Hob3p is a BAR-family protein, ortholog of human BIN3 and *S. cerevisiae* Rvs1p, that regulates *S. pombe* actin organization and cytokinesis. Hob3p binds to the C-terminus of Gef1p and can be co-immunoprecipitated with Gef1p. Additionally, Hob3p interacts directly with Cdc42p bound to either GTP or GDP, and this interaction is independent of Gef1p, suggesting that Hob3p is a Cdc42 adaptor. Genetic data suggests that Hob3p is required for the Gef1p activation of Cdc42p, since *hob3Δ scd1Δ* double deletion is not viable, as *gef1Δ scd1Δ* deletion.

Hob3p localizes to patches forming a ring at the division area, and to some patches at the growth poles. Its localization to the medial ring requires actin polymerization and Cdc15p but is independent of the Septation Initiation Network function. GFP-Cdc42p localizes to the division area and the growth poles. We show that Hob3p is required to localize Cdc42p to the division area. By contrast, Hob1p, the other BAR-family protein in *S. pombe* is not required. Other proteins required for cell separation such as Mid2p, Rho4p, Eng1p, Agn1p or the exocyst complex were properly localized in *hob3Δ* cells. Therefore, the role of Hob3p to localize Cdc42p is specific. The rate of actin ring contraction in *hob3Δ* and wild type cells was analyzed using either Cdc15-GFP or Cdc4-GFP. In both cases, ring contraction was slower in *hob3Δ* than in wild-type cells. We propose that Hob3p is an adaptor required to recruit Cdc42p to the cell division site where this GTPase is activated by Gef1p, and play a role in the contraction of the actomyosin ring.



**Pas1, a G1 cyclin, may link nutrient availability to cell cycle progression downstream of Tsc1/Tsc2 in *S. pombe***

Marjon van Slegtenhorst, Aladdin Mustafa and Elizabeth Petri Henske  
Fox Chase Cancer Center

Tuberous sclerosis complex (TSC) is a genetic disorder characterized by the development of a variety of benign tumors (hamartomas), lung cysts, lung collapse and severe neurological problems. In mammalian cells, the TSC1 and TSC2 gene products function as complex to negatively regulate mTOR signaling. We and others have previously shown that  $\Delta tsc1$  and  $\Delta tsc2$  in *S. pombe*, have defective uptake of amino acids, causing conditional lethality on minimal medium and resistance to canavanine, a toxic analog of arginine.

We report here the results of a genetic screen, in which we identified a G1 cyclin,  $pas1^+$ , that can rescue the amino acid uptake defect in  $\Delta tsc1$  and  $\Delta tsc2$ . To test the hypothesis that  $pas1^+$  functions downstream of  $tsc1^+$  and  $tsc2^+$ , we measured arginine uptake in  $\Delta pas1$  single mutants and  $\Delta pas1\Delta tsc1$  and  $\Delta pas1\Delta tsc2$  double mutants. We found that  $\Delta pas1$  single mutants as well as the  $\Delta pas1\Delta tsc1$  and  $\Delta pas1\Delta tsc2$  double mutants have similar defects in the uptake of arginine. In addition we found that  $pas1^+$  mRNA expression is regulated by  $tsc1^+$  and  $tsc2^+$  in *S. pombe*.

In a second screen, we identified a novel G63D/S165N mutant of the small GTPase Rbh1, the target of the Tsc1/Tsc2 protein complex. The Rbh1 mutant suppresses amino acid uptake in  $\Delta tsc1$  yeast, but not in  $\Delta pas1$  yeast. Hence, Pas1 does not regulate amino acid uptake through Rbh1.

In order to determine whether  $pas1^+$  links nutrient availability to cell cycle progression downstream of the Tsc1/Tsc2 complex, we examined G1 arrest in  $\Delta tsc1$  and  $\Delta tsc2$  yeast. After nitrogen starvation,  $\Delta tsc1$  and  $\Delta tsc2$  yeast had a delay in G1 arrest compared to wild type, which was rescued by deletion of  $pas1^+$ . In summary, we identified the G1 cyclin, Pas1, as a novel regulator of amino acid uptake. Our data support a model in which Pas1 inhibits G1 arrest downstream of Tsc1 and Tsc2, linking nutrient uptake and cell cycle progression in yeast.

## **SREBP Regulates Anaerobic Gene Expression in Fission Yeast**

Bridget Todd, Adam Hughes, Emerson Stewart, John Burg and Peter Espenshade

Department of Cell Biology, Johns Hopkins University School of Medicine

Cholesterol and fatty acid synthesis in mammals are controlled by SREBPs, a family of membrane-bound transcription factors. Our studies identified homologs of SREBP and its binding partner SCAP in *Schizosaccharomyces pombe*, named sre1+ and scp1+. Like SREBP, Sre1 is cleaved and activated in response to sterol depletion in a Scp1-dependent manner. Sre1 is required for anaerobic growth and is rapidly activated in response to oxygen depletion. Microarray analysis reveals that Sre1 is a principal activator of anaerobic gene expression, controlling non-respiratory, oxygen consumptive pathways such as ergosterol, heme, sphingolipid, and ubiquinone biosynthesis. Low oxygen induction of glycolytic genes and repression of mitochondrial oxidative phosphorylation genes largely does not require Sre1. Using chromatin immunoprecipitation, we demonstrated that Sre1 acts directly at promoters of target genes and stimulates its own transcription under anaerobic conditions. Promoter analysis of sre1+ identified a DNA element required for oxygen-dependent, Sre1-dependent transcription in vivo that is homologous to the sterol regulatory element (SRE) from the human LDL receptor promoter. Sre1 bound to both SREs in vitro highlighting the evolutionary conservation between Sre1 and SREBP. Based on these findings, we conclude that Sre1 and Scp1 monitor oxygen-dependent sterol synthesis as an indirect measure of oxygen supply and mediate a hypoxic response in fission yeast. This mechanism represents a new paradigm for oxygen sensing in eukaryotes.



## **Investigation of the Tsc2/Rheb/Tor Signaling Pathway in *S. pombe*: Identification of hyperactive Rheb mutants**

Mellisa Comiso, Urano, J., Guo, L., Aspuria, P.J., Deniskin, R., Tabancay, Jr., A.P., Kato-Stankiewicz, J. and Tamanoi, F.

Department of Microbiology, Immunology and Molecular Genetics,  
University of California, Los Angeles

The Tsc2/Rheb/Tor signaling pathway controls cell cycle and cell size in mammalian cells. The Tor pathway is regulated by various inputs, including energy, nutrients and amino acids. In this pathway, Tsc1 and Tsc2 form a regulatory complex that inhibits the GTPase Rheb, which subsequently activates mTor, the key regulator of protein synthesis. Deregulation of this pathway has been shown to result in human disease such as Tuberous Sclerosis Complex, which leads to the development of benign tumors in the brain, lung and kidney. Homologous components of the mTor pathway exist in *S.pombe*, including the Tsc1/Tsc2 complex, Rheb (Rhb1), as well as two Tor kinases, Tor1 and Tor2, making fission yeast an ideal genetic system for studying this pathway. In this presentation, I will discuss our recent project to identify novel Rhb1 hyperactive mutants. We designed a screen based on the expected hyperactive phenotype of decreased arginine uptake, due to the finding that inhibition of Rhb1 conversely leads to increased arginine uptake. Screening a randomly mutagenized Rhb1 mutant library using high concentrations of canavanine, the toxic analogue of arginine, led to the identification of four different hyperactivating Rhb1 mutations: V17G, S21G, K120R and N153T. These residues are conserved in all Rheb proteins and are within or adjacent to G-box domains that are involved in the recognition of guanine nucleotide binding and GTPase activity. These mutants have an increased GTP/GDP binding ratio compared to wildtype in vitro, as well as in vivo. One of the exciting results concerns the use of these mutants to investigate the interaction of Rhb1 with downstream effectors. Sucrose gradient sedimentation showed that the majority of hyperactive Rhb1 mutants migrated to higher density fractions in a similar profile to Tor2 sedimentation. In contrast, Rhb1wt migrated to the lower density fractions. The interaction of the hyperactive mutant Rhb1K120R with TAP-tagged Tor2 was confirmed by demonstrating that Rhb1K120R is pulled down with Tor2. Furthermore, Tor2 did not appear to interact with the guanine nucleotide-free Rhb1S20N mutant, indicating that Tor2 interaction with Rhb1 is indeed GTP-dependent. This is the first report exhibiting evidence of Rhb1 interaction with Tor2 in a GTP-dependent manner. In order to identify other components of the Tor2/Rhb1 complex, we are currently using TAP-Tor2 for large scale purifications. Mass spectrometry analysis leading to the identification of novel components in the Tor2 complex could contribute to our understanding of how Rheb regulates Tor signaling and could furthermore greatly expand what is currently known of the Tor signaling pathway in *S.pombe* as well as in mammalian cells.



## Genome-Wide Characterization of Fission Yeast DNA Replication Origins

Christian Heichinger (1,3), Christopher J. Penkett (2), Jürg Bähler (2) and Paul Nurse (1,3,4)

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We have mapped the origins of DNA replication (ORIs) during mitotic S-phase and pre-meiotic S-phase of the fission yeast *Schizosaccharomyces pombe*. A total of 401 efficient ORIs were identified which were used on average 29 percent of the time during mitotic S-phase and were spaced on average every 31 kilobases. The same ORIs were used during pre-meiotic S-phase, although with lower efficiency in most chromosomal regions. A further 503 potential ORIs were used less efficiently at eight percent of the time during mitotic S-phase, reducing on average inter-origin distance to 14 kilobases. This data supports the idea of a continuum of ORI activity. ORIs were not defined by a strict sequence consensus but were characterized by A+T-richness, the presence of AT-hook binding sequences and of extended intergenic regions. When the initiation factors *cdc18* and *cdt1* were over-expressed, regions of DNA containing particularly efficient ORIs became over-amplified, suggesting that interactions between these factors and efficient ORIs may be important for the mechanism ensuring that an ORI only fires once in each S-phase.



## **Locations of Replication Origins in the Fission Yeast Genome**

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Previous analyses of DNA replication origins in fission yeast have relied on ARS assays (measurements of the efficiency with which specific DNA sequences support autonomous plasmid replication) and 2D gel assays. Although both assays produce reliable information, each is limited to examination of just a few DNA sequences at a time. For that reason, until now only a few replication origins have been identified and characterized in fission yeast.

Here we report the application of microarray technology to the study of replication origin locations throughout the fission yeast genome. For these experiments, wild-type and checkpoint (*cds1* and *rad3*) mutant cells were blocked in G2, then permitted to reenter the cell cycle in the presence of hydroxyurea (HU). This inhibitor of ribonucleotide reductase blocks the synthesis of new dNTPs and thus blocks DNA synthesis once the pre-existing pool of dNTPs is exhausted. Under these conditions, when wild-type cells enter S phase, replication forks move out from early-firing origins, thus increasing the relative DNA copy number near the origins. In checkpoint-mutant cells exposed to HU, the copy number was also expected to increase near early-firing origins—and also near late-firing origins that are ordinarily prevented by a checkpoint-dependent mechanism from firing in HU-treated cells.

Although our results are still in the early stages of analysis, several conclusions seem evident. First, the sequences previously predicted by Francisco Antequera's laboratory to serve as replication origins do, in fact, frequently function as origins. However, some of them do not serve as origins. In addition many other sequences—not predicted by Antequera—also serve as origins. Some of these appear to be strong origins, and many are weak origins. The total number of detectable origins in the genome, strong and weak, appears to exceed 1000. We are attempting to decipher the features of DNA sequence that enable origin function.

