Functions of the Cdc14-family Phosphatase Clp1p in the Cell Cycle Regulation of *Schizosaccharomyces pombe*

A Dissertation Presented

By

SUSANNE TRAUTMANN

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

MAY 20, 2005

MOLECULAR GENETICS AND MICROBIOLOGY

Interdisciplinary Graduate Program

COPYRIGHT INFORMATION

Parts of chapters II, III and appendix A have appeared in separate publications:

Trautmann, S., Wolfe, B.A., Jorgensen, P., Tyers, M., Gould, K.L., and McCollum, D. (2001). Fission yeast Clp1p phosphatase regulates G2/M transition and coordination of cytokinesis with cell cycle progression. Curr Biol *11*, 931-940.

Trautmann, S., and McCollum, D. (2002). Cell cycle: new functions for Cdc14 family phosphatases. Curr Biol *12*, R733-735.

Trautmann, S., Rajagopalan, S., and McCollum, D. (2004). The S. pombe Cdc14-like phosphatase Clp1p regulates chromosome biorientation and interacts with Aurora kinase. Dev Cell *7*, 755-762.

Mishra, M., Karagiannis, J., Trautmann, S., Wang, H., McCollum, D., and Balasubramanian, M.K. (2004). The Clp1p/Flp1p phosphatase ensures completion of cytokinesis in response to minor perturbation of the cell division machinery in Schizosaccharomyces pombe. J Cell Sci *117*, 3897-3910.

Trautmann, S. and. McCollum, D. Subcellular targeting of S. pombe Cdc14-like phosphatase Clp1p/Flp1p reveals distinct nuclear and cytoplasmic functions and suggests a role for nuclear shuttling in Clp1p regulation. Curr Biol, accepted.

Functions of the Cdc14-family Phosphatase Clp1p in the Cell Cycle Regulation of *Schizosaccharomyces pombe*

A Dissertation presented by

SUSANNE TRAUTMANN

Approved as to style and content by

Nick Rhind, Ph.D., Chairman of Committee

Fred Chang, Ph.D., Member of Committee

William Theurkauf, Ph.D., Member of Committee

Craig Mello, Ph.D., Member of Committee

Kirsten Hagstrom, Ph.D., Member of Committee

Dannel McCollum, Ph.D., Thesis Advisor

Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

Interdisciplinary Graduate Program IGP

May 20, 2005

To Dirk Hönicke and my parents

for their love and support

ACKNOWLEDGEMENTS

I am very thankful for the great atmosphere I enjoyed during my graduate studies with such great colleagues and advisors at the third floor of Biotech IV. Thanks to all who have supported me with ears, food and exercise. This thesis would not have been possible without you!

Most of all, I would like to thank my advisor Dr. Dannel McCollum for being the best advisor one could possibly wish for: for teaching me science as well as life lessons and being a great example in patience and generosity.

Special thanks go to the members of the McCollum lab for fruitful discussions and making this place fun to be at every day. Enjoy your time at BiotechIV and good luck. I especially thank my former lab members Dr. Ming-Chin Hou and Dr. David Guertin for their support during the early phase.

LOTS of thanks to my friends at Biotech IV, Cheryl, Satoe, Jess, Shelly (I will miss the lunch ladies!), Yumi, Maddathia, Matt, Rachel, Yi-Shuian and Barbara. What would I have done without chat-Panera-breaks with you!

I would also like to acknowledge Dr. Nick Rhind and Dr. Peter Pryciak and their lab members for fruitful discussions in our joint lab meetings. I want to express my gratitude to the members of my thesis committee, Dr. William Theurkauf, Dr. Craig Mello, Dr Kirsten Hagstrom and Dr. Nick Rhind for being supportive during thesis research and postdoctor hunting as well as Dr. Greenfield Sluder for letters of recommendation.

Special thanks also go to our collaborators Dr. Mohan Balasubramanian and his lab members, especially Mitilesh Mishra and Srivydia Rajagopalan, as well as Dr Kathleen Gould and Ben Wolfe in the Gould lab and Drs. Paul Jorgensen and Mike Tyers for generously sharing reagents and data prior to publication.

I would also like to thank my friends in Germany and Seattle for their support to get me to and through Graduate School, especially my former advisor Dr. Gerold Schubiger.

Finally, my deepest gratitude is dedicated to my parents, my grandmother, my sister and Dirk Hönicke for their endless patience, love and support.

Abstract

In order to generate healthy daughter cells, nuclear division and cytokinesis need to be coordinated. Premature division of the cytoplasm in the absence of chromosome segregation or nuclear proliferation without cytokinesis might lead to aneuploidy and cancer.

The cyclin dependent kinases, CDKs, are a main regulator of the cell cycle. Timely increase and decrease in their activity is required for cell cycle progression. To enter mitosis, mitotic CDK activity needs to rise. CDK activity stays elevated until chromosome segregation is completed and exit from mitosis requires decrease in CDK activity.

Observations in several experimental systems suggest that coordination of cytokinesis with the nuclear cycle is regulated through CDK activity. Prolonged high CDK activity, as it occurs when chromosome segregation is delayed, was found to oppose cytokinesis. Prevention of cytokinesis through high CDK activity may therefore provide a mechanism to prevent precocious cell division in the absence of chromosome segregation. To prevent polyploidy when cell division is delayed, progression through the next nuclear cycle should be inhibited until cytokinesis is completed, presumably by the inhibition of CDK activity.

In the fission yeast *Schizosaccharomyces pombe*, a signaling cascade called Septation Initiation Network (SIN) is required for the coordination of cytokinesis with the nuclear cycle. The SIN is essential for cytokinesis, triggering the execution of cell division through constriction of the actomyosin ring. The activation of the SIN signaling

cascade, and thus cytokinesis, is opposed by high CDK activity, preventing precocious cytokinesis.

S. pombe delay entry into the next nuclear division in response to delayed cytokinesis due to defects in the contractile ring until cytokinesis is completed thereby preventing the accumulation of multinucleate, non viable cells. This safeguard against multinucleate cells is termed the cytokinesis checkpoint. The cytokinesis checkpoint keeps CDK activity low, preventing nuclear cycle progression. The SIN is required for the cytokinesis checkpoint and therefore is a key coordinator between nuclear cycle and cytokinesis. How the SIN functions in the cytokinesis checkpoint was not known.

Cdc14-family phosphatases are highly conserved from yeast to humans, but were only characterized in *Saccharomyces cerevisiae* at the time this thesis was initiated. Cdc14 had been identified as the effector of a signaling cascade homologous to the SIN, called the mitotic exit network (MEN), which is required for exit from mitosis. This thesis describes the identification of the *S. pombe* Cdc14-like phosphatase Clp1p as a component of the cytokinesis checkpoint. Clp1p opposes CDK activity, and Clp1p and the SIN activate each other in a positive feedback loop. This maintains an active cytokinesis checkpoint and delays mitotic entry. We further found that Clp1p regulates chromosome segregation.

Concluding, this thesis describes discoveries adding to the characterization of the cytokinesis checkpoint and the function of Clp1p. While others found that Cdc14-family phosphatases, including Clp1p, have similar catalytic functions, we show that their biological function may be quite different between organisms, possibly due to different biological challenges.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

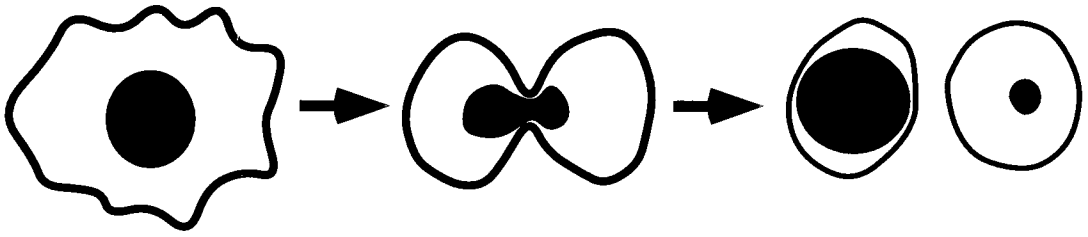
General Introduction

In order for any organism to develop, cells must multiply by growth, genome duplication and nuclear division, followed by cytoplasmic division, which ultimately generates a new cell. Coordination of cytoplasmic division and nuclear division is crucial for healthy cell proliferation. Precocious cytokinesis, before chromosome segregation might lead to the abscission of unsegregated chromosomes (Figure 1-1A). If cytokinesis is delayed, mitotic reentry prior to cytokinesis will lead to multinucleate cells and complications during mitosis due to multiple centrosomes and polyploidy (Figure 1-1B). Defects caused by the lack of coordination between cytokinesis and mitosis can therefore cause aneuploidy, which has been associated with cancer (Jallepalli and Lengauer, 2001).

Coordination of cytokinesis with the nuclear cycle requires cross talk between the nuclear cycle regulators and the cytokinesis regulators. The nuclear cycle regulators should be able to delay cytokinesis when chromosome segregation is delayed and the progression of the nuclear cycle regulators should react to delays in cytokinesis and stop mitotic entry to prevent polyploidy. A large amount to data suggest that higher as well as lower eukaryotes have evolved mechanisms to ensure proper order of cell cycle events, linked to cytokinesis. Understanding of coordination between nuclear cycle and cytokinesis requires knowledge about the regulation of the nuclear cycle and cytoplasmic division themselves.

Figure 1-1: Coordination of mitosis and cytokinesis

A: precocious cytokinesis



B: delayed cytokinesis

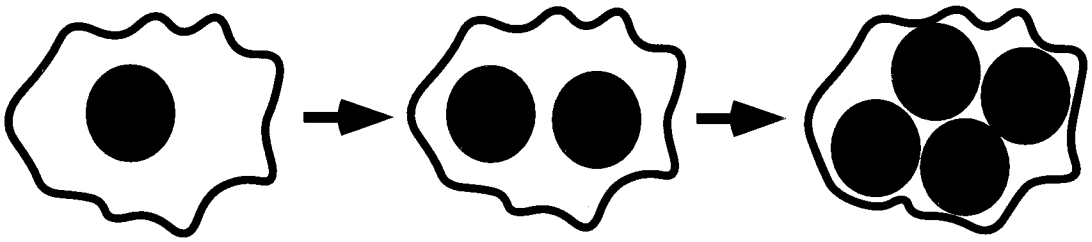


Figure 1-1: Coordination of mitosis and cytokinesis

A: Precocious cytokinesis prior to successful chromosome segregation can lead to a daughter cells with incorrect genetic material.

B: Cell cycle progression in the absence of prior cell division results in cells with polyploid genomes.

The cell cycle machinery is highly conserved between higher and lower eukaryotes such as the unicellular yeast. This along with the ease of genetics, biochemistry and cell biology in the lower eukaryotic yeast make it an ideal model organism to study basic cell cycle regulatory mechanisms. The fission yeast *Schizosaccharomyces pombe*, which was used in this study, offers a particularly useful system for the study of cytokinesis, since this rod shaped yeast divides by medial fission through an actomyosin ring, similar to mammalian cells.

Nuclear cycle regulation

Nuclear cycle events are often regulated by phosphorylation. A key regulator of nuclear cycle progression are the Cyclin-Dependent-Kinases, CDKs. This conserved kinase family is controlled by the activating regulatory subunits, the cyclin family proteins, and through activating and inhibitory phosphorylation. Most cells express several cyclins and binding of CDKs to different cyclins changes the function of CDKs during the cell cycle (Morgan, 1997). The fission yeast possesses only one essential cyclin, Cdc13p.

The nuclear cycle starts with the duplication of the genetic material by replication during S-phase. The cell stays in G2-phase until this requirement is fulfilled and the cell is big enough and harbors copies of other organelles (Figure 1-2 A). Only then does mitotic CDK (referred to as Cdk1in the following) activity rise to allow entry into mitosis. Cdk1 activity increases through the accumulation of the mitotic B-type cyclin subunit and activating dephosphorylation and phosphorylation of Cdk1.

In *S. pombe*, the mitotic cyclin Cdc13p accumulates during G2 phase and binds the CDK Cdc2p, the fission yeast Cdk1. This complex is maintained inactive due to inhibitory phosphorylation of Cdk1 on Tyr15 in the ATP binding domain by the kinases Wee1p and to a minor extend by Mik1p kinase, until replication is finished and sufficient cell size is reached (Creanor and Mitchison, 1996; Gould and Nurse, 1989; Russell and Nurse, 1987). Only then is the inhibitory phosphorylation reversed by the essential phosphatase Cdc25p in a rate-limiting step and Cdk1 activated (Nilsson and Hoffmann, 2000; Russell and Nurse, 1986) (Figure 1-2 B). *wee1* loss of function mutants enter mitosis prematurely at a smaller size (hence: "wee") (Russell and Nurse, 1987). Conversely, lack of Cdc25p leads to failure in Cdk1 activation and a G2 arrest (Russell and Nurse, 1986). Additionally, Cdk1 is activated through phosphorylation at its T-loop Threonine T167. The kinases Mcs6p, Mcs2p and Csk1p are responsible for this phosphorylation (Buck et al., 1995; Damagnez et al., 1995; Hermand et al., 1998; Lee et al., 1999b). Translocalization of the Cdk1/Cyclin complex Cdc2p/Cdc13p to the nucleus, where its primary substrates are localized, enhances the sudden increase in Cdk1 activity (Alfa et al., 1989; Audit et al., 1996). The regulation of mitotic entry through mitotic cyclin accumulation and the activation of the Cdk1/Cyclin complex by dephosphorylation of the ATP-binding domain Tyrosine through Cdc25 and phosphorylation at the T-loop is conserved among other eukaryotes (Morgan, 1997). It was shown in *Xenopus laevis* egg extracts, that the initial Cdk1 activation is enforced by a positive feedback loop, where Cdk1 phosphorylates and inhibits its inhibitor, the Wee1p kinase and activates its activator the Cdc25p phosphatase (Izumi and Maller, 1993). In *S. pombe,* Cdc25p is hyperphosphorylated and activated upon mitotic entry and dephosphorylation of the

critical site reduces its activity, suggesting a similar positive feedback loop (Coleman and Dunphy, 1994; Kovelman and Russell, 1996). Whether the feedback loop functions through Wee1p kinase in fission yeast is not known, although Wee1p activity decreases during mitosis (Coleman and Dunphy, 1994). As suggested from data shown here and elsewhere, the Cdc14-like phosphatase Clp1p can delay mitotic entry in a Wee1p dependent manner (Cueille et al., 2001; Trautmann et al., 2001). As with other Cdc14-family phosphatases, Clp1p specifically dephosphorylates proline directed phosphorylation sites, such as CDK sites and may therefore disrupt a positive feedback loop for CDK activation. Indeed, dephosphorylation of Cdc25p by Clp1p reduces Cdc25p activity (Figure 2 B)(Esteban et al., 2004; Wolfe and Gould, 2004). Clp1p may also dephosphorylate Wee1p and thereby promote its inhibitory function. Whether Clp1p directly regulates Wee1p and whether the G2/M regulatory function of the Cdc14-family phosphatase Clp1p is conserved in higher eukaryotes is not known (Figure 1-2 B).

During mitosis, chromatin condenses and is evenly segregated to opposite cell ends. In order for cells to proceed beyond chromosome segregation and to exit mitosis and divide, mitotic CDK activity needs to decrease. The most conserved mechanism for CDK inactivation is degradation of the mitotic cyclin; expression of non-degradable cyclins prevents mitotic exit and cytokinesis in a variety of organisms (Surana et al., 1993; Wheatley et al., 1997; Yamano et al., 1996). The mitotic cyclin is ubiquitinated through the E3-ubiquitin ligase complex APC/C and targeted to the proteasome for destruction (Peters, 2002). *S. pombe* mitotic cyclin Cdc13p is also degraded through ubiquitin-mediated proteolysis (Yamano et al., 1996). Additionally, Cdk1 might be partly inactivated by inhibitory phosphorylation, caused by the destabilization of Cdc25p

through Clp1p (Esteban et al., 2004; Wolfe and Gould, 2004). Some active Cdk1 may remain after mitotic exit to regulate further cell cycle progression. Additional inhibition of Cdk1 through further degradation and direct inhibition is only required for G1 arrest in response to starvation and mating pheromones. This G1 inhibition is mediated by specialized cyclin degradation, which uses the APC/C specificity factor Ste9p, and through the Cdk1 inhibitor Rum1p; mutants in *rum1* and *ste9* are sterile (Kitamura et al., 1998; Labib and Moreno, 1996).

In addition to ubiquitin mediated degradation of Cyclin, Cdk1 activity is down regulated by CDK inhibitors in several organisms and cell types (Morgan, 1997). Reversal of CDK activity by dephosphorylation of substrates may accelerate the switch from high to low CDK activity. In budding yeast *S. cerevisiae*, the phosphatase Cdc14, the homolog of Clp1p, dephosphorylates substrates of Cdk1/Cdc28 and is essential for exit from mitosis. Cdc14 triggers Cyclin degradation through activation of APC/C specificity factor Cdh1, a homolog of *S. pombe* Ste9p, and activates the Cdk1 inhibitor Sic1, which carries homology to *S. pombe* Rum1p. This secondary step of Cdk1 inhibition is essential in budding yeast, but does not appear to be required for mitotic exit in fission yeast. Whether this mitotic exit regulatory mechanism is conserved in other eukaryotes remains an unanswered question.

Figure 1-2: *S. pombe* cell cycle

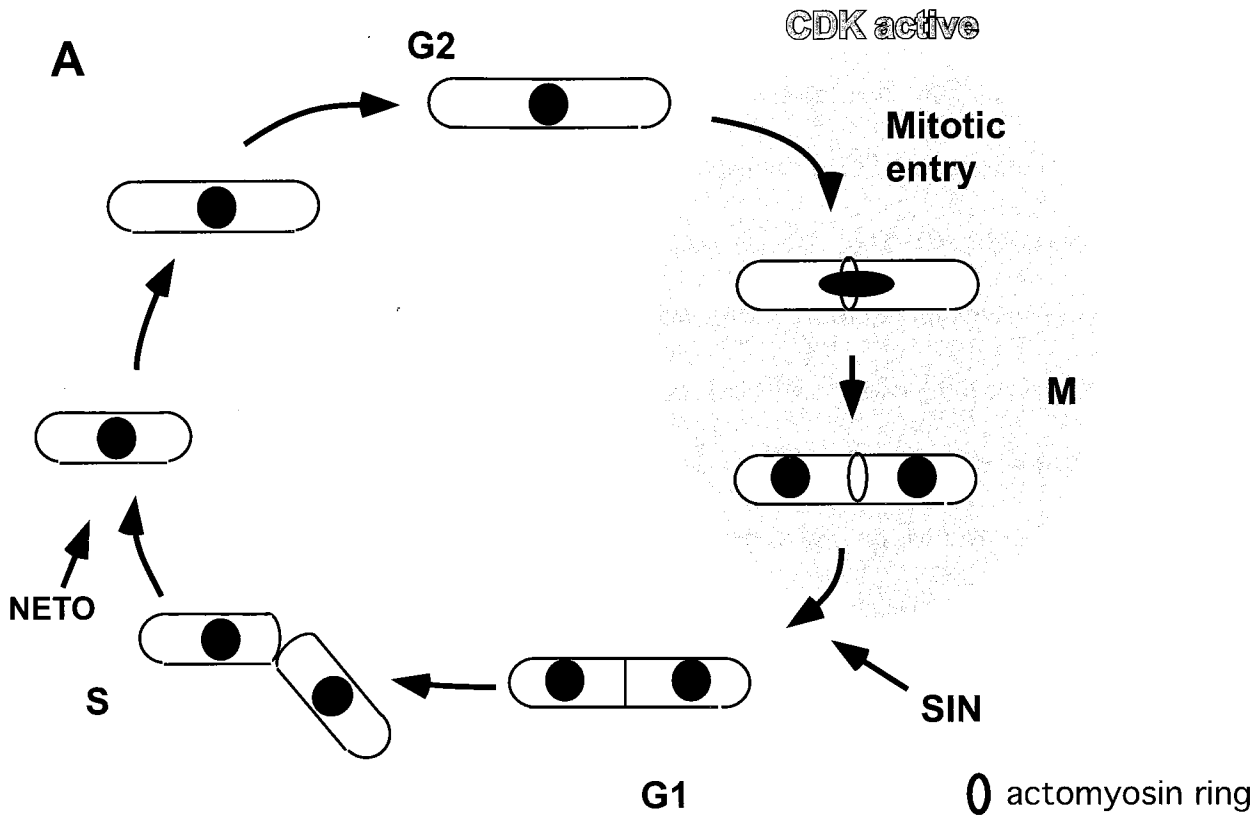


Figure 2: *Schizosaccharomyces pombe* cell cycle

A: Starting in G2 phase, fission yeast grows from the cell tips to reach the required size for cell division in G2 phase. Then, CDK activity rises and the cell enters mitosis (M-phase). Precocious mitotic entry reduces the time of growth in G2 and results in shorter cells, while block in G2 phase allows the cells to grow longer. At the beginning of mitosis, an actomyosin ring assembles on the cortex proximal to the nucleus. After chromosome segregation and CDK downregulation, the SIN signaling cascade triggers ring constriction and cytoplasmic division. Together with ring constriction, a septum is formed between the two daughter cells. After a cell wall is built at the new cell ends, this septum is degraded, allowing cell separation in G1 or S phase. During S phase, DNA replicates and the cell grows first from the old until it reaches the new-end take-off stage (NETO) and starts to grow at both tips to the required size for mitotic entry.

B: Activation of the mitotic cdk/cyclin complex Cdc2p/Cdc13p is inhibited by phosphorylation through Wee1p kinase and activated through dephosphorylation of the same phosphate by Cdc25p. When CDK activity rises, Wee1p kinase may be inhibited through phosphorylation by Cdc2p/Cdc13p and Cdc25p activated through the same mechanism. This positive feedback loop is thought to be disrupted by the phosphatase Clp1p. Clp1p dephosphorylates the Cdc2p/Cdc13p phosphorylation sites of Cdc25p and possibly Wee1p, activating and inactivating them respectively.

Regulation of Cytokinesis

As described in several reviews (Balasubramanian et al., 2004; Guertin et al., 2002a), cell division events start with the determination of the division site and the assembly of the structure that executes separation, such as the contractile ring in animal cells and fission yeast. This contractile ring consists of actin and myosin and several of their assembly factors. After chromosome segregation, the ring constricts and the plasma membrane ingresses, forming the cleavage furrow. Together with the remnants of the spindle microtubules, the constricting ring forms the midbody at the narrow cytoplasmic bridge. Finally, this bridge between the two cytoplasmic compartments is sealed off by mechanisms involving membrane fusion and fission and two entirely separated daughter cells are generated.

Cytokinesis in fission yeast

Work from a number of labs has contributed to a good understanding of cell division in *S. pombe* (Krapp et al., 2004b; McCollum and Gould, 2001). At the beginning of mitosis a ring composed of actin and myosin is assembled around the medial cell cortex, proximal to the nucleus. A screen for mutants with misplaced actomyosin rings has implicated the anillin homolog Mid1p and the polo kinase Plo1p in determination of the division site at mitotic entry (Bähler et al., 1998a; Chang and Nurse, 1996; Sohrmann et al., 1996). Several proteins were found to be essential for ring assembly. Among those are profilin Cdc3p, the formin Cdc12p, tropomyosin Cdc8p, type II myosin Myo2p and Myp2 and the light chain Cdc4p and Rlc1p, Cdc15p, a SH3-domain containing protein and the IQ-GAP protein Rng2p (Balasubramanian et al., 1992; Balasubramanian et al., 1994;

Balasubramanian et al., 1998; Bezanilla et al., 1997; Chang et al., 1997; Eng et al., 1998; Fankhauser et al., 1995; Kitayama et al., 1997; Le Goff et al., 2000; May et al., 1997; McCollum et al., 1995). Collectively, they are called the ring (rng) genes (Gould and Simanis, 1997). An essential signaling cascade termed the septation initiation network (SIN) triggers the constriction of the actomyosin ring after chromosome segregation, followed by the deposition of septum material to build a new cell wall. After completion of cytoplasmic division, the septum is digested to allow cell separation (Figure 1-2 A).

The SIN

The septation initiation network was first identified through the discovery of several complementation groups of mutants with either of two phenotypes. SIN loss of function mutants were not defective in the assembly of the actomyosin ring in early mitosis, but fail in ring maintenance, constriction and septum deposition (Balasubramanian et al., 1998; Mishra et al., 2004; Wu et al., 2003a). The other phenotype showed several uncontrolled rounds of septation events without cell cleavage, resulting in a multiple septa phenotype (Fankhauser et al., 1993; Minet et al., 1979; Song et al., 1996). The genes of those complementation groups encode activators or inhibitors of the cytokinesis inducing signaling network SIN.

Molecularly, the SIN is a Ras-like signaling cascade anchored at the spindle pole body (SPB, the functional homolog of the centrosomes in higher eukaryotes)(Figure 1-3). The two scaffolding proteins Sid4p and Cdc11p bind several SIN signaling molecules to the SPB and mutations in *sid4* or *cdc11* lead to loss of SIN function (Chang and Gould,

2000; Krapp et al., 2001; Morrell et al., 2004; Tomlin et al., 2002). The most upstream activator of the SIN cascade is a small GTPase, Spg1. Spg1p localizes to the SPB throughout the cell cycle and is thought to be converted from its GDP form in interphase to its GTP form at mitotic entry (Schmidt et al., 1997; Sohrmann et al., 1998). In anaphase, Spg1p on the old SPB is converted from its GTP-bound from to its GDP bound form (Grallert et al., 2004; Sohrmann et al., 1998). This asymmetry in nucleotide conversion is regulated through the two-component GAP Cdc16p and Byr4p (Furge et al., 1998). Lack of this change to Spg1p-GDP through mutations in *cdc16* or *byr4* leads to uncontrolled septum formation in all cell cycle stages as well as multiple septation events; the asymmetry might therefore ensure that cytokinesis happens only once per cell cycle (Furge et al., 1998; Guertin et al., 2000; Song et al., 1996). After establishment of the Spg1-GTP/GDP asymmetry, the kinase Cdc7p localizes asymmetrically to the new SPB, which carries Spg1pGTP (Grallert et al., 2004; Sohrmann et al., 1998). Like Cdc7p, the kinase complex Sid1p/Cdc14p becomes localized to the new SPB in response to Spg1p conversion (Guertin et al., 2000). The activated Sid1p/Cdc14p kinase induces a rise in Sid2p kinase activity and the translocalization of Sid2p/Mob1p complex from the SPB to the actomyosin ring, where they are thought to trigger ring constriction (Hou et al., 2000; Sparks et al., 1999). The substrates of Sid2p at the ring and the molecular mechanism for the induction of ring constriction remain elusive.