Second, as predicted, some origins that do not fire in HU-treated wild type cells do fire in HU-treated checkpoint-mutant cells. Where studied, these correspond to origins known on the basis of previous studies to fire in late S phase in untreated cells. Surprisingly, however, some origins that fire in HU-treated wild-type cells seem not to fire in HU-treated checkpoint mutant cells. Elucidating the molecular mechanisms that make some origins dependent on a functional replication checkpoint pathway is a challenging problem for the future.

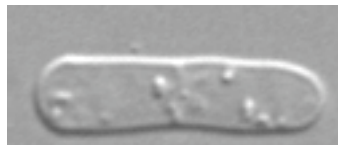
## Origin Regulation In Fission Yeast

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DNA replication in eukaryotic cells initiates with activation of multiple origins along the chromosome during each cell cycle. Origins in humans and fission yeast are inefficient, firing only in fraction of S phases. Thus, in any cell, only a subset of potential origins fire. This observation raises important question about origin selection and regulation. For example, whether origin activation is globally coordinated to ensure that origin firing is distributed evenly across the genome. We have used DNA combing techniques to study the distribution of origin firing and its regulation in *S. pombe* genome. Our results suggest that early origins activation is distributed randomly across the genome. Further more, origin firing is random from one cycle to next, i.e. an origin firing in one cell cycle has no enhanced efficiency to fire in the next cell cycle. The random distribution of origin firing suggest that there is no mechanism that specifically selects origins to ensure even distribution.

However, very little is known about the different factors that are responsible for such observation. A simple hypothesis that accounts for random origin firing is that there is one or more stochastic, rate-limiting steps in the activation of individual origins. It could be any one of the proteins involved in origin firing, from origin recognition to replication initiation. Using a candidate protein approach, we have tested proteins required for origin licensing and firing. Our results show that manipulation of the activity of Hsk1-Dfp1 influences origin firing efficiency. Lower activity of Hsk1 kinase reduced the efficiency of individual origins, whereas over expression of Dfp1 elevated the same. As a direct test for the role of Hsk1/Dfp1 complex in regulating the efficiency of origin firing, we have tethered these proteins close to specific origins. Preliminary data shows that efficiency increased for the origins close to the tethered complex.



## **Converging checkpoint pathways in the regulation of the G2/M transition**

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The control over the transition from G2 into mitosis centers on the regulation of inhibitory Y15 phosphorylation of Cdc2. In response to DNA damage, the Chk1 protein kinase prevents mitotic entry, allowing time for DNA repair. Chk1 is activated by Rad3-mediated phosphorylation, and hyper-phosphorylated Chk1 interacts with the 14-3-3 homologs Rad24 and Rad25. Activated Chk1 functions via the phosphorylation of Cdc2 regulators; Chk1 both inhibits the Y15 phosphatase Cdc25, and activates the Y15 kinase Wee1 through enhanced stability. Similarly, strong overexpression (multicopy wildtype nmt1 promoter) of Chk1 elicits a G2 arrest independently of extrinsic DNA damage via these effectors, whereas a more moderate level of overexpression (attenuated nmt1 promoters or a single copy of wildtype nmt1::chk1) results in a G2 delay.

We used the latter condition to screen for mutants that had altered sensitivity to Chk1 overexpression using a single copy of nmt1::chk1 integrated using the sup3-5 tRNA suppressor. This strain shows a modest G2 delay in the absence of thiamine, and following mutagenesis of cells we screened for two phenotypes: complete resistance to Chk1 (rto phenotype; white colonies on phloxin plates lacking thiamine); and supersensitive to Chk1 (stc phenotype; dead colonies on plates lacking thiamine. 12 rto mutants were obtained, 11 of which were alleles of rad24. Three strong stc mutants were obtained, and each were shown to be allelic and designated stc1-B3, -C47 and -D8. The stc phenotype was suppressed by deletion of wee1 or rad24, both of which substantially reduce cells sensitivity to Chk1. Moreover, each stc1 allele was shown to be dominant. Whilst each allele formed normal colonies in the absence of Chk1 overexpression, and had a normal doubling time, they divided at a length ~25% longer than wildtype cells. Moreover, each allele exhibited a changed division response phenotype: upon nitrogen starvation, wildtype cells arrest as small G1 cells, where cdr mutants arrest in G2.

Through a combination of a positional cloning and plasmid shuffling, stc1 was mapped to a locus on chromosome 1. This locus mapped closely to cdr1, and sequencing confirmed that each stc1 allele resulted in amino acid substitutions in invariant residues within the kinase sub-domains; thus the dominance of these mutants is likely to be a dominant negative effect. cdr1 was originally identified as nim1, a high-copy suppressor of cdc25-22. Cdr1 has been shown to phosphorylate the C-terminal catalytic domain of Wee1 and inhibit Wee1 kinase activity. This is in contrast to Chk1, which phosphorylates residues outside the catalytic domain.

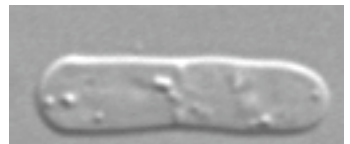
These alleles of cdr1 partially suppress the DNA damage sensitivity of several checkpoint mutants, while Cdr1 overexpression results in a wee phenotype and a hypersensitivity to DNA damage. These data suggest that in the absence of starvation signals, Cdr1 and Chk1 antagonize each other. Although we are yet to see regulation of Cdr1 by DNA damage, the activation of Chk1 following irradiation changes the balance between these Wee1 regulators favoring G2 arrest. These findings further our understanding of the regulatory balances to negatively control cell cycle checkpoints.

## **Involvement of Fission Yeast Clr6-HDAC in Regulation of the Checkpoint Kinase Cds1**

Tatsuki Kunoh, Toshiyuki Habu and Tomohiro Matsumoto

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Post-translational modification of histones plays important roles in maintenance of the genome integrity. Phosphorylation of histone H2AX, which occurs shortly after introduction of double strand break (DSB) of DNA, is required for DNA repair as well as regulation of the damage checkpoint. It was also shown that acetylation of lysine residues at the N-terminal tail of H4 is required for DSB repair in budding yeast. A recent study demonstrated an important role of acetylation of histone H3 lysine 56 in generation of a favorable chromatin structure for DNA repair. In this study we show that fission yeast Clr6-HDAC (histone deacetylase) regulates the checkpoint kinase Cds1 when DNA replication encounters a stressful condition. Overexpression of Clr6 or removal of the acetylation sites from histone H4 shortened the duration of the Cds1-dependent cell cycle arrest. In contrast, Cds1 was prolongedly activated in a mutant deficient in the Clr6-HDAC after the completion of DNA replication. Remarkably, Cds1 remained active in the *clr6* mutant even when the checkpoint upstream kinases, ATM/ATR, were inhibited. These results indicate that Cds1 is regulated by a dual mechanism. ATM/ATR monitors the status of DNA replication and a novel surveillance mechanism involving Clr6-HDAC possibly monitors global chromatin environment. These two mechanisms independently regulate Cds1 and maintain the chromosomal integrity in eukaryotes.



## **Stress MAPK in cellular response to toxic cations**

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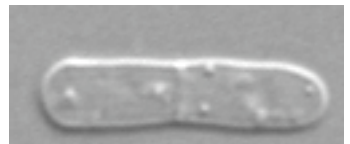
Stress-activated protein kinases (SAPKs), members of a mitogen-activated protein kinase (MAPK) subfamily, are highly conserved among eukaryotes. Studies in yeasts demonstrated that SAPKs play pivotal roles in survival responses to high osmolarity, oxidative stress, and heat shock. We report a novel physiological role of the fission yeast Spc1/Sty1 SAPK in cellular resistance to certain cations, such as Na<sup>+</sup>, Li<sup>+</sup> and Ca<sup>2+</sup>. Strains lacking Spc1 or its activator, Wis1 MAPKK, are hypersensitive to these cations. Spc1 positively regulates expression of *sod2<sup>+</sup>* encoding a Na<sup>+</sup>/H<sup>+</sup> antiporter through Atf1 and other transcription factors. In addition, we have identified a novel Spc1-interacting protein, Hal4, which is highly homologous to the budding yeast Sat4/Hal4 protein kinase. Like its budding yeast counterpart, the fission yeast Hal4 kinase is essential for cellular resistance to Na<sup>+</sup>, Li<sup>+</sup> and Ca<sup>2+</sup>. The *hal4* null phenotype is complemented by overexpression of the Trk1 potassium transporter or increased K<sup>+</sup> in the growth medium, suggesting that Hal4 promotes K<sup>+</sup> uptake, which consequently increases cellular resistance to other cations. Interestingly, the Spc1-Hal4 interaction appears to be required for cellular resistance to Ca<sup>2+</sup> but not Na<sup>+</sup> and Li<sup>+</sup>. We propose that Spc1 SAPK and Hal4 kinase cooperatively function to protect cells from the toxic cations.

## **Sap1 is required for the replication stress response pathway**

Chiaki Noguchi and Eishi Noguchi

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College of Medicine

To achieve faithful DNA replication, eukaryotic cells are equipped with a dedicated sensor-response system, termed the replication checkpoint. This checkpoint is activated by stalled replication forks and is required for accurate transmission of genetic information. Stalled forks are inherently unstable structures that are prone to collapse, regression and recombination, posing serious threats to genomic integrity. In humans, defects in this checkpoint cause genetic instability, leading developmental and neurological defects and a predisposition to cancer. Recently, our investigations revealed that two proteins, Swi1 and Swi3, form a replication fork protection complex (FPC) that plays a central role in the activation of the replication checkpoint and the stabilization of stalled forks. To further understand the mechanisms of the replication checkpoint, we have carried out screenings to identify genes that genetically interact with FPC. Here we describe one of FPC interacting factors, Sap1, a protein bound to DNA sequences required for switching of sexual identity in the fission yeast *Schizosaccharomyces pombe*. We show that Sap1 is required for replication checkpoint signaling. Inactivation of Sap1 causes DNA damage represented by Rad22 DNA repair foci during S-phase, indicative of fork damage. A pulsed-field gel electrophoresis analysis shows that the sap1 mutants have a defect in resumption of DNA replication after fork arrest. Our blast search reveals that Sap1 is conserved throughout evolution. We propose that Sap1 is required for the maintenance of replication forks in eukaryotic cells.



## **Csk1, A CDK-activating kinase, promotes homologous recombination in fission yeast**

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<sup>1</sup>Molecular Biology Program, Memorial Sloan-Kettering Cancer Center,

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Cyclin-dependent kinases (CDKs) require activating phosphorylation on the T-loop by a CDK-activating kinase (CAK) to promote progression through the cell division cycle. Fission yeast, unlike metazoans and budding yeast, contains two CAKs, each of which can directly activate Cdk1, the CDK that drives the cell cycle. We have uncovered a unique and unexpected role in the DNA damage response for one of these CAKs, Csk1. Cells deleted for *csk1+* are hypersensitive to DNA damage caused by UV light, ionizing radiation and alkylating agents. We observed epistatic interactions between *csk1d* and mutations in the homologous recombination (HR) genes *rhp51+* and *rhp54+*, and *csk1d* strains had a reduced frequency of spontaneous recombination. Removal of *srs2+*, which negatively regulates recombination, suppressed the UV-hypersensitivity of a strain lacking *csk1+* in an *rhp51+*-dependent manner, suggesting that Csk1 and Srs2 act in opposition through Rhp51 to promote and inhibit HR, respectively. Consistent with this model, deletion of *csk1* strongly suppressed the hyper-recombination phenotype of an *srs2* deletion mutant. In addition, synthetic interactions observed between *csk1* and the *rhp51* paralogs *rhp55* and *rhp57* suggest that Csk1 acts in a pathway parallel to Rhp55/57 and upstream of Rhp51 and Rhp54. Taken together, our data point to a role for a CAK in supporting normal levels of HR during normal cell cycle progression and in the face of UV-induced DNA lesions. Csk1 exclusively activates Cdk9 and Mcs6, and has overlapping specificity with the other *S. pombe* CAK, Mcs6, for Cdk1. The DNA damage sensitivity of strains lacking Csk1 cannot be fully accounted for by loss of phosphorylation of Mcs6 or Cdk9, suggesting that activation of Cdk1 might be important for DNA damage repair. We hypothesize a specific requirement for Csk1 in a specialized pathway of T-loop phosphorylation that continues to function when bulk CDK activation is suppressed by the DNA damage checkpoint.