Other SIN regulators include polo kinase Plo1p and Dma1p, a protein with a FHA domain and an E3 ubiquitin ligase-like RING finger domain and ubiquitin ligase activity (Guertin et al., 2002b). Polo kinase expression can induce septation out of interphase and lack of Plo1p in anaphase leads to a SIN mutant phenotype (Ohkura et al., 1995). It has

been hypothesized that Plo1p inhibits Cdc16/Byr4p and thereby activates Spg1p (Hu et al., 2001; Krapp et al., 2004a; Ohkura et al., 1995; Tanaka et al., 2001). Plo1p kinase localization to the SPB in early mitosis may displace Cdc16p/Byr4p from the SPB so that Spg1p changes to its GTP form (Bähler et al., 1998a; Mulvihill et al., 1999). Absence of Plo1p in anaphase may revert Spg1p at one SPB to its GDP form, activating the SIN. Plo1p downregulation was shown to be regulated by Dma1p. Dma1p may ubiquitinate Plo1p and thereby facilitate Plo1p degradation. Supporting this model, Dma1p over production inhibits SIN activity (Guertin et al., 2002b).

Coordination of cytokinesis with the nuclear cycle in *S. pombe*

Regulation of cytokinesis by the nuclear cycle

Studies in many biological systems have shown that high CDK activity caused by the expression of non-degradable Cyclins arrests cells shortly after anaphase initiation and inhibits cytokinesis (Surana et al., 1993; Wheatley et al., 1997; Yamano et al., 1996). This dependency is crucial to prevent cell division prior to the completion of chromosome segregation. The same is true in *S. pombe* (Yamano et al., 1996). In *S. pombe*, the SIN induces ring constriction and septum formation only after chromosome segregation, when Cdk1 activity is lowered. Further, the activity of the E3-ubiquitin-ligase complex APC/C is required for SIN activation (Chang et al., 2001). This observation suggested that the SIN might be inhibited by high CDK activity prior to anaphase. Indeed, it was found that high CDK activity reduces the localization of Sid1p/Cdc14p to the SPB, leading to suppression of SIN signaling. Conversely, when

CDK activity is inappropriately lowered in metaphase, Sid1p localizes to the SPB prior to chromosome segregation (Guertin et al., 2000). As described here and elsewhere, absence of the CDK-activity opposing phosphatase Clp1p, reduces SIN activity (Cueille et al., 2001; Trautmann et al., 2001). This suggests that dephosphorylation of a SIN component by Clp1p supports SIN activity. Whether Sid1p/Cdc14p or other SIN members are substrates of CDK and Clp1p remains unknown. SIN might also be regulated by CDK phosphorylation on multiple components (Figure 1-3 and 1-4 A).

Regulation of nuclear cycle progression through cytokinesis regulators

Delay in cytokinesis can lead to polyploidy if nuclear proliferation continues. *S. pombe* possesses a checkpoint, termed cytokinesis checkpoint, that monitors cytokinesis and delays mitotic reentry when cytokinesis is delayed (Le Goff et al., 1999b; Liu et al., 2000; Liu et al., 1999). The cytokinesis checkpoint is activated when septum formation, actomyosin ring assembly or ring stability are affected (Le Goff et al., 1999b; Liu et al., 2000; Mishra et al., 2004; Trautmann and McCollum, 2005). This is the case in mutants in a 1,3-β-glucan synthase subunit *cps1*, which are impaired in septum formation, as well as in rng mutants and after treatment of cells with low dosages of the actin depolymerizing drugs Latrunculin A or B (Le Goff et al., 1999b; Liu et al., 2000; Liu et al., 1999; Mishra et al., 2004; Trautmann and McCollum, 2005; Trautmann et al., 2001). All of those perturbations result in the accumulation of binucleate cells with nuclei in G2 phase.

Although mutants in the SIN cascade encounter defects in ring stability as well, their phenotypes are quite different from the above. SIN loss of function mutants fail in

cytokinesis, but do not delay in G2 as binucleate cells. Instead they reenter mitosis rapidly and become polyploid. This suggested that the SIN is required for the cytokinesis checkpoint (Liu et al., 2000) (Figure 1-4 B). Further, SIN signaling is maintained active when cytokinesis and mitotic entry are delayed and may keep cells in a cytokinesis competent state. While wild type cells are resistant to treatment with low dosages of the actin depolymerizing drug Látrunculin A (or B), which slows down cytokinesis, thermo sensitive SIN mutants at semi permissive temperatures cannot form colonies, possibly due to loss of ability to trigger cytokinesis (Mishra et al., 2004). SIN may also inhibit CDK activity. This model is supported by the observation that ectopic activation of the SIN through over production of the Sid1p binding protein Cdc14p results in G2 delay (Fankhauser and Simanis, 1993).

Another protein required for the cytokinesis checkpoint is the CDK inhibiting kinase Wee1p (Liu et al., 2000). In the absence of Wee1p, wild type cells enter mitosis precociously and delay in cytokinesis leads to rapid polyploidization.

Our studies showed that the phosphatase Clp1p is also required for the cytokinesis checkpoint (Cueille et al., 2001; Trautmann et al., 2001). Clp1p inhibits G2/M transition in a Wee1p dependent manner by promoting tyrosine phosphorylation of Cdc2p. Furthermore, Cdc2p is tyrosine phosphorylated in the cytokinesis checkpoint, suggesting that Clp1p mediates delayed mitotic entry in the absence of cytokinesis. It is not clear, however, whether Clp1p regulates mitotic entry in the cytokinesis checkpoint through Cdc25p or Wee1p.

Further indications for how Clp1p functions in the cytokinesis checkpoint originated from its localization. Clp1p localizes to the nucleolus and the spindle pole

body during interphase. Upon mitotic entry, Clp1p leaves these sites and is released from the nucleolus throughout the cell and appears at the actomyosin ring and the kinetochores and at the spindle midzone in anaphase. Once cytokinesis is complete, Clp1p relocalizes to the nucleolus and the SPB. When the cytokinesis checkpoint is activated, Clp1p does not return to the nucleolus as in normal interphase nuclei, but is retained in the cytoplasm. This cytoplasmic localization is required for Clp1p function in the cytokinesis checkpoint (Trautmann and McCollum, 2005; Trautmann et al., 2001). Active SIN signaling can retain Clp1p in the cytoplasm once it has been released from the nucleolus at mitotic entry and the SIN is required for the cytoplasmic retention of Clp1p during active cytokinesis checkpoint as shown in Chapter II (Cueille et al., 2001; Trautmann et al., 2001). In summary, binucleate cells arrested due to the cytokinesis checkpoint maintain SIN activity and Clp1p in the cytoplasm and Clp1p cytoplasmic localization and SIN activity depend on each other.

It is therefore of interest to determine how Clp1p nucleolar relocalization is prevented by the SIN. Two mechanisms might be envisioned: the SIN might reduce the binding affinity to a yet unknown nucleolar Clp1p binding partner. Alternatively, Clp1p nucleolar accumulation might be prevented by the regulation of nuclear import/export mechanisms by SIN. As shown here, the release of Clp1p from the nucleolus at G2/M transition is independent of the SIN and the SIN does not regulate the affinity of Clp1p for the nucleolus to keep Clp1p cytoplasmic (Cueille et al., 2001; Trautmann and McCollum, 2005; Trautmann et al., 2001). This suggests regulation of Clp1p by the SIN through nuclear import/export mechanisms. How in detail the SIN facilitates this regulation is an open question. As discussed in Chapter IV, a likely candidate of a

mediator between Clp1p and the SIN is the 14-3-3 protein Rad24p (Mishra et al., ; Trautmann and McCollum, 2005).

Which mechanisms Clp1p triggers to block the nuclear cycle in G2 in the cytokinesis checkpoint is not known. As mentioned above, one way may be by inhibition of Cdk1p by tyrosine phosphorylation. As a second possibility, cytoplasmic Clp1p might function in the cytokinesis checkpoint through stabilizing the actomyosin ring. Deletion of *clp1* impairs the maintenance of the actomyosin ring after mild treatment with actin depolymerizing drugs Latrunculin A or B (Mishra et al., 2004)(Figure 1-4).

The SIN is also required for ring stability and has been reported to delay G2/M transition (Balasubramanian et al., 1998; Fankhauser et al., 1993; Minet et al., 1979; Schmidt et al., 1997). Therefore, Clp1p might function entirely through maintaining SIN activity in a positive feedback loop and SIN would execute the cytokinesis checkpoint through unknown mechanisms. In support of a function of Clp1p upstream of the SIN, nuclear cycle delay in the SIN hyperactivating mutant *cdc16-116* is independent of Clp1p (Mishra et al., 2004) and localization of Clp1p to the cytoplasm through fusion to a nuclear export signal does not rescue the cytokinesis checkpoint defect in SIN mutants (Trautmann and McCollum, 2005)(Figure 1-4).

The involvement of SIN and Clp1p in both the prevention of precocious cytokinesis and the cytokinesis checkpoint emphasizes their importance in the coordination of the nuclear cycle with cytokinesis. Higher eukaryotes and other yeast encode homologs of both components of the SIN and Clp1p, suggesting conservation of their function in coordinating cytokinesis with the nuclear cycle (Figure 1-4 B and Table 1-1).

Figure 1-3 SIN Model

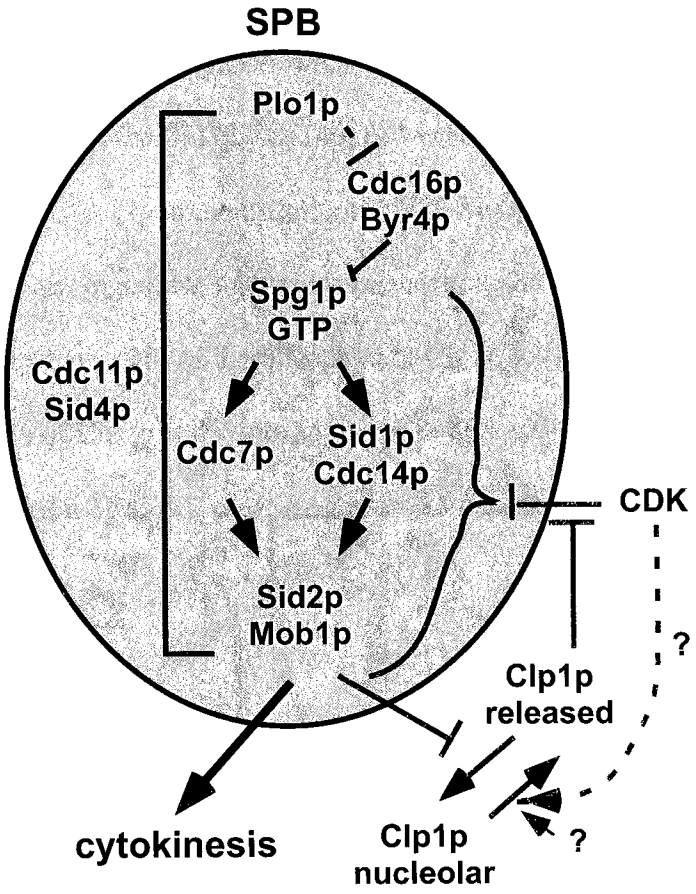


Figure 1-3: Septation initiation network

The SIN is anchored at the SPB (pink) through the scaffold proteins Cdc11p and Sid4p. The polo kinase Plo1p activates the GTPase Spg1p through inhibition of the GAP Cdc16p/Byr4p. After anaphase, when CDK activity is reduced, Spg1p recruits the kinases Cdc7p and the Sid1p/Cdc14p, followed by the activation of the kinase complex Sid2p and Mob1p. Upon the upstream signal, Sid2p/Mob1p translocate from the SPB to the actomyosin ring and trigger ring constriction and septum formation. Additionally, the SIN prevents the return of the phosphatase Clp1p to the nucleolus. This cytoplasmic maintenance of Clp1p is thought to promote SIN activity by reversing inhibitory phosphorylation by Cdk1, creating a positive feedback loop between Clp1p and the SIN.