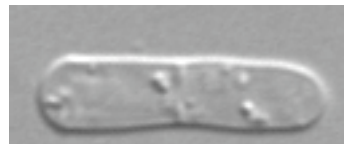


## **Role of the p21-Activated Kinase Complex in Regulating Microtubule Cytoskeletal Organization and Polarized Cell Growth in Fission Yeast**

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Dept. of Molecular Genetics, Univ. of Texas M.D. Anderson Cancer Center

The essential p21-activated kinase (PAK) homolog, Shk1 (a.k.a. Pak1 and Orb2), plays a central role in the regulation of polarized cell growth and cytokinesis in *S. pombe*. We have previously provided genetic evidence that Shk1 interacts functionally with the microtubule plus end and cell tip localized cell polarity factor, Tea1, to regulate polarized growth and cytokinesis in *S. pombe*. We have also shown that Tea1 is directly phosphorylated by Shk1 in vitro and phosphorylated in a Shk1-dependent fashion in *S. pombe* cells, thus implicating Tea1 as substrate-effector of the Shk1 kinase. Here we show that Tea1 associates with a second Shk1 interacting cell polarity factor, Scd2 (a.k.a. Ral3), in *S. pombe* cells and that the two proteins bind directly in vitro. While in cultures of *scd2Δ* and *tea1Δ* single mutants relatively low frequencies of cells exhibit microtubules that curve around the cell tips (a phenotype rarely observed in wild type *S. pombe* cells), greater than 90% of *scd2Δ tea1Δ* double mutant cells exhibit highly curved microtubules. Similarly, mutants carrying the *tea1Δ* mutation together with a kinase defective allele of *shk1*, *shk1K415R*, also exhibit high frequencies of microtubule curving. *scd2Δ tea1Δ* double mutants, but not the respective single mutants, are also temperature sensitive for growth, exhibit severe cell polarity and cytokinesis (cell separation) defective phenotypes, and are hypersensitive to the microtubule disrupting drugs thiabendazole (TBZ) and methyl 2-benzimidazolecarbamate (MBC). We show further that Scd2, like Tea1, is directly phosphorylated by Shk1 in vitro. Interestingly, in *shk1K415R* cells, Scd2 is hyperphosphorylated, suggesting that Shk1 functions antagonistically to a second Scd2 kinase in *S. pombe* cells. We provide evidence that this Scd2 kinase activity is stimulated by Rho1. These and additional data to be described suggest that Shk1, Scd2, and Tea1 function in cooperative fashion to regulate polarized growth and cytokinesis in *S. pombe*, and, specifically, that they function cooperatively to destabilize microtubules at the cell tips, thus preventing microtubule curving and promoting the antipodal pattern of growth characteristic of *S. pombe* cells. Our findings strongly implicate Scd2 and Tea1 as major components of a Shk1 substrate-effector complex in *S. pombe*.



## **Regulation of cell cycle-dependent microtubule morphology by nucleocytoplasmic protein shuttling**

Takashi Toda and Masamitsu Sato

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UK

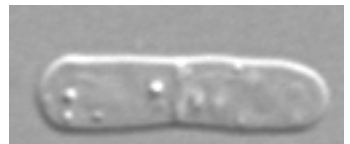
The microtubule (MT) plays a pivotal role in diverse aspects of cellular functions. It forms various morphologies in a cell cycle- and developmental stage-specific fashion. In fission yeast, MT forms distinct filamentous arrays along the cell axis in the cytoplasm during interphase. Upon entry into mitosis, on the other hand, these cytoplasmic MTs disappear and are replaced by nuclear spindles that emanate exclusively from the nuclear side of the SPBs (spindle pole bodies). During meiotic prophase, a bundle of horsetail-astral arrays associated with the SPB is formed in the cytoplasm. We have been investigating roles of two conserved MAPs (MT associated proteins), Alp7 and Alp14, in MT morphogenesis and dynamics. Alp7 is a homologue of vertebrate TACC (transforming coiled-coil containing protein), whilst Alp14 is a member of the Dis1/MAP215 family. In this presentation, we will present the evidence that nucleocytoplasmic shuttling of these two proteins play a key role in formation of cytoplasm MTs during interphase and nuclear spindles during mitosis.

## **Morphogenesis network from spindle pole body**

Dai Hirata

Hiroshima University

Cell morphogenesis is of fundamental significance in all eukaryotes for development, differentiation, and cell proliferation. In fission yeast, *Drosophila* Furry-like Mor2 plays an essential role in cell morphogenesis in concert with the NDR/Tricornered kinase Orb6. Mutations of these genes result in the loss of cell polarity. Here we show that the conserved essential proteins, MO25-like Pmo25, GC kinase Nak1, Mor2, Orb6, and Mob2, constitute a morphogenesis network that is important for polarity control and cell separation. Pmo25 was localized at mitotic SPBs and then underwent translocation to the dividing medial region upon cytokinesis. Our results indicated that Pmo25 interacts with Nak1 at SPBs and is required for both the localization and kinase activity of Nak1. Pmo25 and Nak1 were essential for Orb6 kinase activity. Further, the Pmo25 localization at the SPBs and the Nak1-Orb6 kinase activities during interphase were under the control of the Cdc7 and Sid1 kinases in septation initiation network (SIN), suggesting a functional linkage between SIN on SPBs and the network for cell morphogenesis following cytokinesis.



## **Mcp5, a meiotic cell cortex protein, is required for dynein-mediated oscillatory nuclear movement**

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During meiotic prophase I of the fission yeast *Schizosaccharomyces pombe*, oscillatory nuclear movement occurs. This promotes homologous chromosome pairing and recombination and involves cortical dynein, which plays a pivotal role by generating a pulling force with the help of an unknown dynein anchor. Here we show that Mcp5, the ortholog of the budding yeast dynein anchor Num1, may be this putative dynein anchor. mcp5<sup>+</sup> is predominantly expressed during meiotic prophase and GFP-Mcp5 localizes at the cell cortex. Moreover, the mcp5 $\Delta$  strain lacks the oscillatory nuclear movement. Accordingly, homologous pairing and recombination rates of the mcp5 $\Delta$  strain are significantly reduced. Furthermore, the cortical localization of dynein heavy chain 1 (Dhc1) appears to be reduced in mcp5 $\Delta$  cells. Finally, the full function of Mcp5 requires its coiled-coil and PH domains. Our results suggest that Mcp5 localizes at the cell cortex through its PH domain and functions as a dynein anchor, thereby facilitating nuclear oscillation.

## **Analysis of the Orb6 Kinase Pathway in *S. pombe***

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Department of Molecular and Cellular Pharmacology, University of Miami  
School of Medicine, \*Department of Chemistry, Purdue University

We have previously identified Orb6, a serine/threonine kinase in *Schizosaccharomyces pombe*, which functions in the control of cell morphology. Orb6 is conserved in higher eukaryotic organisms, and is related to mammalian NDR1 and NDR2 kinases, which have a role in tumorigenesis and in the control of cell proliferation. To explore the physiological role of Orb6 kinase activity, we have employed a chemical genetic approach involving site-directed mutagenesis of the kinase active site, which creates a new pocket where specific compounds bind to inhibit kinase activity (Alaimo et al., 2001). Therefore, we have chemically inhibited the function of the Orb6 kinase in vivo by altering the ATP binding site so that it can accept the kinase inhibitor 1-NA-PP1 (1-naphthylmethyl-pyrazolo pyrimidine-1). We found that when the Orb6 kinase mutant (orb6-as2) cells are exposed to the inhibitor, the actin cytoskeleton is rapidly altered. Actin cables quickly disappear and actin patches become uniformly distributed throughout the cell cortex. Furthermore, we found Orb6 kinase inhibition does not affect actin ring assembly during mitosis in synchronized cells, however it prevents post-telophase actin reorganisation and cell separation. These studies suggest that Orb6 kinase activity controls actin cable and actin patches dynamics, and promotes actin reorganization after mitosis. To identify potential effectors of the Orb6 pathway, we have employed a number of techniques, including Mass Spectrometry analysis of the Orb6-associated protein complex and 2-hybrid and genetic screens. Several proteins that are potential effectors of the Orb6 pathway have been identified.



## **Cell Polarity Determinants Tea1p, Tea4p, and Pom1p Inhibit Cell Division at Cell Ends in Fission Yeast**

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Cell Division Laboratory, Temasek Life Sciences Laboratory and the Department of Biological Sciences, National University of Singapore

The mechanisms positioning the plane of cell division are crucial to all cells. While bacteria appear to choose their division plane by negative regulatory mechanisms that prevent cell division at inappropriate sites, most eukaryotic cells do so by signals that positively influence this process. Cells of the fission yeast *Schizosaccharomyces pombe* are cylindrically shaped with hemispherical ends and divide through the use of an actomyosin based contractile ring. The metazoan anillin-related Mid1p is required for selection of the division site in fission yeast. Intriguingly, we find that, although mid1 mutants misplace the division septa, the misplaced septa are occluded from the cell ends. This process, referred to as tip-occlusion, is essential for viability of Mid1p-defective cells and requires tip-localized kelch repeat protein Tea1p, SH3 domain protein Tea4p / Wsh3p, and the Dyrk-kinase related Pom1p (tip-complex). In the absence of tip-complex proteins and Mid1p, resumption of growth at cell tips is aborted, leading to lethality. We propose that tip-complex provides a secondary mechanism of placing the division site in cells lacking Mid1p and enhances the fidelity of ring placement in some instances even when Mid1p is present. The fission yeast tip-complex might function by negatively regulating cell division ring assembly / attachment at cell tips in a manner similar to the bacterial Min proteins.

## **Regulation of F-actin cytoskeleton organization by the Kin1 kinase**

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In *S. pombe*, cell growth occurs in interphase at ends of cells where cell polarity factors and cytoskeletal elements are concentrated. In early G2, cell growth is restricted to the old pre-existing end. Then, cell growth is activated in the newly-formed cell tip and cells grow in a bipolar fashion. Distribution of polymerized F-actin structures such as patches and cables coincides with active growth sites.

The conserved Kin1 kinase is involved in morphogenesis, cell polarity and cell division. Using a kin1-deleted allele, we show that Kin1 is required for F-actin polarization in interphase cells as well as for F-actin relocation after cell division. However, in kin1-deleted mitotic cells, F-actin ring formation is not significantly perturbed, indicating that Kin1 may be dispensable for F-actin organization during mitosis.