Figure 1-4 Coordination of Cytokinesis with Cdk1 in *S. pombe*

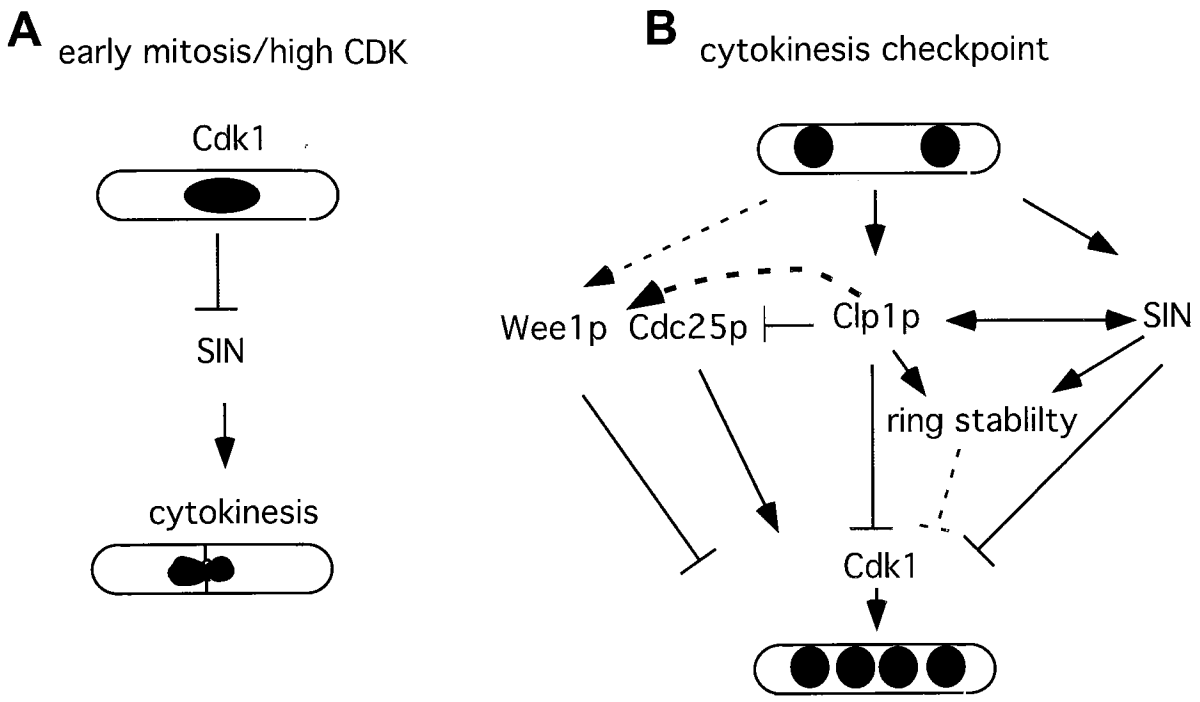


Figure 1-4: Coordination of cytokinesis and CDK in *S. pombe*

(A) Cytokinesis prior to completion of proper chromosome segregation is inhibited through CDK inhibition of SIN. Until chromosomes are segregated, CDK activity is high and inhibits the SIN from executing ring constriction.

(B) In the event of delayed cytokinesis due to perturbations in the cytokinesis apparatus, mitotic reentry is delayed. This delay requires the SIN, Clp1p and the CDK inhibitory kinase Wee1p. In late mitosis, the SIN is active and Clp1p phosphatase is released from the nucleus throughout the cytoplasm. When the actomyosin ring is perturbed, the SIN signal stays active even though cells exit mitosis and Clp1p is kept in the cytoplasm. These two events are interconnected with SIN maintaining Clp1p cytoplasmic and active and Clp1p maintaining SIN active. The SIN and Clp1p, as well as Wee1p are capable of CDK inhibition, which leads to a delay in mitotic entry. While it is known that Wee1p kinase can execute CDK inhibition directly, Clp1p and the SIN might do so indirectly. Clp1p might either dephosphorylate CDK targets and thereby prevent mitotic entry or function through tyrosine phosphorylation of Cdk1 through the Wee1p/Cdc25p pathway. The SIN might then function through Clp1p. Both the SIN and Clp1p are further required for actomyosin ring stabilization, which is required for the cell to try and undergo cytokinesis for a healthy cell division. This stable ring might be monitored through an unknown mechanism and prevent mitotic reentry.

Functions of Clp1p in chromosome segregation

Because Clp1p dephosphorylates CDK substrates (Wolfe and Gould, 2004) and localizes to a variety of cellular locations (Cueille et al., 2001; Trautmann et al., 2001), it is not surprising that Clp1p regulates multiple cellular processes. As shown in Chapter III, Clp1p localizes to the kinetochores and regulates chromosome segregation by ensuring sister chromatid bi-orientation (Trautmann et al., 2004). *clp1* has genetic, cell biological and biochemical interaction with members of the conserved protein family of chromosomal passenger proteins. Chromosomal passenger proteins localize to the kinetochores and the spindle mid zone, similar to Clp1p, and are implicated in the regulation of sister chromatid segregation in several experimental systems (Carmena and Earnshaw, 2003; Tanaka, 2002). Therefore, Clp1p might regulate chromosome segregation together with the chromosomal passenger proteins in *S. pombe*.

Homologies to other Organisms

Budding yeast *S. cerevisiae*

S. cerevisiae possesses a signaling network homologous to the SIN, which is required for exit from mitosis and is termed the mitotic exit network (MEN). Despite the high degree of molecular conservation (see Table 1-1), the SIN is not essential for mitotic exit. While loss of SIN signaling leads to cytokinesis failure and multinucleate cells, loss of MEN signaling prevents mitotic exit, arresting cells with an elongated spindle after anaphase. Interestingly, several MEN proteins localize to the mother-bud neck in late anaphase and telophase or display cytokinesis defects when rescued for their mitotic exit defect,

suggesting a requirement of MEN for cytokinesis (Frenz et al., 2000; Lippincott et al., 2001; Luca et al., 2001; Menssen et al., 2001; Shou et al., 1999; Song et al., 2000; Song and Lee, 2001; Xu et al., 2000; Yoshida and Toh-e, 2001). Conversely, the SIN may regulate mitotic exit through the phosphatase Clp1p (Esteban et al., 2004; Wolfe and Gould, 2004).

The MEN

Like the MEN the SIN, is a G-protein-coupled signaling cascade, organized at the SPB by the scaffolding protein Nud1, a homolog of Cdc11p (Grueneberg et al., 2000) (Figure 1-5). Genetic and biochemical experiments suggest that the GTPase Tem1 functions near the top of the pathway (Jaspersen et al., 1998; Lee et al., 2001; Shirayama et al., 1994b; Visintin and Amon, 2001). Tem1 is the homolog of Spg1p and is negatively regulated through the GAP-complex Bub2 and Bub2/Bfa1, the homologs of Cdc16p and Byr4p (Alexandru et al., 1999; Hoyt et al., 1991; Krishnan et al., 2000; Lee et al., 1999a). The inhibitory Bub2/Bfa1 complex is itself inhibited by polo kinase Cdc5 (Hu et al., 2001). Cdc5 phosphorylates Bub2/Bfa1, which reduces its binding to Tem1. Tem1 is activated at the future daughter SPB when the mitotic spindle elongates into the bud where a putative GEF Lte1 localizes (Bardin et al., 2000; Pereira et al., 2000; Shirayama et al., 1994a). This dependence of MEN signaling on chromosome segregation is thought to coordinate the two. The asymmetric signal on the bud-SPB then localizes the kinase Cdc15, a Cdc7p homolog, at the SPB (Cenamor et al., 1999; Grueneberg et al., 2000; Menssen et al., 2001; Xu et al., 2000) and Cdc15 activates the kinase complex

Dbf2/Mob1, the homologues of Sid2p/Mob1p (Komarnitsky et al., 1998; Luca and Winey, 1998; Mah et al., 2001).

The effector of mitotic exit and the key component of the MEN is the phosphatase Cdc14, the homolog of the fission yeast protein Clp1p. Upon activation of Cdc14 through the MEN, Cdc14 engages in its essential function, the downregulation of CDK activity to allow mitotic exit. Cdc14 activates the degradation of mitotic cyclins (Clb1-4) through dephosphorylation and activation of the APC/C specificity factor Cdh1/Hct1 (Jaspersen et al., 1999; Schwab et al., 1997; Visintin et al., 1998; Visintin et al., 1997; Zachariae et al., 1998). Dephosphorylated Cdh1/Hct1 facilitates ubiquitination of the cyclins by the APC/C in late anaphase. Further, Cdc14 stabilizes the CDK inhibitor Sic1 through dephosphorylation and promotes Sic1 expression by activating the transcription factor Swi5 (Jaspersen et al., 1999; Knapp et al., 1996; Moll et al., 1991; Toyn et al., 1997; Visintin et al., 1998). Dephosphorylation of other CDK substrates by Cdc14 reverses the effect of high mitotic CDK activity and thus accelerates CDK down regulation (Bembenek and Yu, 2001; Bembenek and Yu, 2003; Gray and Barford, 2003; Gray et al., 2003; Wang et al., 2004). Cdc14 also dephosphorylates the inhibitory CDK sites on Cdc15 (Jaspersen and Morgan, 2000). Thus, Cdc14 and MEN activate each other in a positive feedback loop, similar to Clp1p and the SIN.

Cdc14

The phosphatase Cdc14 is more intensely investigated than its homolog Clp1p. This allows us to learn from the discoveries made in budding yeast and to test whether *S. cerevisiae* Cdc14 functions are conserved.

Regulation of Cdc14

From G1 phase to early anaphase, Cdc14 is bound by its inhibitor Net1/Cfi1, and localizes to the nucleolus (Shou et al., 1999; Visintin et al., 1999). Cdc14 inhibition and nucleolar localization are first relieved in early anaphase through the Fourteen-Early-Anaphase-Release network (FEAR) and through the phosphorylation of Net1/Cfi1 by the mitotic CDK Cdk1/Cdc28. This allows Cdc14 to localize throughout the nucleus (Azzam et al., 2004; Stegmeier and Amon, 2004). Subsequently, Cdc14 is released form the nucleus to the cytoplasm in a MEN dependent manner (Stegmeier et al., 2002). The essential function of Cdc14 in mitotic exit requires the MEN, while non-essential functions of Cdc14 in early anaphase depend on the FEAR and Cdk1/Cdc28.

Regulation of Cdc14 through the FEAR network and Cdk1/Cdc28

The FEAR network is comprised of five activators and two inhibitors of Cdc14 release. The Separase Esp1, polo kinase Cdc5, Slk19 and Spo12 as well as its paralog, Bns1, are required for Cdc14 release in early anaphase (Stegmeier et al., 2002; Visintin et al., 2003). Securin (Pds1), the inhibitor of separase and the nucleolar replication fork block protein Fob1 function as inhibitors of FEAR-dependent Cdc14 release (Cohen-Fix and Koshland, 1999; Stegmeier et al., 2004; Sullivan and Uhlmann, 2003; Tinker-Kulberg and Morgan, 1999).

The Separase Esp1 is known for its function in promoting sister-chromatid separation. After degradation of the Esp1 inhibitor Securin (Pds1), Esp1 cleaves a subunit of Cohesin, the complex that keeps sister chromatids together. This cleavage

allows chromosome segregation (Nasmyth, 2002). Although Esp1 function in the FEAR is independent of its separase activity, activation of Esp1 through Securin Pds1 degradation is required for the FEAR network (Stegmeier et al., 2002; Sullivan and Uhlmann, 2003). Therefore, the FEAR regulatory mechanism ensures the timely release of Cdc14 not prior to chromosome segregation to prevent premature CDK down regulation. Spo12 and Bns1 are nuclear phosphoproteins and Spo12 binds the FEAR inhibitor Fob1, which is a component of the Cdc14 inhibiting RENT complex in the nucleolus, including Net1/Cfi1. Phosphorylation of Spo12/Bns1 might promote the disassembly of the RENT complex, releasing Cdc14 in anaphase (Stegmeier et al., 2004). *S. cerevisiae* Cdc5 is a member of the polo kinase family and over production of Cdc5 correlates with Cdc14 and Net1 phosphorylation in vivo (Visintin et al., 2003). Cdc5 is the only FEAR factor that can release Cdc14 out of interphase. Because Cdc5p is also an activator of the MEN, Cdc5 over production might generate a positive feedback loop of MEN activation and Cdc14 release and it is therefore not clear whether Cdc5 releases Cdc14 as part of the FEAR network of or the MEN.

A recent study shows that Cdk1/Cdc28 phosphorylates Net1 and relieves Cdc14 binding to Net1/Cfi1 (Azzam et al., 2004). The FEAR is required for full activity of this release mechanism, such that the FEAR might inhibit premature release of Cdc14 at the beginning of mitosis, when CDK activity is already high. How in detail the FEAR components interact with each other and function with Cdk1/Cdc28 to release Cdc14 from the nucleolar inhibitor is not understood.

Cdc14 regulation by the MEN

After Cdc14 release from the nucleolus through the FEAR network and Cdk1/Cdc28, the MEN is required to release Cdc14 into the cytoplasm and to keep Cdc14 from returning to the nucleolus. How in detail the MEN prevents binding of Cdc14 to Net1/Cfi1 is not understood. The model preceding the finding that the FEAR/Cdk1/Cdc28 releases Cdc14 by phosphorylation of Net1/Cfi1 proposed that polo kinase Cdc5 or Dbf2, the Sid2 homolog, were the kinases phosphorylating Net1/Cfi1 and responsible for Cdc14 release By this model the MEN might regulate Cdc14 cytoplasmic maintenance through loss of affinity to the nucleolar Net1/Cfi1. Alternatively, Cdc14 might be kept in the cytoplasm through nuclear shuttling. In support of this model, mutations in two importins, Mtr10 and Kap104, have been identified as suppressors for MEN mutants (Asakawa and Toh, 2002; Shou and Deshaies, 2002). The MEN might inhibit these importins from localizing Cdc14 to the nucleus, where it would bind to Nef1/Cfi1.

FEAR dependent functions of Cdc14

With the initiation of chromosome segregation and preparation for mitotic exit, the cell faces changes on multiple levels, such as the coordination of microtubules and chromatin dynamics. As many of these changes are connected to decrease in Cdk1/Cdc28 activity, it is not surprising to find some of them dependent on Cdc14 and its release through the FEAR/Cdk1.