Overexpression of Kin1 also promotes F-actin delocalization. The N-terminal catalytic kinase domain but not the highly conserved C-terminus of Kin1 is required for this phenotype. When Kin1 is overexpressed in G1 or G2 arrested cells, F-actin is delocalized showing that Kin1 overexpression can perturb F-actin organization throughout interphase.

Overexpression of higher eukaryotic related PAR-1/MARK kinases also disrupts F-actin organization, suggesting the existence of potentially conserved targets.



## **Clp1p phosphatase targets substrates at the contractile ring through Mid1p-mediated localization**

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1. Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, 2. Department of Cell Biology, Scripps Research Institute, 3. Howard Hughes Medical Institute

Strictly regulated alterations in cyclin-dependent kinase (cdk) activity control cell cycle progression in all eukaryotes studied. Reversal of cdk-dependent phosphorylation events involves the highly conserved Cdc14-family of phosphatases. The fission yeast Cdc14 homolog, Clp1p (also known as F1p1p), is sequestered in the nucleolus and the spindle pole body during interphase. Early in mitosis Clp1p disperses into the nucleus localizing to the kinetochores and spindle, and into the cytoplasm concentrating at the contractile ring. Consistent with Clp1p's complex localization pattern, the phosphatase plays multiple roles in regulating cell division, including entry into mitosis, chromosome segregation, the cytokinesis checkpoint and mitotic exit. While Clp1p participates in several mitotic events, few *in vivo* substrates have been identified. Therefore, we utilized biochemical methods to identify proteins that associate with Clp1p during mitosis. Here we present evidence that Clp1p associates with Mid1p, a protein required for correct positioning of the contractile ring. Cells lacking mid1 form contractile ring structures that are non-centered and/or tilted leading to unequal nuclear and cellular division. The interaction between Clp1p and Mid1p does not depend on the catalytic activity of Clp1p or the phosphorylation status of these phosphoproteins. We find that Mid1p localization to the contractile ring is unaffected by the absence of clp1. However, live cell microscopy reveals that Clp1p localization to the contractile ring requires mid1. Based on these findings, we propose that Mid1p recruits Clp1p to the contractile ring permitting Clp1p to dephosphorylate ring component substrates. In support of this model, we observe a defect in Clp1p-mediated dephosphorylation of Cdc15p, an essential ring component, in mid1Δ cells. Although the loss of Clp1p-dependent dephosphorylation of critical ring components may not be essential for normal contractile ring function, it is possible that dephosphorylation of these substrates during mitosis ensures proficiency of the cytokinesis checkpoint in response to ring perturbation.

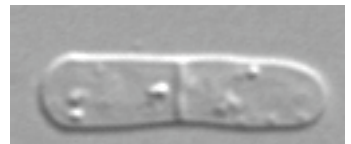


## **Role of the spindle pole body in regulating cytokinesis**

V. Magidson, R. Daga, A. Khodjakov and Fred Chang

Wadsworth Center, New York Public Health and Microbiology  
Department, Columbia Univeristy

Many cell cycle regulatory proteins are located at microtubule organizing centers (MTOCs), and so it has been proposed that one of the major roles of MTOCs is in cell cycle progression. In fission yeast, SIN pathway components, which are required for regulating cytokinesis, localize to the spindle pole bodies (SPBs) and are thought to be active primarily on the daughter SPB. We tested the function of SPBs by laser microsurgery in fission yeast cells. Ablation of both SPBs in metaphase (in a *mad2* background) or in anaphase caused a *sin*-like phenotype: a failure in septation and maintenance of the contractile ring. However, ablation of one of the SPBs did not cause a cytokinesis defect. Interestingly, ablation of the daughter SPB often led to activation of the pathway on the mother SPB, suggesting that the daughter SPB usually “inhibits” the mother one. Displacement of the spindle relative to the ring by cell centrifugation showed that proper positioning of the SPB is not monitored. Our results show that a SPB, but not its position, drives cytokinesis during late mitosis and septation.



## **Comprehensive analysis of heterochromatin- and RNAi-mediated epigenetic control of the fission yeast genome**

Hugh P. Cam, Tomoyasu Sugiyama, Ee Sin Chen, Xi Chen, Peter C. FitzGerald, Shiv I. S. Grewal

National Institute of Health/National Cancer Institute

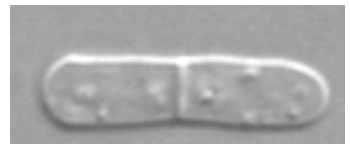
The organization of eukaryotic genomes into distinct structural and functional domains is important for the regulation and transduction of genetic information. Here, we investigate heterochromatin and euchromatin profiles of the entire fission yeast genome and explore the role of RNAi in genome organization. Histone H3 methylated at lysine 4, which defines euchromatin, is not only distributed across the majority of chromosomal landscape, but also is present at the centromere core, the site of kinetochore assembly. In contrast, histone H3 methylated at lysine 9 and its interacting Swi6/HP1 protein, which define heterochromatin, coat extended domains associated with a variety of repeat elements, and small islands that correspond to meiotic genes. Remarkably, RNAi components distribute throughout all these heterochromatin domains, and their localization depends upon Clr4/Suv39h histone methyltransferase. Sequencing of siRNAs associated with the RITS RNAi effector complex identifies “hot-spots” of siRNAs, which map to a diverse array of elements within these RNAi/heterochromatin domains. We find that Clr4/Suv39h predominantly silences repeat elements whose derived transcripts, transcribed mainly by RNA polymerase II, serve as a source for siRNAs. Our analyses also reveal an important role for the RNAi machinery in maintenance of genomic integrity.

## **F-Box-Directed CRL Complex Assembly and Regulation by the CSN**

Michael W. Schmidt<sup>1,2</sup>, Susan Wee<sup>1,4</sup>, Kay Hofmann<sup>3</sup>, Dieter A. Wolf<sup>1,2</sup>

<sup>1</sup> Department of Genetics and Complex Diseases and <sup>2</sup> NIEHS Center for Environmental Health Proteomics Facility, Harvard School of Public Health, <sup>3</sup> Memorec Biotec GmbH, <sup>4</sup> Novartis Biomedical Research Institute

The COP9 signalosome (CSN) stimulates cullin-RING ubiquitin ligase (CRL) assembly by preventing the autocatalytic destruction of substrate adapters as documented for a few F-box and BTB proteins. In a systematic survey of ten F-box proteins (FBPs) of the fission yeast *S. pombe*, we unexpectedly found that only five required CSN for stability. The CSN-insensitive FBPs contain a variant F-box lacking a critical proline residue required for efficient incorporation into CRL1 complexes and regulation by the CSN. Unlike the CRL3 adapter Btb3p, none of the CSN-sensitive FBPs proteins were affected by deletion of *ubp12*. Similarly, *Can1p*, the putative orthologue of mammalian CAND1, was dispensable for adapter stabilization and CRL1 function. Our results suggest that distinctive features of the F-box motif specify the assembly of FBPs into CRL1 complexes thus destining them for regulation by the CSN through a mechanism, which can principally function independently of CAND1.



## **Ubiquitin ligase component Cul4 associates with Clr4 histone methyltransferase to assemble heterochromatin**

Songtao Jia<sup>1</sup>, Ryuji Kobayashi<sup>2</sup> and Shiv I. S. Grewal<sup>1</sup>

<sup>1</sup>Laboratory of Molecular Cell Biology, National Cancer Institute,

<sup>2</sup>Department of Molecular Pathology, University of Texas MD Anderson Cancer Center

In eukaryotes, heterochromatin mediates diverse processes including gene silencing and regulation of long-range chromatin interactions. The formation of heterochromatin involves a conserved array of histone modifications; in particular, methylation of histone H3 at lysine 9 (H3K9me) is essential for recruiting HP1/Swi6 proteins. In fission yeast, the Clr4 methyltransferase is responsible for H3K9me across all heterochromatic domains. However, the mechanism of Clr4 recruitment to these loci is poorly understood. We show that Clr4 associates with Cul4, a cullin family protein that serves as scaffold to assemble ubiquitin ligases. Mutations in Cul4 result in defective localization of Clr4 and loss of silencing at heterochromatic loci. This is accompanied by severe reduction in H3K9me and Swi6 levels and accumulation of transcripts corresponding to naturally silenced repeat elements within heterochromatic domains. Moreover, heterochromatin defects in Cul4 mutant could not be rescued by expression of Cul4 protein lacking Nedd8 modification, which is essential for its ubiquitin ligase activity. Rik1, related to DNA damage binding protein DDB1 and required for H3K9me, also interacts with Cul4, the association of which serves to target Clr4 to heterochromatic loci. These analyses uncover a role for Cul4-mediated protein ubiquitination in regulating H3K9me and heterochromatin formation.

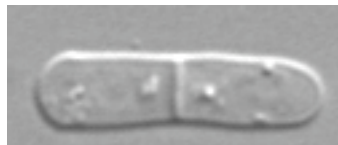
## **The Many PHD fingers of Msc1 each act as E3 ubiquitin ligases**

Barbara E. Dul, Shakil Ahmed, Nancy C. Walworth

Department of Pharmacology, Robert Wood Johnson Medical School,  
University of Medicine and Dentistry of New Jersey and Rutgers, The State  
University of New Jersey

Eukaryotic cells go to great length to ensure that progeny cells receive accurate copies of parental genomes. A complex series of controls ensure ordered progression through the cell cycle. Incomplete DNA replication, the presence of unrepaired DNA, or incorrect spindle assembly initiates an arrest of cell cycle progression through various checkpoint mechanism. Chk1 is required for the proper block of cell cycle progression when DNA damage occurs (Walworth, et al., 1993). In the presence of DNA damage, Chk1 becomes phosphorylated (Walworth and Bernards, 1996). Chk1 kinase then phosphorylates specific components of the cell cycle machinery that blocks entry into mitosis until genomic integrity is restored (O'Connell et al., 2000).

A screen for suppressors of a *S. pombe* chk1 mutant identified a fission yeast protein, Msc1, which shows similarity to the mammalian protein RBP2 (Ahmed et al., 2004), a protein that binds the tumor suppressor RB (Defeo-Jones et al., 1991). Yeast cells that have a deletion of the Msc1 gene show a striking alteration in the pattern of histone acetylation (Ahmed et al., 2004) and a dramatic elevation in the rate of chromosome loss (Ahmed et al., unpublished). Both RBP2 and Msc1 contain 3 PHD (plant homeodomain domains) fingers, motifs characteristically defined by one histidine and seven cysteine residues that are spatially arranged in a C4HC3 consensus with intervening sequences of varying length and composition (Aasland et al., 1995), similar to RING finger domains. PHD finger domains in viral proteins and in the cellular protein kinase MEKK1, have been implicated as ubiquitin E3 protein ligases that affect protein stability. The close structural relationship of PHD fingers to RING fingers suggests that other PHD proteins might share this activity. We have evidence showing that each of the three PHD fingers of Msc1 can act as ubiquitin E3 ligases, and ubiquitinate histones in vitro. Strikingly, deletion of *msc1* confers complete suppression of the slow growth phenotype and HU sensitivity seen in a deletion of the ubiquitin conjugating enzyme, *rhpf*. Experiments addressing the in vivo significance of the E3 ligase activity of the multiple PHD fingers of Msc1 will be presented.