Function of Cdc14 in microtubules dynamic

At the transition from metaphase to anaphase, spindle microtubules dynamics change dramatically from kinetochore attaching microtubules to an elongating spindle, segregating the sister chromatids. Recently, an elegant study by Higuchi and Uhlmann (2005) proved that Cdc14 is required for the regulation of the changes in microtubules dynamics on the spindle in anaphase, using Fluorescence-Recovery-After-Photobleaching (FRAP) on spindle microtubules. During metaphase microtubules are highly dynamic, while anaphase spindles show a reduced degree of FRAP and are therefore less dynamic. Lack of Cdc14 maintained the high metaphase microtubules dynamics throughout anaphase, destabilizing the anaphase spindle. This study further implicated several microtubule regulatory proteins to be regulated by Cdc14 (Higuchi and Uhlmann, 2005). Pereira and Schiebel (2003) showed localization of Cdc14 to the kinetochores and the spindle mid zone and that the localization of chromosomal passenger proteins to the spindle mid zone is dependent on FEAR and Cdc14. The chromosomal passenger proteins have been implicated in the regulation of spindle stability (Buvelot et al., 2003) and mutations in *cdc14* caused reduced anaphase spindle stability which was rescued by the expression of a non-phosphorylatable form of $Sli15^{6A}$ (Pereira and Schiebel, 2003). Therefore Cdc14 may regulate anaphase spindle dynamics through the chromosomal passenger proteins.

The anaphase spindle is not the only case where Cdc14 regulates microtubules. In budding yeast, the astral microtubules first generate asymmetric forces at the centrosomes to position the nucleus in the bud neck. Then symmetric forces help spindle elongation and chromosome segregation by pulling the spindle poles to opposite ends. Mutants in *esp1* can not separate or segregate sister chromatids, but move the whole nucleus to the

bud (Bardin et al., 2000). Further, the change in astral microtubules dynamics is dependent on the FEAR in general and expression of a hyperactive allele of *cdc14*, *cdc14-TAB6*, rescues the defect in *esp1* mutants on nuclear positioning. It is therefore thought that Cdc14 regulates astral microtubules dynamics (Ross and Cohen-Fix, 2004; Shou et al., 1999).

Further, Cdc14 regulates meiotic spindle dynamics (Buonomo et al., 2003; Marston et al., 2003). In meiosis, failure in destabilization of the anaphase I spindle results in lack of equatorial sister chromatid segregation during meiosis II and for formation of 2 dyads instead of four spores. Similar to the events in mitosis, Cdc14 is periodically released in early anaphase I and anaphase II in a FEAR dependent manner. Release of Cdc14 in anaphase I is required for the destabilization of the meiotic spindle (Buonomo et al., 2003; Marston et al., 2003). In absence of this destabilization, the meiosis I spindle persists and the second meiotic division fails. Despite the similarity in release of Cdc14 in mitosis and meiosis, Cdc14 has opposite effects on the spindle in the meiotic versus the mitotic division. The reason for this curious difference has yet to be uncovered.

Function of Cdc14 in rDNA segregation

Another FEAR dependent function of Cdc14 is the regulation of rDNA segregation. In the absence of Cdc14, rDNA segregation is delayed due to defects in its condensation (D'Amours et al., 2004; Sullivan et al., 2004). How in detail the FEAR and Cdc14 regulat rDNA segregation is not known. One model suggests that the degradation of condensin through sumoylation is regulated by Cdc14 (D'Amours et al., 2004).

Figure 1-5 mitotic exit network

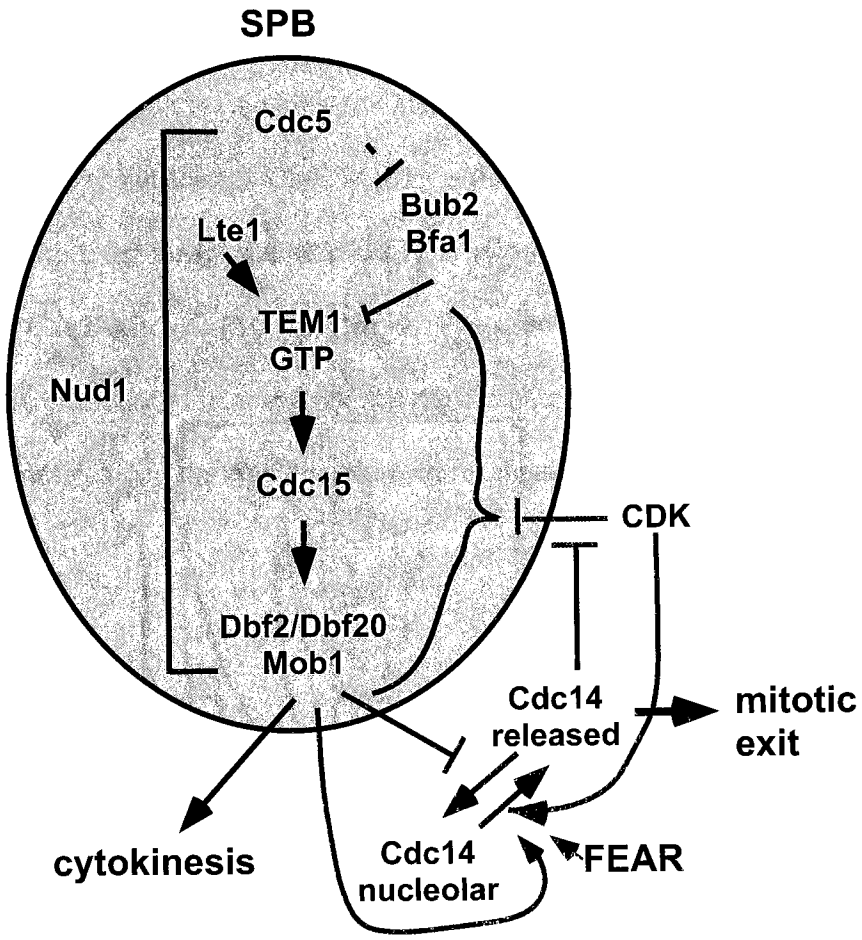


Figure 1-5: *S. cerevisiae* mitotic exit network

The MEN is anchored at the SPB (pink) through the scaffold protein Nud1. The polo kinase Plo1p inhibits the GAP complex Bub2/Bfa1. This together with the GEF Lte1p promotes the change of the GTPase TEM1 from its GDP bound form to the GTP bound, active form. TEM1-GTP localizes the kinases Cdc15 followed by the activation of the kinase complex Dbf2/Dbf20/Mob1p. Dbf2/Mob1p is presumed to maintain release of the phosphatase Cdc14 from the nucleus, which has been released in early anaphase from the nucleolus by Cdk1/Cdc28 and the FEAR network. Released Cdc14 then opposes Cdk1 and facilitates exit from mitosis. Aside from mitotic exit, the MEN appears to be required for cytokinesis, albeit through an unknown mechanism.

Comparison between Clp1p and Cdc14

Cdc14 and Clp1p share 36% identity and are therefore highly conserved. In how far their regulation and functions are conserved will be discussed in this section (Table 1-1).

Clp1p localization to the nucleolus during interphase is similar to *S. cerevisiae* Cdc14 (Cueille et al., 2001; Shou et al., 1999; Trautmann et al., 2001; Visintin et al., 1999). Like the MEN in *S. cerevisiae*, SIN activity is required to maintain Clp1p in the cytoplasm at the end of mitosis (Bardin et al., 2000; Cueille et al., 2001; Trautmann et al., 2001). The cytoplasmic retention of Clp1p does not appear to be regulated through nucleolar affinity, as proposed for Cdc14, but through a Rad24p dependent nuclear import and export mechanism as suggested in Chapter IV (Mishra et al., ; Trautmann and McCollum, 2005). The mechanism for Cdc14 cytoplasmic retention by MEN is not well understood, but nuclear import mechanisms have also been suggested and thus Cdc14-family phosphatase regulation by the SIN/MEN through nuclear import or export may be conserved.

Regulation of the signaling networks SIN and MEN by Cdc14 family phosphatases may also be conserved. Lack of Clp1p reduces the localization of Sid1p. Clp1p may promote Sid1p localization and therefore SIN activity by dephosphorylating of Sid1p or several SIN components (Cueille et al., 2001; Trautmann et al., 2001). Similarly, Cdc14 promotes MEN activity through the dephosphorylation of Cdc15 (Jaspersen and Morgan, 2000).

Release of Clp1p from the nucleolus seems to be regulated differently from Cdc14. While Cdc14 is released from the nucleolus in early anaphase, Clp1p is released

upon G2/M transition. The FEAR network and Cdk1/Cdc28 are required for Cdc14 early anaphase release. Although the FEAR components are conserved, the Securin homolog Cut2p and the Separase Cut1p are not active at G2/M transition, when Clp1p is released, nor is the separase Cut1p required for Clp1p release (Trautmann, Chen and McCollum, unpublished results).

The requirement for Cdk1/Cdc28 might however be conserved. Mitotic CDK activity rises at G2/M transition and, if no FEAR network is required for Clp1p release, this Cdk1/Cdc2 activity at mitotic entry might allow Clp1p nucleolar release. Clp1p nucleolar localization strongly suggests that it has a nucleolar binding partner, similar to Net1/Cfi1 in *S. cerevisiae*. Knowledge of such a binding partner would greatly contribute to our understanding of Clp1p regulation. To date, our biochemical and genetic approaches to identify such a binding partner have proven unsuccessful.

The answer to why Clp1p is released at G2/M and not early anaphase might be in the difference between the budding and fission yeast cell cycles. The G2/M transition is not as defined in *S. cerevisiae* as in *S. pombe*. For example, DNA damage results in the prevention of anaphase, not in prevention of mitotic entry (Cohen-Fix et al., 1996). Thus, a Cdc14-release in early anaphase in *S. cerevisiae* might translate into a G2/M release of Clp1p in *S. pombe*.

As shown in Chapter III, Clp1p localization to the kinetochores in early mitosis and the spindle midzone in anaphase is similar to Cdc14 localization (Pereira and Schiebel, 2003; Trautmann et al., 2004). Further, both Clp1p and Cdc14 exist in a complex with chromosomal passenger proteins. However, the homologs seem to function differently at first glance: Cdc14 is required for the localization of the

chromosomal passengers to the spindle midzone and thereby stabilizes the anaphase spindle while deletion of *clp1* has no impact on chromosomal passenger localization to the spindle mid zone (Pereira and Schiebel, 2003; Trautmann et al., 2004). However, Clp1p localization of the chromosomal passenger proteins aurora kinase Ark1p and survivin Bir1p/Cut17p to the kinetochores is reduced in *clp1* deletion mutants (Trautmann et al., 2004). One possible explanation for the difference between fission and budding yeast could be that Cdc14-family phosphatases may required to maintain a stable chromosomal passenger complex when CDK activity is high. In *S. cerevisiae,* complete CDK downregulation to allow mitotic exit requires the MEN and Cdc14 (Irniger et al., 1995). The CDK activity in anaphase is therefore still relatively high even in the presence of initial Cyclin degradation. In fission yeast mitotic Cyclin is efficiently degraded with the initiation of anaphase (Alfa et al., 1989) and overall CDK activity in anaphase might be lower in fission yeast compared to budding yeast. In *S. pombe,* Cdk1p activity might be low enough in anaphase to ensure a chromosomal passenger protein complex stability, but dephosphorylation through Clp1p would be required in metaphase. Whether chromosomal passenger localization to the kinetochores is affected in *cdc14* mutants in *S. cerevisiae* has not been addressed.

Cdc14 has additional roles in astral microtubules regulation and meiotic spindle disassembly. Whether Clp1p regulates astral microtubules dynamics remains to be addressed. A meiotic role for Clp1p is unlikely since deletion of *S. pombe clp1* shows no obvious defects in meiosis (Trautmann and McCollum, unpublished observations). Cdc14 has been reported to localize to the SPB during anaphase and bind Bfa1. Correlative evidence suggests that the dephosphorylation of Bfa1 by Cdc14 activates

Bfa1/Bub2, thereby inhibiting MEN when mitotic exit is completed (Pereira et al., 2002). Whether Clp1p inhibits the SIN has not been addressed. Clp1p is absent from the SPB during mitosis until after septation (Trautmann and McCollum, 2005) and Clp1p might therefore inhibit the SIN during interphase.

The essential function of cdc14 is mitotic exit regulation. Clp1p is not essential, but opposes CDK activity as well, albeit through somewhat different mechanisms (Cueille et al., 2001; Esteban et al., 2004; Trautmann et al., 2001; Wolfe and Gould, 2004). Clp1p dephosphorylates and destabilizes the Cdk1 activator Cdc25p (Esteban et al., 2004; Wolfe and Gould, 2004) and Cdc25p itself activates Cdk1 by dephosphorylation on Tyrosine15 (Nilsson and Hoffmann, 2000). Destabilization of Cdc25p through Clp1p therefore inhibits Cdk1 by promoting its inhibitory phosphorylation. Interestingly, this regulation of Cdc25p does not only regulate mitotic entry, but also exit from mitosis (Esteban et al., 2004; Wolfe and Gould, 2004). The CDK inhibition through Clp1p is however not essential for *S. pombe*. Conversely, Cdc14 regulates cytokinesis indirectly through the MEN as some MEN mutants exhibit cytokinesis defects.