**NatB N $\alpha$ -acetylation activity required for Bsu1p pyridoxine transporter function in *S. pombe*.**

Austin Nam\*, Gordon Chua\*\*, Brian Owen, Paul G. Young\*

\*Dept. of Biology, Queen's University, \*\*Banting and Best Research Institute, University of Toronto

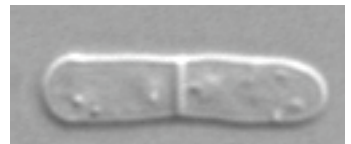
Sensitivity to amiloride, a diuretic drug toxic to the fission yeast *Schizosaccharomyces pombe*, requires the activity of the plasma membrane vitamin B6 transporter, Bsu1p. *bsu1* mutants are amiloride resistant. We have identified a new locus conferring amiloride resistance, termed *arm1* (for amiloride resistant mutant). Loss of Arm1p also results in failed pyridoxine transport. By homology, Arm1p is part of an N $\alpha$ -acetyltransferase complex (NatB) that acetylates a wide range of targets. Disruption of *arm1+* results in a range of phenotypes including the disruption of actin cables, thick misoriented septa, failure to undergo fluid-phase endocytosis, amiloride resistance and failed pyridoxine transport. Loss of Arm1p does not affect the transcription or translation of Bsu1p, nor prevent it from moving to the plasma membrane. By sequence, Bsu1p is not a direct target of Arm1p.

## **Rqh1 regulates the process of DSB repair and prevents crossovers from forming during homologous recombination**

Justin C. Hope<sup>1</sup> and Greg A. Freyer<sup>1,2</sup>

<sup>1</sup>Graduate program in Anatomy and Cell Biology and <sup>2</sup>Environmental Health Sciences, Columbia University

Double strand breaks (DSBs) are repaired by either non-homologous end joining (NHEJ), or homologous recombination (HR). The basic mechanisms of these repair processes have been determined, yet many questions remain concerning the details of these processes and the exact roles of individual proteins involved remain ambiguous. One protein whose role in HR has only recently become apparent is that of the RecQ helicase, explaining at least in part its role in maintaining genomic stability. Using a system that creates a unique DSB in a non-essential chromosome in *Schizosaccharomyces pombe* (*S. pombe*) we studied the role of its RecQ helicase, Rqh1, in repair of this DNA damage. We found that Rqh1 plays an apparent early role in HR, promoting DSB repair by gene conversion (GC) between homologous chromosomes. In cells lacking Rqh1 DSBs are predominantly repaired by HR with their sister chromatid (sister chromatid repair, SCR). Interestingly, when SCR was blocked, DSB repair in  $\Delta$ rqh1 cells switched to NHEJ. These data suggest that Rqh1 acts to direct DSB down a GC pathway as opposed to blocking SCR. We also found that contrary to the conclusions of previous studies, NHEJ in *S. pombe* appears to be faithful, at least when the end is a DSB containing a 3' extension. We have also analyzed colonies where DSBs are repaired by GC for the presence of crossovers. We found that crossovers are rare in wild type cells, occurring in 9% of cells, while 50% of GCs in cells with a  $\Delta$ rqh1 background had crossovers. These crossovers in  $\Delta$ rqh1 cells are dependent on Rhp51 and Rhp57. We found that in  $\Delta$ rhp57 cells that were *rqh1*<sup>+</sup>, crossovers occurred frequently (30% of cells had crossovers). These crossovers depended on Mus81-Eme1. We next asked if crossovers formed in a temperature sensitive mutant of top3 (*top3-15*). At semi-permissive temperatures crossovers formed in 25% of cells. The majority of these crossovers formed independently of Mus81-EME1. These data show that Rqh1 not only suppresses crossovers but also regulates the process of DSB repair. Also, for the first time we provided data that supports a role for Mus81-Eme1 promoting crossovers in mitotic cells. This demonstrates that at least two mechanisms for crossover formation in mitotic cells exists in *S. pombe*.



## **Modular basis of a La protein activity in the maturation of structurally-impaired pre-tRNA that is distinguishable from 3' end protection**

Ying Huang, Mark A. Bayfield and Richard J. Maraia

Laboratory of Molecular Growth Regulation, National Institute of Child Health and Human Development, National Institutes of Health

La protein is a major autoimmune antigen. La proteins are abundant, ubiquitous and multifunctional, involved in the metabolism of coding and noncoding RNAs. For precursor-tRNAs and other small nuclear RNAs, La protects their 3'-UUU-OH ends from exonucleolytic digestion. Rrp6p is a 3'-5' exonuclease component of a nuclear surveillance system that degrades structurally impaired pre-tRNAs. In La-minus cells, deletion of rrp6 restores suppression by tRNAs that bear moderate defects, indicating that these benefit from 3' protection by La. Surprisingly, rrp6-deletion does not restore suppression by more structurally impaired pre-tRNAs indicating that these require an activity distinct from 3' end protection. We show that point mutants in the RNA binding surface of the La RRM specifically impair this activity but not UUU-OH recognition or 3' end protection, providing the first data that map this activity to the La RRM. Consistent with a modular nature of the two distinguishable activities, mutants in the 'La motif' do impair UUU-OH recognition and 3' protection. Thus although it was believed that the La motif and the RNA-binding surface of the RRM might cooperate for UUU-OH recognition, and it was suspected that 3' end protection alone might account for La's ability to promote the maturation of structurally-impaired pre-tRNAs, our data indicate these as distinguishable functions and moreover, that they differentially map to the two conserved motifs of La.

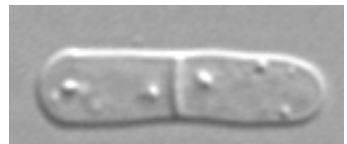


## **Semiquantitative analysis of the fission yeast proteome**

Michael W. Schmidt, Andres Housman, Dieter A. Wolf, Alexander R. Ivanov

Department of Genetics and Complex Diseases, NIEHS Center for Environmental Health Proteomics Facility, Department of Biostatistics, Harvard School of Public Health

Using a shotgun proteomics approach based on multidimensional prefractionation and liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC ESI MS/MS), we have detected ~30% of the theoretical proteome of the fission yeast *Schizosaccharomyces pombe*. Applying statistical modelling to normalize spectral counts to the number of predicted tryptic peptides, we obtained a semiquantitative measure of the relative abundance of ~1400 proteins. The fission yeast data showed highly significant correlations with mRNA expression levels and with protein abundance in budding yeast. Unsupervised hierarchical clustering revealed several groups of proteins with distinct patterns of transcriptional and posttranscriptional control that are highly conserved in the two yeasts. Our data suggest that relative quantitation of LC ESI MS/MS proteomics data based on normalized spectral counts has the potential of providing valid information on the control of gene expression.



## **New Insights into Regulating the Mitotic Mechanism.**

Joe Batac, Paul Shih, Jason Filopei, Adrianna S. Rodriguez, Kriti Mohan, Christina Mayer and Janet L. Paluh., Biology Department, Boston College

Chromosome segregation failures result in aneuploidy that is most often a direct consequence of aberrant spindle function and inappropriate mitotic exit. Microtubule motor kinesin-like proteins are ubiquitous in eukaryotes and are vital to maintaining the fidelity of mitosis. These proteins participate in spindle microtubule organization and microtubule dynamic properties and in doing so contribute actively to chromosome capture, alignment and segregation. Kinesin-like proteins are also integrated into signaling networks within the spindle, including vital checkpoint pathways that arrest mitotic progression if errors arise in the process. These critical delays allow correction mechanisms to operate and reduce the likelihood of aneuploidy. At kinetochores, kinesin-like proteins are important regulators of correct chromosome attachment, segregation and in the case of CenpE, integrated into the Mad2p-spindle assembly checkpoint. Mitotic stage transitions necessitate the modification of parameters of microtubule nucleation, minus-end dynamics, and post-metaphase signaling towards mitotic exit. At spindle poles, what contributions kinesin-like proteins play outside of pole organization of microtubules has not been determined. However, members of the ubiquitous kinesin-14 family are implicated in regulation of microtubule number, organization, minus-end dynamics and  $\gamma$ -tubulin function. Two exciting areas of research are providing us with new insights into the mitotic mechanism:

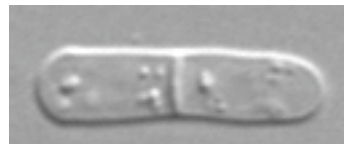
**Kinesin/Tubulin interactions:** A defining feature of kinesin-like proteins is a globular ATPase domain that docks with the microtubule lattice. We are determining the precise nature of kinesin-like protein binding to tubulin at the molecular level using protein modeling, site-directed mutagenesis and genetic and cytological analysis. We identified a novel feature of tubulins for directing motor protein interactions, a subset of this domain is shared with  $\gamma$ -tubulin and may favor kinesin-14 proteins at poles. A second component of this work is to establish fission yeast strains with human  $\beta$ -tubulin isoforms in lieu of the yeast protein. Despite the highly conserved sequence and structure of tubulins, necessitated by functional constraints, at least six isoforms of  $\beta$ -tubulin exist in human cells. We hope to further evaluate the mitotic impact of these isoforms and reveal novel controls on tubulin dynamics.

**Monitoring post-metaphase mitotic progression:** In yeast and human cells, checkpoint protein Mad2 undergoes a conserved transitional relocalization from kinetochores to spindle poles, immediately preceding the metaphase/anaphase transition. We determined that *pombe* Mad2p associates with the microtubule organizing center complex found at the juncture of microtubule ends at poles. At least two additional transitions of Mad2p localization occur during anaphase and telophase. Loss of two microtubule motor proteins, kinesin-14 family Pkl1p and dynein, disrupt these latter transitions. To define those mitotic processes relying on post-metaphase positioned Mad2p, surface domains of Mad2p are being targeted to disrupt its pole interactions while leaving kinetochore association intact. However Mad2p may not represent the sole regulator of APC/C function. In *S. pombe*, as in human cells, we have identified a Mad2-related protein that may represent additional levels of post-metaphase control of mitotic progression.

## **Regulation of nucleolar localization of the Cdc14-family phosphatase Clp1p/Flp1p**

Chun-Ti Chen, Young Sam Shim and Dannel McCollum  
UMass Medical School

Cdc14-family phosphatases are highly conserved and play an important role in coordinating late mitotic events in both budding and fission yeast. These proteins are regulated in part through sequestration in the nucleolus. Budding yeast Cdc14p is released from its nucleolar inhibitor Net1/Cfi1 in early anaphase by the cdc-fourteen early anaphase release (FEAR) network. The mitotic exit network (MEN), then takes over to keep Cdc14p released from the nucleolus until after mitotic exit through an unknown mechanism. The *S. pombe* Cdc14-family phosphatase, Clp1p (also known as Flp1p) is also kept out of the nucleolus in mitosis through a two-step mechanism. In contrast to budding yeast Cdc14p, but similar to human Cdc14B, Clp1p is released from the nucleolus in early mitosis. Then, like in budding yeast, a homologous pathway to the MEN, the septation initiation network (SIN), acts independently to keep Clp1p out of the nucleolus. The mechanism of nucleolar release and cytoplasmic maintenance by the SIN are not understood. Here, we show that the FEAR-homologs in fission yeast (Plo1p, Cut1p, Spo12p, and Mialp/Alp7p) are not required for nucleolar release of Clp1p. We are also investigating the role of the SIN in regulating Clp1p release. Recent work suggests a model where the SIN acts by affecting nuclear shuttling of Clp1p. We will present our initial efforts to test this model.



## **The *S. pombe* Cdc14 phosphatase, Clp1p and the *S.pombe* INCENP homolog Pic1p**

Sung Hugh Choi<sup>1</sup>, Ian McLeod<sup>2</sup>, John Yates<sup>2</sup>, Susanne Trautmann<sup>1</sup>,  
Dannel McCollum<sup>1</sup>

<sup>1</sup>Department of Molecular Genetics and Microbiology, University of  
Massachusetts Medical School <sup>2</sup>Department of Cell Biology, The Scripps  
Research Institute

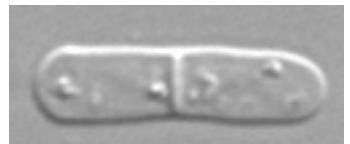
The *S. pombe* Cdc14-like phosphatase, Clp1p also known as Flp1, regulates G2/M transition mitotic exit, and coordinates mitosis with cytokinesis. Clp1p also regulates chromosome bi-orientation through interaction with Ark1p, the *S. pombe* aurora kinase B homolog. We are studying how Clp1p regulates chromosome segregation during mitosis. In order to find proteins that function with Clp1p for chromosome segregation, we carried out tandem affinity purification with metaphase-arrested cells expressing Clp1C286S-TAP, a phosphatase inactive form of Clp1p. Analysis of purified complex using mass spectrometry showed that Clp1p exists in a co-complex with the INCENP homolog, Pic1p and the survivin homolog, Bir1p, which are both highly phosphorylated on Cdk site. The chromosome passenger proteins aurora-B, survivin, and INCENP are known to function together in coordinating chromosome segregation with cytokinesis. Pic1p localized in the nucleolus in interphase, at kinetochores in prometaphase and at the spindle midzone in anaphase. Cells lacking *pic1+* were not viable and showed stretched chromatin, “cut” or uneven chromosome segregation. When chromosomal *pic1+* was tagged with 13myc, cells expressing Pic1-13myc surprisingly showed a weak mutant phenotype, 33% of *pic1*-13myc cells in anaphase showed lagging chromosome. Genetic analysis showed that *pic1*-13myc and the survivin mutant *bir/cut17-275* were synthetically lethal with *clp1Δ*. In cells lacking *clp1+*, kinetochore localization of aurora B Ark1p and survivin Bir1p was reduced whereas Clp1p did not affect localization of Pic1p to kinetochores. Taken together, our results suggest how Clp1p may function in chromosome segregation; Clp1p may dephosphorylate Pic1p to facilitate recruitment of Ark1p and Bir1p to kinetochores and/or Clp1p may dephosphorylate Ark1p and/or Bir1p for their proper localization to kinetochores to form a chromosome passenger protein complex with Pic1p.

## **Revisiting the role of Mcs6 in transcription and cell cycle regulation**

Maitreyi Das and Tomi P. Mäkelä

Molecular Cancer Biology Program, institute of Biomedicine, Biomedicum Helsinki, University of Helsinki

Mcs6, the CDK7 homolog in *Schizosaccharomyces pombe*, has been suggested to be involved in cell cycle regulation and transcription. Genetic evidence reveals that Mcs6 phosphorylates Cdc2 and is required for cell cycle regulation. Also a large body of evidence mostly from other species demonstrates that CDK7 a component of TFIIF is essential for transcription. More recently studies in fission yeast as well as other species suggest that Mcs6 has only a specific role in transcription. In an attempt to address these issues we are studying Mcs6 of *Schizosaccharomyces pombe*. Using Mcs6 mutants we are attempting to study its role in both cell cycle regulation as well as transcription. Our preliminary results suggest that the kinase activity of Mcs6 is not essential for cell cycle regulation or transcription initiation. Information from additional studies conducted to establish this finding will also be presented.



## **Transcriptional response during activation of S/M replication checkpoint in *S. pombe***

Chaitali Dutta<sup>1</sup>, Anna Oliva<sup>2</sup>, Janet Leatherwood<sup>2</sup> and Nicholas Rhind<sup>1</sup>  
1UMass Med School, 2SUNY Stony Brook

The S/M checkpoint in *S. pombe* is defined as the checkpoint that prevents mitosis if replication is blocked by hydroxyurea (HU). HU is a competitive inhibitor of ribonucleotide reductase, which blocks replication after initiation by preventing nucleotide synthesis. There are three different known responses in presence of HU: Inhibition of mitosis, stabilization of replication forks, and transcriptional maintenance of S-phase transcripts. Our study focuses on the third aspect.

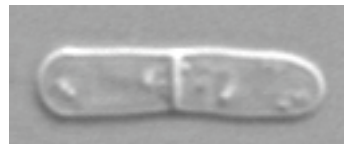
In *Saccharomyces cerevisiae*, transcriptional induction in response to replication block by HU induces only the enzyme that is needed to produce more deoxynucleotides. In *S. pombe*, the activated checkpoint up-regulates many genes normally expressed during S phase including the genes that are needed to produce more deoxynucleotides, suggesting that the checkpoint acts through the S-phase transcription factor DSC1. The DSC1 (DNA synthesis control) is a transcriptional factor complex binding to common repeated DNA sequences in the promoters of S-phase genes. We hypothesize that in response to HU, the activated Cds1 kinase is responsible of maintaining higher level of S phase transcripts by regulating DSC1 activity.

In order to learn about HU induced intra S-phase checkpoint, we looked at the global transcriptional induction in response to HU by gene array in collaboration with Dr. Janet Leatherwood (Stony Brook NY). Our gene array data suggests that all S-phase transcripts are affected in response to HU. We have shown that maintenance of high level of all S-phase transcripts in response to HU is Cds1 dependent. Furthermore, our data suggests that checkpoint has no effect of S-phase transcription when DSC1 activity is compromised, corroborating the hypothesis that DSC1 is the checkpoint target. Currently, our study is focused on regulation of DSC1 activity by checkpoint.

## **Cloning the *Schizosaccharomyces pombe* lys2+ gene and construction of new molecular genetic tools**

Richard L. Hoffman and Charles S. Hoffman  
Biology Department, Boston College

Molecular genetic analyses in the fission yeast *Schizosaccharomyces pombe* rely on selectable markers that are used in cloning vectors or to mark targeted gene deletions and other integrated constructs. In this study, we describe the cloning of the *S. pombe* lys2+ gene, which is homologous to the *Saccharomyces cerevisiae* LYS4+ gene, along with the construction of the lys2+-based cloning vector pRH3. In addition, we demonstrate the ability to delete the *S. pombe* his7+ gene with a lys2+-marked PCR product as well as the ability to delete the *S. pombe* lys2+ gene with a his7+-marked PCR product. In doing so, we have constructed deletions of both lys2+ and his7+ that can be used in the future to improve the efficiency of deleting other *S. pombe* genes with either of these two selectable markers.



**The *Schizosaccharomyces pombe* git1+ gene encodes a novel adenylate cyclase regulator required for glucose-triggered cAMP signaling**

Richard S. Kao, Eric Morreale, Lili Wang, F. Douglas Ivey and Charles S. Hoffman

Biology Department, Boston College

The fission yeast *Schizosaccharomyces pombe* senses environmental glucose through a cAMP-signaling pathway that activates the cAMP-dependent protein kinase A (PKA). This process requires nine git genes (git=glucose insensitive transcription) that encode adenylate cyclase, the PKA catalytic subunit and seven “upstream” proteins required for glucose-triggered adenylate cyclase activation, including three subunits of a heterotrimeric G protein and its associated receptor. We describe here the cloning and characterization of the git1+ gene. Git1 is distantly related to a small group of proteins in the Genbank database, but is functionally distinct from a second *S. pombe* protein that is a member of this family. Mutations in git1+ demonstrate functional roles for the two most highly conserved regions of the protein, the C2 domain and a carboxy-terminal domain. Cells lacking Git1 (git1Δ) are viable, but display phenotypes associated with cAMP signaling defects, even in the presence of a mutationally activated Gpa2 Gα subunit, which activates adenylate cyclase. These git1Δ cells possess reduced basal cAMP levels and fail to mount a cAMP response to glucose. In addition, Git1 and adenylate cyclase physically interact and co-localize in the cell. Thus, Git1 is a critical component of the *S. pombe* glucose/cAMP pathway.

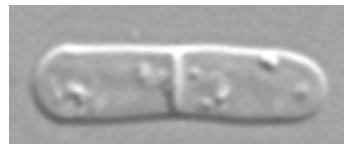


## **Using Spheroplasts to Investigate Cell Shape in Fission Yeast**

Felice Kelly and Paul Nurse

Rockefeller University

The fission yeast has a cylindrical rod shape which grows by elongation at the tips at each end of the cell. To investigate the roles of the pre-existing rod-shape and of the cell wall in establishing and maintaining the rod shape we monitored growth during the transition from rod to spherical shape and back again to rod shape. We have disrupted rod shape of cells by enzymatically digesting the cells' walls to make spherical spheroplasts and then have followed their recovery. Once spherical, a wild-type cell maintained in isotonic media will re-form its cell wall, establish a new growth zone, and divide to produce a rod-shaped daughter cell. When mutants that form bent or branched cells in normal growth conditions regrow from spheroplasts their morphology defects are more penetrant and pronounced. When wild-type cells regrow in the presence of MBC, they form bent cells similar to the morphology mutants. Observations of mutant and wild-type cells recovering from cell wall digestion may provide a way to test cell-shape models.



## **Cdc25 is not required for the intra-S-phase DNA damage checkpoint in fission yeast**

Naveen Kommajosyula and Nicholas Rhind

Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School

The intra-S-phase checkpoint slows replication when damage occurs during S phase. Cdc25, which dephosphorylates and activates Cdc2, has been shown to be a downstream target of the checkpoint in metazoans, but in fission yeast its role is not clear. The dephosphorylation of Cdc2 by Cdc25 in fission yeast has been associated with the beginning of mitosis. Dephosphorylation has been assumed not to play a role in S-phase regulation because the M phase cyclin can initiate S phase in the absence of S phase cyclin or Cdc25. This observation suggests that phosphorylated Cdc2 is sufficient for S phase. However, it has been reported recently by Kumar et al. (2004) that Cdc25 is a target in fission yeast suggesting a modulator role for Cdc2 dephosphorylation in S phase.

We have investigated the role of Cdc25 in intra-S-phase checkpoint and our results are not compatible with the conclusion that Cdc25 is a target of the checkpoint. We use MMS to induce damage in various mutant strains and measure bulk replication by flow cytometry. The checkpoint was not abrogated in a Cdc25 overexpressing strain. We have also done experiments in strains where the role of Cdc25 has been bypassed by overexpressing Pyp3. We are presently working on a Cdc25ts strain to look at the intra-S checkpoint in the absence of Cdc25 to confirm our results.

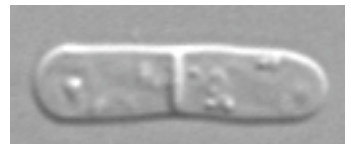
Our results are consistent with a Cdc2-Y15 phosphorylation independent mechanism of the checkpoint. We are now in the process of identifying the targets for this checkpoint.