Table 1-1: Comparison of *S. pombe* Clp1p and *S. cerevisiae* Cdc14 Localization and Function

Localization	Clp1p		1. Cdc14	
	localization	function	localization	function
nucleolus	interphase	unknown	G1 to anaphase	rDNA condensation and segregation
SPB	interphase	unknown	late mitosis	?
kinetochores	early mitosis	sister chromatid bi-orientation	ana-/ telophase	chromosomal passenger regulation
spindle midzone	anaphase	Unknown	anaphase	spindle stabilization
actomyosin ring	mitosis	ring stabilization	?	?
cytoplasm	mitosis cytokinesis checkpoint	Cytokinesis checkpoint	late anaphase to G1	mitotic exit
meiosis	?	?	nucleolus/ spindle	anaphase I spindle destabilization

Homologs of SIN/MEN and Cdc14 in higher eukaryotes

Models for SIN/MEN and Cdc14 function are primarily derived from yeast. Whether these finding apply to higher eukaryotes is an exciting question. A number of SIN and MEN components and Clp1p/Cdc14 have homologs in higher eukaryotes (Table 1-2). A GTPase-activating protein (GAP) GAPCenA, which localizes to the centrosomes and carries sequence similarity to Cdc16p in *S. pombe* and Bub2 in *S. cerevisiae* was identified in humans (Cuif et al., 1999). A substrate for GAPCenA is the GTPase Rab6. Interestingly, the yeast GTPase Spg1p/Tem1 shows sequence similarity to the RAB family protein Rab6. This suggests that GAPCenA might function in similarly to Cdc16p/Bub2. Whether GAPCenA functions in cytokinesis or mitotic exit and whether Rab6 is a functional homolog of Spg1p/Tem1 remains unknown.

Serine/Threonine kinases of the Sid2/Dbf2 family of proteins have been reported in higher eukaryotes. In flies, a Sid2p/Dbf2 homolog was identified as a tumor suppressor and named LATS for "large tumor suppressor" (Justice et al., 1995; Xu et al., 1995). Mutations in *lats* cause tumors in flies. Two human and mouse homologs, *LATS1* and *LATS2*, were found (St John et al., 1999; Tao et al., 1999; Yabuta et al., 2000). Mice deficient for *Lats1* develop tumors and human tumor cells were found to carry point mutations in LATS1 (Hisaoka et al., 2002). The human Lats1 has been reported to localize to the centrosomes in interphase, the spindle and midbody during anaphase and telophase and to the cytokinetic furrow (Hirota et al., 2000; Yang et al., 2004). Human Lats1 has been described to regulate cytokinesis through the LIM-kinase LIMK1 (Yang et al., 2004). Therefore, the function of the SIN/MEN in cytokinesis might be conserved in higher eukaryotes.

In addition to Dbf2/Sid2p itself, the regulatory subunits of the MOB-protein-family have homologs in higher eukaryotes. Mob1 proteins bind to Sid2p/Dbf2p family kinases and enhance the activity of the kinase in humans and *D. melanogaster* (Bichsel et al., 2004; Devroe et al., 2004; Lai et al., 2005). Mutations or down regulation of the MOB homologs further shows phenotypes consistent with the reported kinase functions (Lai et al., 2005).

The kinases Cdc7p, Sid1p and Cdc15 in fission and budding yeast are members of the Ste20 family of kinases. Cdc15 activates Dbf2p by phosphorylation(Mah et al., 2001). Proteins of the Ste-20 family of protein kinases, Mst2 in humans and Hippo in *D. melanogaster*, have been shown to phosphorylate LATS1 proteins (Chan et al., 2005; Harvey et al., 2003; Jia et al., 2003; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003b). Mst2 was further shown to activate hLATS (Chan et al., 2005). Activation of Sid2p by Sid1p has also been suggested in *S. pombe* (Hou et al., 2004). The human centrosomal protein centriolin shows sequence homology to the SIN/ MEN scaffold protein Cdc11p and Nud1p in fission and budding yeast respectively and was reported to regulate cytokinesis and cell cycle progression. Its ability to bind budding yeast Bub2p makes it a candidate for the Cdc11/Nud1 homolog (Gromley et al., 2003; Grueneberg et al., 2000). These experimental data suggest at least a structural, if not a functional conservation of SIN/MEN is higher eukaryotes.

Table 1-2. *S. pombe* SIN and *S. cerevisiae* MEN components

2. SIN	Protein function	MEN	Higher eukaryotes
Plo1p	Protein kinase	Cdc5	Polo like kinase
Byr4p	GAP component	Bfa1	?
Cdc16p	GAP component	Bub2	GAPCenA?
Spg1p	GTPase	Tem1	?
Sid4p	Scaffold protein	?	?
Cdc11p	Scaffold protein	Nud1	Centriolin
Cdc7p	Protein kinase	Cdc15	?
Sid1p	Protein kinase	?	?
Cdc14p	Sid1 binding protein	?	?
Sid2p	Protein kinase	Dbf2/Dbf20	Warts/Lats
Mob1p	Kinase regulator	Mob1	Mob1
Clp1p	Protein Phosphatase	Cdc14	Cdc14A/B

Homologs of Cdc14 have also been identified in *X. laevis*, *C. elegans* and humans. Recent studies on the *Xenopus laevis* homologs of CDC14, Xcdc14α and Xcdc14ß reveal localization to the nucleolus and the centrosome respectively. A functional study used the injection of antibodies which detect both isoforms and resulted in cell division defects (Kaiser et al., 2004).

The genome of the nematode *C. elegans* encodes one CDC14 homolog, CeCDC14. The characterization of a putative null allele of *cdc-14*, confirmed by RNAi, showed that CeCDc14 is not essential, but is required for cell cycle arrest in G1 in specific cell lineages of later developmental stages (Saito et al., 2004). The affected cell lineages underwent extra divisions in the absence of CeCDC14. Based on genetic experiments CeCDC14 functions in G1 arrest through the CDK inhibitor CKI-1. Dephosphorylation might stabilize CKI-1 until sufficient CDK inhibitor is present to arrest the cell in G1 phase. This bears much resemblance to the regulation of Sic1 by Cdc14 in budding yeast. Expression of *cecdc14-GFP* revealed localization to the centrosome in early mitosis and the spindle and astral microtubules in anaphase. Differentiating cells localize CeCDC14 to the nucleus and nucleolus, possibly to sequester it (Saito et al., 2004). Another study found that CeCDC14 RNAi results in embryonic lethality in kinesin ZEN4-GFP transgenic worms (Grueneberg et al., 2002). Here, RNAi resulted in spindle assembly, midzone formation and cytokinesis defects. Zen4-GFP genetic background might be sensitized for CeCdc14 RNAi defects. The effect of CeCDC14 RNAi might suggest that CeCDC14 has a non-essential function in mitotic spindle assembly, possibly similar to *S. cerevisiae*.

In humans, two isoforms of Cdc14 phosphatase, hCdc14A and hCdc14B have been reported. As in the two yeasts, at least hCDC14A preferably dephosphorylates CDK phosphorylation sites (Gray and Barford, 2003; Gray et al., 2003; Kaiser et al., 2002). While hCdc14B localizes to the nucleolus, hCdc14A is localized to the centrosomes during interphase and to the spindle midzone and the centrosomes during mitosis, suggesting that the functions of Cdc14 family phosphatases were divided in human cells (Gruneberg et al., 2004; Kaiser et al., 2002; Mailand et al., 2002). Localization of hCdc14A to the centrosome is dependent on its endogenous nuclear export signal (NES) and deletion of this NES results in nucleolar localization of hCdc14A (Kaiser et al., 2002; Mailand et al., 2002). This suggests that localization and function of hCdc14A might be regulated through nuclear shuttling, similar to Clp1p and possibly Cdc14 (Mishra et al., 2005; Trautmann and McCollum, 2005). Localization of hCdc14A to the spindle suggested a function in chromosomal passenger localization. Spindle localization of both Aurora B and hCdc14A requires the kinesin MKlp2, and hCdc14A can dephosphorylate INCENP in vitro (Gruneberg et al., 2004). Whether hCDC14A regulates chromosomal passenger proteins in human tissue culture cells has not been reported.

siRNA and over expression studies revealed functions for hCDC14A in centrosome splitting or separation, microtubule nucleation or stability and spindle formation, which might be the cause of observed cytokinesis defects (Kaiser et al., 2002; Mailand et al., 2002). How hCDC14A regulates these processes is unknown and will require the identification of substrates. Biochemical experiments showed that hCDC14A can dephosphorylate hCdh1 and increase its activity in vitro. Evidence for an impact of

hCDC14A on CDH1 regulation in vivo is however missing (Bembenek and Yu, 2001). Another biochemical study showed that hCDC14A and B bind p53 and dephosphorylate its S315 phosphorylation site (Li et al., 2000). The physiological function of this interaction is unaddressed.

To what degree regulation of Cdc14 –family phosphatases is conserved throughout eukaryotes is not known, although proteins of FEAR and MEN/SIN pathways are conserved in higher eukaryotes. Human hCDC14B and Xenopus XCDC14α localize to the nucleolus in interphase and are released around entry into mitosis after prophase, similar to *S. pombe*. This release might be dependent on CDK activity. In contrast to yeast, which undergo closed mitosis, higher eukaryotes break down their nuclear envelope and reorganize their nucleus during mitosis. Therefore Cdc14 release from the nucleolus in higher eukaryotes might just be the consequence of their physiology.

Coordination between cytokinesis and nuclear cycle in higher eukaryotes

A considerable amount of evidence suggest that regulatory mechanisms for the coordination of nuclear cycle and cytokinesis also exist in higher eukaryotes.

The inhibition of cytokinesis through CDK activity might be conserved in higher eukaryotes, since overexpression of non-degradable mitotic Cyclins prevents cytokinesis (Surana et al., 1993; Wheatley et al., 1997). The molecular mechanism of this regulation remains obscure. Interestingly, human Lats1 is phoshoporylated by CDK, although the functional relevance of this phosphorylation is not known (Morisaki et al., 2002). This is consistent with the inhibition of SIN/MEN pathways by Cdk1 observed in yeast.

A delay in nuclear cycle progression in response to cytokinesis defects, similar to the *S. pombe* cytokinesis checkpoint, appears to exist in higher eukaryotes. In mammalian cells, cell cleavage failure through treatment with 10μM of the actin depolymerizing drug cytochalasin results in a G1 block, much like the cytokinesis checkpoint blocks the nuclear cycle in *S. pombe* (Andreassen et al., 2001; Martineau et al., 1995). This phenomenon was called the tetraploidy checkpoint.

During this G1 arrest, cells maintain their ability to undergo cytokinesis as judged by the preservation of the midbody and competence to undergo cytokinesis upon removal of cytochalasin (Martineau et al., 1995). This maintenance of "C"-phase (cytokinesis phase) was also observed after treatment of cells with the myosin inhibitor blebbistatin or the microtubules destabilizing drug nocadozole (Canman et al., 2000; Straight et al., 2003). The prolonged C-phase is reminiscent of the maintenance of the SIN in *S. pombe* upon perturbation of the actin ring and reflects conservation in the mechanisms utilized by eukaryotic cells to survive cytokinesis insults. However, it is not know what the tetraploidy checkpoint monitors. Hypotheses range from DNA content, centrosome number and general actin structures to cleavage failure (Andreassen et al., 2001; Meraldi et al., 2002).

Recent data argue against the existence of a tetraploidy preventing checkpoint in response to cleavage failure. When tissue culture cells were treated with lower concentrations of cytochalasin D (0.5 μM) and grown on a fibronectin surface, no G1 arrest was observed, even though treatment with lower doses of cytochalasin D did block cytokinesis (Uetake and Sluder, 2004). In these experiments, the overall actin cytoskeleton might be less perturbed in comparison to the previous experiment. If the

cell senses the state of its actin cytoskeleton, the treatment with lower doses might maintain enough actin cytoskeleton for the cell to reenter the cell cycle.

In *S. pombe*, actin structures play an important role in the induction and maintenance of the cytokinesis checkpoint as well. Actomyosin ring disruption activates the cytokinesis checkpoint and delays cells in interphase. During an active cytokinesis checkpoint, actin is not concentrated at the cell tips, as it usually is in interphase, when the cell grows at its ends. Instead, actin localized all over the cells cortex with concentrations at the medial cortex, depending on the degree of actomyosin ring disruption. When the cytokinesis checkpoint fails and cells become tetranucleate, actin repolarizes to the tips of the cell (Mishra et al., 2004). Whether this is cause or consequence of checkpoint failure is unclear, but it indicates a relationship between overall actin cytoskeleton and the cell cycle arrest.

But is the nuclear cycle block induced by severe actin depolymerization regulated through SIN/Clp1p homologs? The actin depolymerization induced delay was reported to be dependent on p53 (Andreassen et al., 2001; Hirano and Kurimura, 1974) and hCdc14A was reported to bind and dephosphorylate p53 at S315 (Li et al., 2000). p53 phosphorylation at S315 promotes centrosome duplication, while dephosphorylation inhibits it (Tarapore et al., 2001). The lack of hCdc14A might lead to persistent phosphorylation of p53 and lead to overamplification of the centrosome. This might delay the nuclear cycle if the nuclear cycle block checkpoint monitors the number of centrosomes. Thus, hCDC14A might regulate the nuclear cycle arrest following actin disassembly through p53.

Studies on the Dbf2/Sid2p/Lats family of kinase showed that they can inhibit CDK activity. *D. melanogaster* Lats1 was shown to inhibit Cdc2/Cyclin A and mouse Lats2 inhibits Cyclin E/CDK2 kinase activity (Li et al., 2003; Tao et al., 1999). CyclinE/CDK2 are essential for cell cycle entry at G1/S phase and are required for centrosome duplication, such that Lats might regulate the nuclear cycle arrest of binucleate cells through CyclinE/Cdc2 (Hinchcliffe et al., 1999; Lacey et al., 1999). Further, mammalian Lats1 is required for the G1 arrest of binucleate cells in a p53 dependent manner (Iida et al., 2004), but also regulates actin cytoskeleton through the actin binding protein Zyxin (Hirota et al., 2000). Lats might therefore function in multiple ways to coordinate nuclear cycle with cytokinesis.

Given these results, coordination of cytokinese and nuclear division and the underlying molecular mechanisms might be conserved. The discovery of kinase and phosphatase substrates will surely answer some of the open questions (Figure 6).

Figure 1-6: A cytokinesis checkpoint in higher eukaryotes?

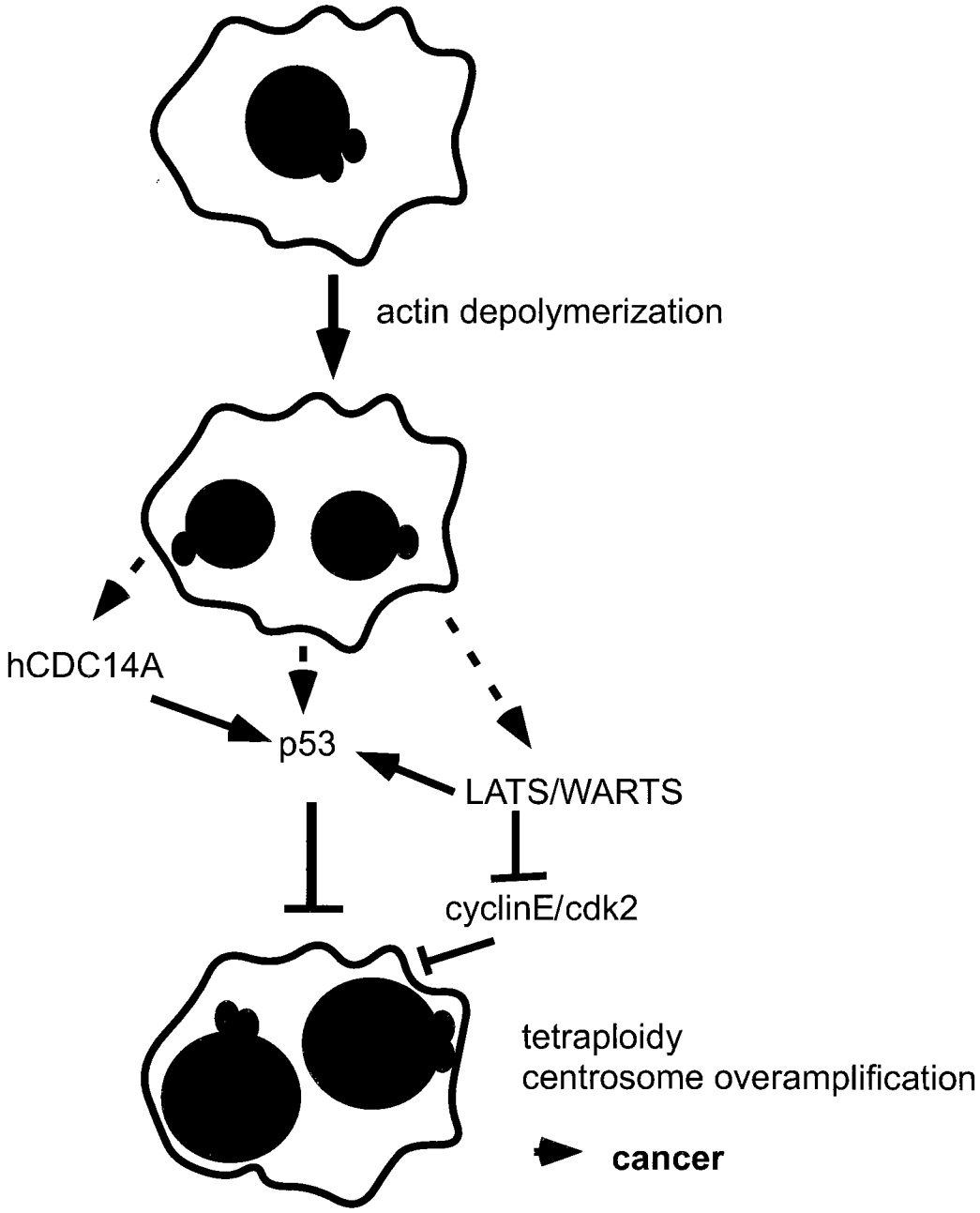


Figure 6: A cytokinesis checkpoint in higher eukaryotes

Treatment of mammalian tissue culture cells with actin depolymerizing drugs can arrest cells in G1, preventing tetraploidy and centrosome overamplifivation, both of which can lead to cancer. The Sid2p/Dbf2/Dbf20 homolog LATS/WARTS and p53 have been found to be required for such an arrest. Since hCDC14A was shown to bind and dephosphorylate p53, the actin perturbation might activate hCDC14A to cause activation of p53. LATS/WARTS have also been shown to affect p53 activity and might inhibit cell cycle progression through p53. Further, LATS/WARTS can inhibit CDK2/CyclinE activity with promotes centrosome duplication. Inhibition of CDK2/CyclinE would prevent centrosome overamplification when cytokinesis is perturbed.

CHAPTER II

The Cdc14-like phosphatase Clp1p in fission yeast regulates G2/M transition and coordination of cytokinesis with cell cycle progression

Figures 2-1 a-d were contributed by Dr. Paul Jorgensen and Dr. Mike Tyers

Figures 2-2, 2-3 d and 2-5d and e were contributed by Ben Wolfe and Kathleen Gould

Figure 2-3 b and c, Figure 5a and b, Figure 2-6 and Figure 2-7 were contributed by Dr Dannel McCollum

The majority of these results was published in:

Trautmann, S., Wolfe, B. A., Jorgensen, P., Tyers, M., Gould, K. L., and McCollum, D. (2001). Fission yeast Clp1p phosphatase regulates G2/M transition and coordination of cytokinesis with cell cycle progression. Curr Biol *11*, 931-940.

Summary

At the time these studies were initiated, *S. cerevisiae* mitotic exit network (MEN) was described to be essential for mitosis exit and to function in anaphase by promoting release of the Cdc14 phosphatase from the nucleolus. The release of Cdc14 was shown to cause mitotic exit via inactivation of the cyclin dependent kinase (Cdk) (Shou et al., 1999; Visintin et al., 1999). Cdc14p like proteins are conserved from yeast to humans, however it was unclear if these proteins regulate mitotic exit as in *S. cerevisiae*. In the fission yeast *Schizosaccharomyces pombe* a signaling pathway homologous to the MEN, termed the septation initiation network (SIN), is required not for mitotic exit, but for initiation of cytokinesis (Gould and Simanis, 1997). The high degree in conservation between the SIN and the MEN suggested that a Cdc14 homolog might function downstream of the SIN in *S. pombe*. We examined the function and regulation the Cdc14-like phosphatase Clp1p in fission yeast and showed that Clp1p is not required for mitotic exit. Instead, Clp1p promotes activation of the SIN by keeping Cdk activity low until cytokinesis is complete. This ensures that cytokinesis is completed prior to initiation of the next cell cycle and might maintain low CDK activity to prevent mitotic entry prior to cytokinesis. Like Cdc14, Clp1p localizes to the nucleolus in interphase, but Clp1p is released in G2/M transition and Clp1p exit from the nucleolus does not depend on the SIN. This is different from *S. cerevisiae*, where the MEN was reported to be required for the release of Cdc14 from the nucleolus. Instead, the SIN is required to keep Clp1p out of the nucleolus until cytokinesis is complete. Subsequently, research in *S. cerevisiae* identified a MEN independent mechanism for Cdc14 release through Cdc28 and a network named

the FEAR for Fourteen-Early-Anaphase-Release (Stegmeier et al., 2002). This suggests that the regulation of Clp1p cytoplasmic maintenance may be similar to Cdc14. In addition, we found that Clp1p regulates the G2/M transition since cells deleted for *clp1* enter mitosis precociously and cells over expressing Clp1p delay mitotic entry. Furthermore, unlike Cdc14p, which down regulates CDK activity by Cyclin degradation and activation of CDK inhibitors, Clp1p appears to antagonize Cdk activity by promoting tyrosine phosphorylation of Cdc2p.

Introduction Chapter II

Cytokinesis is the final crucial event of the cell cycle. In fission yeast, the timing of cytokinesis is regulated through the SIN. SIN loss of function mutants continue nuclear division cycles without cell division. The SIN is intimately involved in coordinating cytokinesis with nuclear cycle events. Inactivation of the fission yeast CDK, Cdc2p, in late mitosis is essential for initiation of cytokinesis (He et al., 1997; Yamano et al., 1996). This effect appears to be mediated, at least in part, through the Sid1p kinase, whose localization to the SPB and activating function in the SIN are inhibited by high CDK activity (Guertin et al., 2000). Since Cdc2p inactivation occurs coincidently with chromosome segregation (Yamano et al., 1996), having the initiation of cytokinesis coupled to Cdc2p inactivation ensures that cell division does not initiate before chromosomes have been segregated. However, this mechanism renders cytokinesis sensitive to Cdc2p activity, which begins to rise as the next cell cycle initiates around the time of septation (Martin-Castellanos et al., 1996). If cytokinesis is delayed, the rising CDK activity could inhibit the SIN and cytokinesis unless the cell has a way to inhibit CDK activity until cytokinesis is complete. In addition, rising CDK activity in the absence of cell division could induce entry into mitosis, leading to polyploidy. Delaying mitosis increases the chance to complete cytokinesis before irreversible mistakes. Evidence for a cytokinesis checkpoint ensuring that cytokinesis is complete before the initiation of the next nuclear division cycle was revealed by the characterization of the *cps1-191* cytokinesis mutant (Le Goff et al., 1999b; Liu et al., 2000; Liu et al., 1999). The *cps1-191* mutant arrests in cytokinesis with an actomyosin ring and two interphase nuclei. The block in nuclear division is relieved by the inactivation of either the CDK

inhibitory kinase Wee1p or the SIN (Le Goff et al., 1999b; Liu et al., 2000; Liu et al., 1999). The mechanism of action of the SIN or Wee1p, however, remained unclear at the time.

Since *S. cerevisiae* Cdc14 functions downstream of the SIN homologous signaling network MEN and it is capable of inhibiting Cdk activity in *S. cerevisiae*, we sought to investigate the function of a Cdc14-like phosphatase in *S. pombe*. When the sequence of a protein with 36% sequence identity to Cdc14 was released by the *S. pombe* sequencing consortium, we named this ortholog Clp1p for Cdc14-Like-Phosphatase.

Here we show that Clp1p is not required for mitotic exit. Instead, Clp1p functions together with Wee1p and the SIN to coordinate completion of cytokinesis with initiation of the next cell cycle. In addition Clp1p plays a key role in regulating the timing of initiation of mitosis. Furthermore, unlike Cdc14p, Clp1p appears to antagonize Cdk activity by promoting tyrosine phosphorylation of Cdc2p in a Wee1p dependent manner.

Results Chapter II

Clp1p regulates G2/M transition

After finding the homolog of the *S. cerevisiae* Cdc14 in the Sanger Centre *S. pombe* genome data base, we named the homolog Cdc14-like-posphatase *clp1* (accession # CAB76271). Simultaneously, Viestrus Simanis' group characterized the same gene and called it *flp1* for fourteen-like-phosphatase (Cueille et al., 2001). In this thesis, *clp1/flp1* will be referred to as *clp1*. Collabloratorive efforts with Paul Jorgensen in Dr Mike Tyers' laboratory showed that Clp1p can rescue the *cdc14* allele *cdc14-3* (Figure 2-1).

Deletion of the coding region through replacement with URA4+ gene revealed that Clp1p is, in contrast to Cdc14, not essential. Deletion of *clp1* did not result in either a long and multinucleate SIN loss of function phenotype, as might be suggested if a Cdc14 family phosphatase homolog functions as the effector of the SIN as Cdc14 does with the MEN, nor did *clp1*Δ mutants portray obvious mitotic exit defects. Instead *clp1*Δ cells divided at a smaller size than wild type. In fission yeast, cells grow mostly during G2 interphase and cells blocked in G2 phase before mitosis grow longer, while cells entering mitosis precociously enter mitosis at a smaller size (hence "wee"), as for example mutants in the CDK inhibitor *wee1*. Since the cells do not grow during mitosis, they divide at mitotic entry size. While wild type cells divide at 14 μm, *clp1*Δ cells, divided at 9μm, indicating that *clp1*Δ cells enter mitosis early (Figure 2-1).

In addition, *clp1*Δ mutants displayed defects in cytokinesis. In asynchronous cultures 3% of *clp1*Δ cells displayed two or more closely opposed nuclei which is indicative of failed cytokinesis (Figure 2-7e). This phenotype is similar to that of weak

SIN pathway mutants. Additionally, the *clp1*Δ mutation was synthetically lethal with the SIN mutant *cdc7-24* and had negative interactions with other SIN components (not shown).