## **The CENP-B Homolog, Abp1, Interacts With The Essential DNA Replication Protein Cdc23 In Fission Yeast**

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<sup>1</sup>University of Miami School of Medicine, Department of Molecular and Cellular Pharmacology, <sup>2</sup>Department of Applied Bioscience and Biotechnology, Faculty of Life and Environmental Science, Shimane University, <sup>3</sup>Institute for Virus Research, Kyoto University

A two-hybrid screen identified Abp1 (Ars-binding protein 1) as a protein that can interact with the essential DNA replication protein Cdc23 of *S. pombe*. Abp1, and the closely related Cbh1 and Cbh2 are homologous to the human centromere-binding protein Cenp-B that has been implicated in the assembly of centromeric heterochromatin in mammalian cells. Studies in fission yeast have shown that Abp1 is required for efficient recruitment of Swi6 to chromatin during epigenetic silencing at telomeres. Also, cells lacking Abp1 show an increase in mini-chromosome instability suggesting that Abp1 is important for normal chromosome segregation and/or DNA synthesis. Here we show that Abp1 interacts with the DNA replication protein Cdc23 in a two-hybrid assay, and that a mutant deleted for *abp1+* (a non-essential gene) is synthetically lethal with a *cdc23* temperature-sensitive mutant when grown under semi-permissive conditions. No genetic interactions were observed between *cdc23+* and *cbh1+*, or *cbh2+*, suggesting Cdc23 interacts specifically with Abp1. Moreover, genetic interactions were also observed between *abp1+* and four additional DNA replication initiation genes *cdc18+*, *cdc21+*, *orc1+*, and *orc2+*. Interestingly, we find that S phase is delayed in cells deleted for *abp1+* when released from a G1 block. However, DNA replication is not delayed when are released from an early S phase arrest induced by hydroxyurea suggesting that Abp1 functions prior to, or coincident with the initiation of DNA replication.



## **The dual role of the mitotic kinase *Srk1* in fission yeast**

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In fission yeast, the Chk1 and Cds1(Chk2) checkpoint kinases block mitotic entry by inhibiting the Cdc25 phosphatase in response to replication arrest or DNA damage. In this work we show that the stress-activated kinase, *Srk1*, also inhibits mitotic entry by phosphorylating Cdc25. We find that over-expression of *Srk1* kinase causes cell cycle arrest in late G2 phase, an effect which is dependent on the catalytic activity of *Srk1*. Conversely, cells lacking *srk1* enter mitosis prematurely. The G2/M arrest caused by overexpression of *Srk1* is due to hyperphosphorylation of Cdc2 on Tyr15 and can be attributed, primarily, to inhibition of Cdc25 activity. Consistent with this we find that *Srk1* interacts with Cdc25 in vivo. Most importantly, we find that *Srk1* phosphorylates the N-terminal non-catalytic region of Cdc25 by *Srk1* in vitro, at the same sites phosphorylated by the Chk1 and Cds1(Chk2) kinases, and that phosphorylation of Cdc25 on these sites is necessary for *Srk1* to impose a G2 arrest. Furthermore we show that overexpression of *Srk1* causes Cdc25 to accumulate in the cytoplasm, an effect which is dependent on Rad24, a member of the 14-3-3 protein family. Given these observations, we propose a model in which *Srk1* inhibits Cdc25, avoiding a premature onset of mitosis.

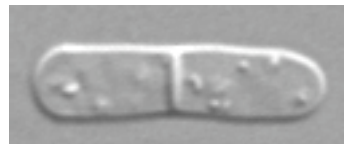
In addition to this role during a normal cell cycle, we show that *Srk1* regulates Cdc25 in response to environmental stress. Treatment of wild type cells with osmotic stress leads to the accumulation of Cdc25 in the cytoplasm and to an increase of the Cdc25-Rad24 binding, whereas this behaviour is not observed in *srk1*-deleted cells. This effect is mirrored in vivo by the stabilization of Cdc25 in the wild type strain compared to the strain lacking *Srk1*. Moreover, the activity of *Srk1* increases dramatically following the exposure of cells to osmotic stress. Taken together, these results suggest that, in a similar manner to Chk1 and Cds1 in response to genotoxic stress, *Srk1* is responsible for the cell cycle arrest and Cdc25 stabilization following a non-genotoxic environmental insult.

## Positioning the Cell Division Plane in Fission Yeast

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In *Schizosaccharomyces pombe*, the position of the pre-mitotic nucleus specifies the cell division plane. Mid1p is a key protein in division site positioning, as *mid1* mutant cells form misplaced contractile rings and septa. Mid1p localizes to the nucleus and to a cortical band of dots overlying the nucleus during interphase. During mitosis, the broad band coalesces with other ring proteins to form the contractile ring. The positioning of the interphase mid1p cortical band is linked to nuclear position. For instance, the mid1p band moves with the nucleus as the nucleus re-centers after cell centrifugation. How mid1p senses the position of the nucleus remains elusive. We hypothesize there exists a physical linkage between the nucleus and the cell cortex which allows mid1p to constantly monitor nuclear position. One candidate for such a linkage is the endoplasmic reticulum (ER). Time-lapse images show that the ER forms dynamic threads between the nuclear envelope and the cell cortex region surrounding the nucleus. We are currently testing the role of the ER in cytokinesis and division site positioning using a number of genetic approaches.



## Analyzing the Role of Rad32 in the Intra-S-Phase DNA Damage Checkpoint

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MRN in *Schizosaccharomyces pombe* is a heterotrimeric complex composed of Rad32 (Mre11 in humans and budding yeast), Rad50 and Nbs1. The MRN complex has many biological functions: formation of double strand breaks in meiosis, homologous recombination, non-homologous end-joining, telomere maintenance and housekeeping functions during replication which are independent of the S-phase checkpoint. The MRN complex also acts in a checkpoint-dependent manner to slow DNA replication in response to DNA damage. Rad32 displays nuclease and DNA binding activities and has been shown to be involved in recombinational repair. Without Rad32, replication progression is not slowed as well as wildtype in response to DNA damage, however the role of specific domains and the cause of replication slowing have not been identified.

Mutations analogous to the mutations most commonly found in the human disease ATLD (ataxia telangiectasia-like disorder), which is caused by mutations in Mre11, have been made in Rad32. However unlike their human analogs thus far the mutations have failed to show separation of function. Mutations made to date have appeared to act just like the Rad32 deletions in *S. pombe*.

One of the potential roles of Rad32 and the rest of the MRN complex is in sister chromatid exchange. The genetic requirements of sister chromatid exchange have been examined using unequal sister chromatid assays which only are able to assay exchanges that are illegitimate and produce changes in the genome. Most sister chromatid exchange must be equal to maintain genomic integrity and thus far there is no good assay for equal sister chromatid exchange. Using a strain containing a human equilibrative nucleoside transporter 1 (hENT1) and a herpes simplex virus thymidine kinase (tk) yeast cells are able to incorporate exogenous thymidine into their DNA. This strain makes it possible for the fission yeast DNA to be labeled with halogenated thymidine analogs. This strain will be used to label one sister with BrdU and then DNA combing will be used to see equal sister chromatid exchange. However, things have been complicated by the difficulty in getting just the right amount of BrdU into the cell so that only one sister is labeled. BrdU blots have been used to look for the correct time point in which the samples should be collected. Also a thymidylate synthase deletion is being made to control the amount of BrdU that is incorporated into the DNA.

## **Novel role of the *S. pombe* nucleolus in cytokinesis checkpoint regulation**

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The fission yeast divides medially through an actomyosin contractile ring (CAR) similar to animal cells. Once the actomyosin ring has formed, the septation initiation network (SIN) regulates ring constriction, septum formation and cytokinesis. However, in the event of a defect in the CAR assembly, a cytokinesis checkpoint is activated that delays further rounds of nuclear division and promotes completion of cytokinesis. This delay depends on the SIN and other regulators like the *S. pombe* homolog of the *S. cerevisiae* Cdc14p phosphatase; Clp1p. Clp1p localizes to the nucleolus and spindle pole body in interphase and is released from the nucleolus in mitosis. If cytokinesis is delayed, Clp1p is retained in the cytoplasm in a SIN dependent manner until the completion of cytokinesis. Cytoplasmic Clp1p in turn prolongs SIN signaling. Thus the SIN and Clp1p are proposed to act in a positive feedback loop to promote completion of cytokinesis. To identify additional regulators of the cytokinesis checkpoint we screened for suppressors of the growth defect of myo2-E1 cdc14-118 cells at 30°C. These cells die because myo2-E1 slows cytokinesis and cdc14-118 compromises SIN signaling which together cause cells to become multinucleate and die. We isolated a suppressor that was named sup6. The sup6 mutation on its own caused temperature sensitivity at 36°C and disrupted nucleolar organization, raising the possibility that it could affect the checkpoint by perturbing the nucleolar localization of Clp1p. However, localization of Clp1p in sup6 cells resembled wt cells. Further, the sup6 cells showed increased Lat B resistance and also suppressed the Lat B sensitivity of the clp1Δ cells suggesting that sup6 acts independent of Clp1p. In our attempt to clone the sup6+ gene we obtained a genomic clone containing the rrn5 gene, which rescued the ts growth defect of sup6. The rrn5 gene is thought to promote RNA pol I specific transcription for the large rRNA synthesis. Mutants in the largest subunit of *S. pombe* RNA polymerase I also exhibit an altered nuclear phenotype at the restrictive temperature similar to sup6. These results suggest that the suppressor is a nucleolar protein and in turn implicate a role for the nucleolus in regulation of SIN and the cytokinesis checkpoint.



## **Cdk1 phosphorylation of Sid2p inhibits cytokinesis in *S. pombe*.**

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Temporal and spatial coordination of nuclear division and cytokinesis is essential for faithful chromosome segregation and genomic stability. Cell cycle transitions are mediated by periodic activation and inactivation of cyclin-dependent kinases (CDKs). Inactivation of Cdk1 triggers mitotic exit and cytokinesis. However, targets of Cdk1 involved in cytokinesis have remained elusive. Sid2p is the most downstream kinase of the Separation Initiation Network (SIN). Sid2p has 6 CDK consensus phosphorylation sites. To identify the significant sites among them, we purified Sid2 protein using a tandem affinity purification approach and analyzed the phosphorylation sites by mass spectrometry. Only 4 sites (S8, S60, S86 and S596) were identified. To determine if the CDK motifs were required for Sid2p function, the serine residues were mutated to alanine or glutamic acid and gene replacement strains were constructed. While the mutation of 4 sites to alanine (4PA) did not inhibit function of Sid2p, the mutation to glutamic acid at amino acid 86 (86E) caused reduced SIN signaling and a cytokinesis checkpoint defect. This suggests that Cdk1 phosphorylation of Sid2p may be one mechanism by which Cdk1 inhibits cytokinesis until Cdk1 is inactivated in anaphase. In addition it suggests that Sid2p must be dephosphorylated to trigger cytokinesis and maintain the cytokinesis checkpoint. The Cdc14-family phosphatase Clp1p dephosphorylates sites phosphorylated by Cdk1, and like Sid2p, is required for the cytokinesis checkpoint. Interestingly Sid2p bound to Clp1p during the cell cycle. The binding was increased at mitosis and decreased at the end of mitosis when the Cdk1 activity is gradually decreased. These results suggest that the Cdk1 activity regulates the timing of cytokinesis through Sid2p, and Sid2p might be a direct target of Cdk1.

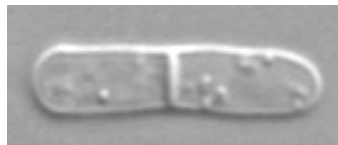


## **Mutation Rate Analysis in *Schizosaccharomyces pombe* replication initiation defective (rid) strains**

Julie M. Sierra-Montes and Gennaro D'Urso

University of Miami School of Medicine, Dept. of Molecular and Cellular Pharmacology and the Dr. John T. MacDonald Center for Medical Genetics

We have recently discovered a novel checkpoint phenotype associated with mutants defective in DNA replication initiation called rid (for replication initiation defective). When grown under semi-permissive conditions, rid mutants display a cell cycle delay that is chk1-dependent, cds1-independent suggesting that a novel checkpoint pathway is activated. By analyzing mutation rates in the presence or absence of chk1, we have determined whether the checkpoint plays a role in either the stimulation or suppression of a mutator phenotype. For each rid mutant, mutation rates were also measured in the absence of cds1+ or in the absence of both chk1+ and cds1+. The forward mutation rate assays using 5-fluoroorotic acid (FOA), which detects mutations inactivating the ura4+ gene, and canavanine, which selects against CAN1+, were used for the identification of the mutator phenotype. Interestingly, we have found that rid mutants, in contrast to mutants that delay S phase progression, do not display a mutator phenotype suggesting that blocks to initiation may be less mutagenic than blocking chain elongation. The data shows that no rid strain at their respective rid-activation temperature displays a mutator phenotype in the presence of both chk1+ and cds1+. However, in the absence of chk1+, some of the rid strains (i.e cdc20-M10) display an increase in mutation rate, consistent with Chk1+ having a role in preventing accumulation of DNA damage when replication initiation is delayed. As expected, no such increase in mutation rates was observed in the absence of cds1+. Therefore, we conclude that chk1+ is required during the cell cycle, either at the G1/S phase transition, or later in G2, to protect cells from DNA damage caused by blocks to DNA replication initiation.



**Cdk9 of fission yeast is activated by the CDK-activating kinase Csk1, overlaps functionally with the TFI<sub>II</sub>H-associated kinase Mcs6 and associates with the mRNA cap methyltransferase Pcm1 in vivo**

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Cyclin-dependent kinase 9 (Cdk9) of fission yeast is an essential ortholog of metazoan positive transcription elongation factor b (P-TEFb) proposed to coordinate capping and elongation of RNA Polymerase (Pol) II transcripts. Here we show that Cdk9 is activated to phosphorylate Pol II and the elongation factor Spt5 by Csk1, one of two fission yeast CDK-activating kinases (CAKs). Activation depends on Cdk9 T-loop residue Thr-212. The other CAK—Mcs6, the kinase component of transcription factor (TF) IIH—cannot activate Cdk9. Consistent with specificities of the two CAKs in vitro, kinase activity of Cdk9 is reduced ~tenfold by *csk1* deletion, and Cdk9 complexes from *csk1Δ* but not *csk1+* cells can be activated by Csk1 in vitro. A *cdk9*-T212A mutant is viable, but phenocopies conditional growth defects of *csk1Δ* strains, indicating a role for Csk1-dependent activation of Cdk9 in vivo. A *cdk9*-T212A *mcs6*-S165A strain, in which neither Cdk9 nor Mcs6 can be activated by CAK, has a synthetic growth defect, implying functional overlap between the two CDKs, which have distinct but overlapping substrate specificities. Cdk9 forms complexes in vivo with the essential cyclin Pch1 and with Pcm1, the mRNA cap methyltransferase. The carboxyl-terminal region of Cdk9, through which it interacts with another capping enzyme, the RNA triphosphatase Pct1, is essential. Together, the data support a proposed model whereby Cdk9/Pch1—the third, essential CDK-cyclin complex described in fission yeast—helps to target the capping apparatus to the transcriptional elongation complex.

## **Nuclear messenger RNA export by Uap56p in *Schizosaccharomyces pombe***

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Center for Cancer Research, National Cancer Institute

*S. cerevisiae* Sub2p or its mammalian homolog UAP56 function as ATP-dependent RNA-helicases with specific roles in pre-messenger RNA splicing and nuclear export of intron-less as well as intron-containing messenger RNA. In the fission yeast, like its homologs, Uap56p functions as an essential mRNA export factor. Recently we demonstrated its role in linking Mlo3p, a putative mRNA adapter, to Rae1p, an essential nuclear pore associated factor. The establishment of a bridging interaction between Rae1p and Mlo3p by Uap56p is critical for targeting of export-ready mRNAs to the nuclear pores in *S. pombe* nucleus. Here we show that unlike its homologs, Uap56p is not essential for splicing. Further, we found that ability to bind RNA rather than ATP is important for mRNA export by Uap56. Finally, we found that a nuclear export activity (NEA) present at the C-terminal half of Uap56 is critical for mRNA export.



## ***S. pombe* as a model system for the investigation of Yersinia outer membrane proteins (Yops)**

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Pathogenic yersiniae (*Y. pseudotuberculosis*, *Y. pestis* and *Y. enterocolitica*) utilize a type three secretion system to inject effector proteins, known as Yersinia outer membrane proteins (Yops), into host cells to evade host immune responses. These Yops are believed to contribute to yersiniae's virulence by directly modulating host factors. However, the identification of specific host factors and the biological significance of their modulation have been hampered by the lack of a good discovery model system. Here, we identify fission yeast, *Schizosaccharomyces pombe*, as an ideal model system to investigate the functions and molecular targets of the Yops. The easily visualized cytoskeleton of *pombe* is especially relevant for the investigation of the Yops since most have been shown to affect the cytoskeleton. We have used *S. pombe* to study the function of the kinase domain of Yersinia protein kinase A (YpkA), which is one of these effector proteins. In mice, the kinase domain of YpkA is important for virulence, however, in model systems with YpkA being expressed at high levels the kinase domain appears to be dispensable. Our results show that the kinase domain of YpkA contributes to the disruption of the actin cytoskeleton in *S. pombe* and demonstrates the importance of expression level and duration when analyzing virulence activities. Moreover, we believe that *S. pombe* is well-suited for biochemical and genetic studies, including mutagenesis and suppression screens, which lend themselves to the application of identifying the pathways for each Yop's role in Yersinia's virulence.

## **Cds1 Activity: Exploring the Mechanisms Underlying Replication Slowing in Response to Damage**

Nicholas Willis and Nicholas Rhind

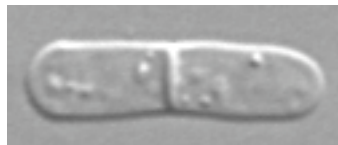
UMass Medical School, Dept. of Biochemistry and Molecular Pharmacology

Cell cycle checkpoints represent mechanisms used by the cell to detect genotoxic stress, slow or arrest the cell cycle, and ensure proper duplication, repair, and segregation of nuclear material to daughter cells upon division. Checkpoint control pathways are activated in response to both intra- and extra-cellular stimuli including products of DNA replication and exposure to DNA damaging agents. When performing properly, players in these pathways facilitate faithful DNA replication during S phase and proper chromosomal segregation during M phase of the cell cycle.

In addition to the S/M and G-2/M checkpoints that prevent mitosis in the presence of damaged and unreplicated DNA, cells employ a checkpoint to slow the rate of DNA replication in response to DNA damage during S phase. In *S. pombe*, slowing is regulated by multiple, Cds1-dependent pathways. Cds1, the downstream checkpoint transducer kinase, is required for signaling during S phase; upon treatment with the alkylating agent MMS, *cds1Δ* mutants display a full checkpoint defect.

Mechanisms invoked for replication slowing in the presence of DNA damage include fork rate slowing and reduction of the number of forks traversing the genome through the regulation of origin firing. Investigating the manner by which Cds1 regulates intra-S checkpoint induced slowing may determine how slowing of forks is employed to slow bulk S-phase progression; over-expression of Cds1 will allow the determination whether fork slowing is a local or global phenomenon. If local, meaning only troubled forks are slowed or have the potential to be slowed, increased Cds1 expression will have no increased slowing effect over that of wild-type cells. If global, meaning that all forks, troubled or otherwise, are slowed, increased Cds1 expression may induce increased slowing in response to MMS.

If over-expression of Cds1 induces additional slowing, this effect may be due to checkpoint-independent, Rad3-independent functions of Cds1 on fork rate or origin regulation. Cds1 overexpression effect on bulk S-phase progression will be investigated on a *rad3Δ* background.



## **Analysis of the dynamics and regulation of DNA replication**

Pei-Yun Wu and Paul Nurse

Rockefeller University

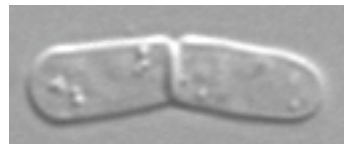
Initiation of DNA replication requires the stepwise assembly of conserved cell cycle regulated proteins to form the pre-replicative complex (pre-RC). The first step of pre-RC formation requires the binding of the six-subunit Origin Recognition Complex (ORC) to origins of replication. In metazoans, levels of ORC, as well as its association with chromatin, are tightly controlled. This regulation restricts pre-RC assembly to the G1 phase of the cell cycle to determine the start of replication and prevent inappropriate re-replication. Previous work in *S. pombe* has shown that both ORC levels and ORC binding to chromatin are constant throughout the cell cycle, although the activity of ORC appears to be modulated by phosphorylation during S phase. In our study, we perform a temporal and spatial analysis of pre-RC formation at specific origins during the cell cycle using chromatin immunoprecipitation and real-time PCR analysis.

## **Checkpoint Activation In Replication Initiation Defective (rid) Mutants Of Fission Yeast**

Ling Yin, Alexandra Locovei and Gennaro D'Urso

University of Miami School of Medicine, Dept. of Molecular and Cellular Pharmacology

We have previously shown that fission yeast mutants deleted for the N-terminal half of DNA polymerase epsilon (*cdc20*) are delayed in the cell cycle and require the checkpoint kinase Chk1 to maintain viability. In contrast, the checkpoint kinase Cds1 that is required for the intra-S phase checkpoint is not essential. We have now identified a number of temperature-sensitive mutants that display a similar phenotype when grown at semi-permissive temperatures. These mutants, all of which are defective in DNA replication initiation, have a cell cycle delay, and are dependent on the presence of the Chk1 kinase for viability. In addition, we have conducted a random mutagenesis screen for cell cycle mutants that are dependent on Chk1 for viability, but are Cds1-independent, and have identified two mutants called *rid1* and *rid2* (for replication initiation defective). The *rid1* gene encodes a novel allele of the *orpl* gene that encodes the Orc1 protein that is essential for DNA replication initiation. These results suggest that our screen is specific for mutants defective in the initiation step. Finally, our analysis of the checkpoint pathway activated in *rid* mutants suggests it responds specifically to initiation blocks, and represents a novel pathway distinct from either the intra-S phase checkpoint or DNA damage checkpoint operating in G2.



### **Ish1p-Ish1p dimerization in vivo by FRET**

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Ish1p is a protein of largely unknown function with orthologues in many other fungal systems. It is expressed strongly in a MAPK dependent fashion during stress response and localizes to the nuclear envelope, subcortical ER, and to a lesser degree in other membrane systems. We have previously shown by yeast two hybrid and IP that Ish1p can homodimerize and also that it can interact with the Bis1p protein (Taricani et al., 2002). Bis1p is highly conserved in all eukaryotes (except budding yeast), localizes to the nucleus and as yet does not have a definitive function associated with it. We do not know to what extent Ish1p is dimerized or oligomerized in vivo or if there is variation in this interaction in different membrane systems or under different physiological conditions. We also do not know where in the nuclear compartment Ish1p interacts with Bis1p although we predict that it is spatially limited to the envelope region.

We have investigated the interaction of Ish1p and Bis1p using CFP/YFP FRET. When Ish1-CFP and Ish1-YFP are co-expressed behind native promoters we can demonstrate a significant FRET signal indicating that these two proteins are in close molecular proximity in the cell. This signal is present in all membrane systems. We interpret this as dimerization or oligomerization of the protein. When tagged Ish1p and Bis1p are co-expressed we are also able to demonstrate a FRET signal between them. These two types of interaction give us tools to investigate the conditions under which they occur and whether they are mutually exclusive.



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