Figure 2-1

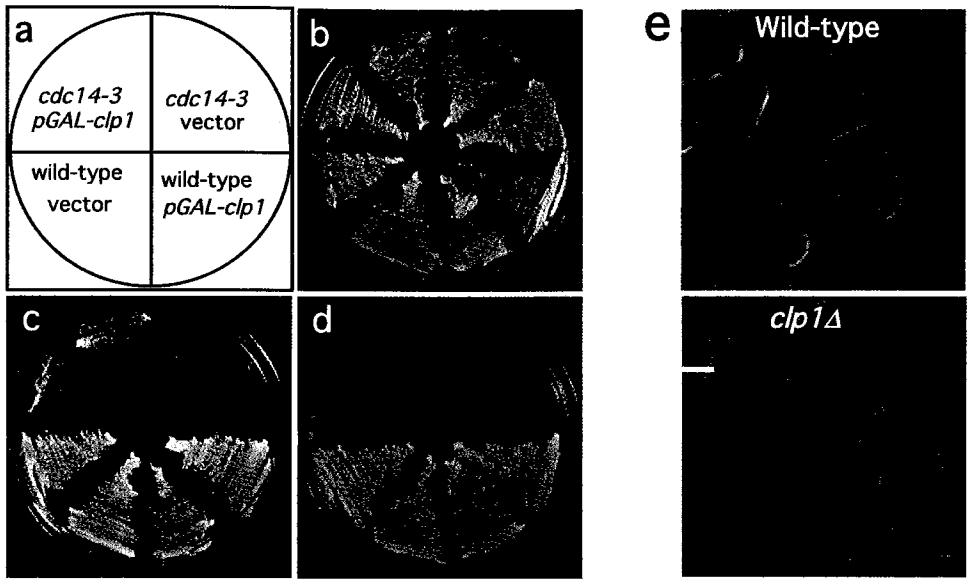

a
cdc14-3
pGAL-clp1
cdc14-3
vector
wild-type
vector
wild-type
pGAL-clp1
b
c
d
e
Wild-type
clp1Δ

Figure 2-1: a-d, Clp1p rescues the growth defect of *S. cerevisiae cdc14-3*. *clp1*$^{+}$ was expressed under control of a gal inducible promoter in *S. cerevisiae cdc14-3* cells. b, Cells grown at 25°C on 2% RAFF, 0.1% GAL. c, Cells grown at 37°C on 2%RAFF, 0.1%GAL expressed *clp1*$^{+}$ at low level, which rescued *cdc14-3* lethality. d, Cells grown at 37°C on 2% Glucose did not express *clp1*$^{+}$ and the *cdc14-3* mutation was not rescued. e, DIC images of wild-type or *clp1*Δ cells grown at 36°C. Scale bar = 5μm.

It is thought that *S. cerevisiae* Cdc14 antagonizes Cdk activity by reversing Cdk dependent phosphorylation events (Visintin et al., 1998). Since Cdk activation is essential for mitotic entry, the premature mitosis in *clp1*Δ mutants supports a role for Clp1p in antagonizing Cdk activity. Incollaboration with Ben Wolfe from Kathleen Gould's lab, we found that overexpression of *clp1*$^+$ from the inducible *nmt1* promoter was lethal and caused cells to delay in the G2 phase and become elongated (Figure 2-2 a, b). To examine this phenotype in more detail, Clp1p expression was induced followed by synchronization of the cells by centrifugal elutriation to isolate G2 cells. These cells became elongated and delayed in G2 for approximately 4 hours, a period of time in which wild-type cells underwent 2 rounds of division (Figure 2-2 a-d). During the delay, Cdc2p kinase activity remained low, similar to cells arrested in G2 as a result of *cdc25* inactivation (Moreno et al., 1990) (Figure 2-2c). Eventually, Cdc2p activity gradually increased (Figure 2-2c) and the *clp1*$^+$ overexpressing cells eventually overcame the G2 block (Figure 2-2 a, b). However Cdc2p activity never reached levels seen in *nda3* mutant cells blocked in metaphase (Figure 2-2c), possibly due to loss of synchrony during this prolonged G2 delay. Although these cells do enter mitosis as judged by the formation of mitotic spindles, elongated spindles are never observed (Figure 2-2b, see arrowheads). Perhaps due to the failure to elongate the spindle, cells were often observed that had septated without segregating the chromosomes to each daughter cell, resulting in one binucleate and one anucleate compartment (Figure 2-2b, see arrow). Because the inhibitory phosphorylation of Cdc2p on tyrosine is maintained at interphase levels in Clp1p overproducing cells (Figure 2-2c), it is possible that the G2 delay could be caused by disruption of a positive feedback loop in which Cdc2p activates its activator Cdc25p, or inhibits its inhibitor, Wee1p. Consistent with this, synchronous wild-type and *wee1*Δ cells overexpressing similar levels of *clp1*$^+$ (Figure 2-2 d, e) exhibited quite different behaviors. The

prolonged G2 delay displayed by wild-type cells was not observed in a *wee1*Δ background (Figure 2-2d). *wee1*Δ cells entered mitosis soon after isolation (Figure 2-2 f) and arrest with one or more septa (Figure 2-2d).

Figure 2-2

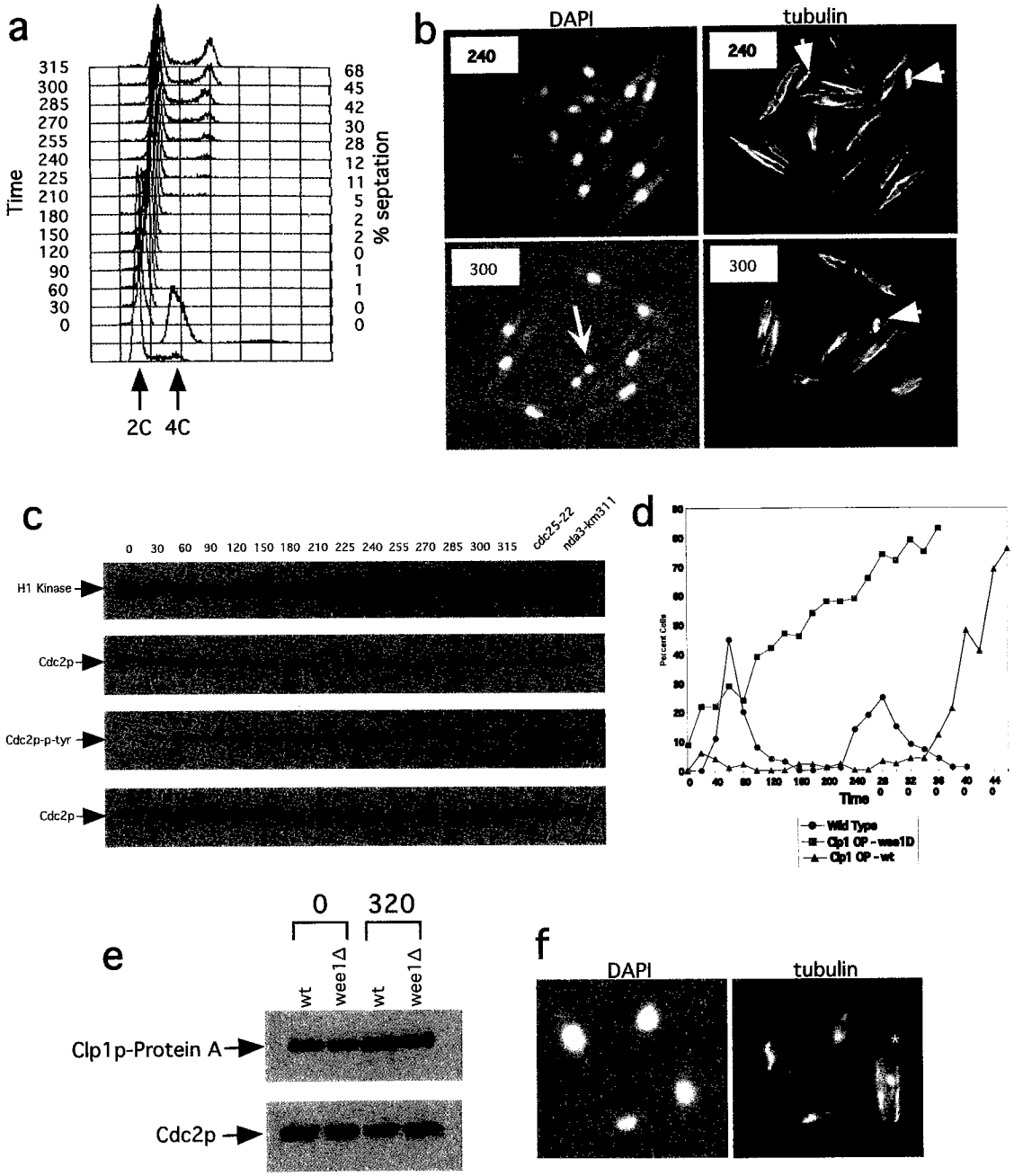

a
Time
315
300
285
270
255
240
225
210
180
150
120
90
60
30
0
68
45
42
30
28
12
11
5
2
2
0
1
1
0
0
% septation
2C
4C
b
DAPI
tubulin
240
240
300
300
c
0 30 60 90 120 150 180 210 225 240 255 270 285 300 315
cdc25-22
nda3-km311
H1 Kinase
Cdc2p
Cdc2p-p-tyr
Cdc2p
d
Percent Cells
Time
Wild Type
Cip1 OP - wee1D
Cip1 OP - wt
e
0
320
wt
wee1Δ
wt
wee1Δ
Clp1p-Protein A
Cdc2p
f
DAPI
tubulin

Figure 2-2 a-e, Overexpression of *clp1*$^+$ results in a failure of cell division. The *clp1*$^+$ cDNA was integrated at the *leu1* locus under control of the *nmt1* promoter, which begins induce 12 hrs after removal of thiamine. Overexpression of *clp1*$^+$ was achieved by incubation of cells in thiamine-free medium at 32°C for 14.5 hr. Small G2 cells were isolated by centrifugal elutriation. Samples were collected every 30 min and analyzed for **(a)** DNA content by flow cytometry, **(b)** microtubule organization by immunofluorescence, **(c)** Cdc2p-associated histone H1 kinase activity as described (Gould et al., 1991), and levels of Cdc2p pTyr, and Cdc2p by immunoblotting. **d,** Wild-type, or cells with *nmt1-clp1-proteinA* in a wild-type (Clp1 OP – wt) or *wee1*Δ (Clp1 OP – *wee1*Δ) background were placed in thiamine free medium for 12 hrs, then synchronized by centrifugal elutriation to collect small G2 cells. Cells were then resuspended in thiamine free medium and monitored for the percentage of cells with septa. **e,** The levels of Clp1p-proteinA in wild-type (wt) and *wee1*Δ cells at time zero and 320 minutes are shown. Arrowheads and arrow indicate cells described in the text. **f,** *wee1*Δ cells at the 40 minute time point were stained with anti-tubulin antibodies and stained with DAPI. A montage of representative cells is shown. Asterisk indicates cell with interphase microtubules.

Clp1p localization

To examine the localization of Clp1p, we constructed a functional *clp1-GFP* fusion gene. In interphase cells Clp1p-GFP was concentrated in the non-DAPI staining region of the nucleus, which corresponds to the nucleolus (Figure 2-3 a, column 1). Nucleolar localization was confirmed using propidium iodide staining, which stains the nucleolus in live cells (Figure 2-3b) (Drummond and Cross, 2000). Nucleolar localization of Clp1p-GFP was also observed in cells blocked in the G1 and G2 phases of the cell cycle using *cdc10* and *cdc25* blocked cells respectively (data not shown). Clp1p-GFP also concentrated at a spot on the nuclear periphery (Figure 2-3 a, column 1) that corresponded to the spindle pole body (SPB) as judged by colocalization with the SPB protein Sad1p (Figure 2-3 c). In early mitosis, Clp1p-GFP nucleolar signal drastically diminished, and Clp1p-GFP concentrated most intensely at the mitotic spindle, the SPB(s) and the medial actomyosin ring (Figure 2-3 a, column 2-6). Faint Clp1p signal was also apparent in the cytoplasm (Figure 2-3a, columns 2-4). Measurements of fluorescence intensities confirmed that cytoplasmic Clp1p-GFP signal in mitotic cells was on average twice as intense as in interphase cells. That Clp1p-GFP is released from the nucleolus early in mitosis was confirmed by examining cells blocked in metaphase using a cold-sensitive ß-tubulin mutant (*nda3-km311*). We found that Clp1p-GFP had been released from the nucleolus in the early metaphase arrest (Figure 2-3 e). In late anaphase, Clp1p-GFP concentrated at the ends and middle of the spindle (Figure 2-3 a, column 5). After spindle disassembly in telophase, Clp1p-GFP remained associated with the actomyosin ring as it constricted and the septum formed (Figure 2-3 a, column 6 (see arrow)). After completion of ring constriction and septum formation, Clp1p-GFP returned to the nucleolus (Figure 2-3 a, column 7). It is unlikely that the

changes in Clp1p signal result from periodic destruction or synthesis of the protein since the levels of the protein did not vary in the cell cycle (Figure 2-3 d).

Exit of Clp1p-GFP from the nucleolus does not depend on the SIN since examination of synchronous *sid2-250* cells after shift to restrictive temperature revealed that Clp1p exited the nucleus normally in mitosis in this and all other *sin* mutants examined (Figure 2-4 a, and data not shown). Thus, in contrast to *S. cerevisiae* Cdc14p, where its exit from the nucleolus depends on the MEN and does not occur until anaphase, *S. pombe* Clp1p exits the nucleolus early in mitosis and this relocalization is independent of the SIN.

Figure 2-3

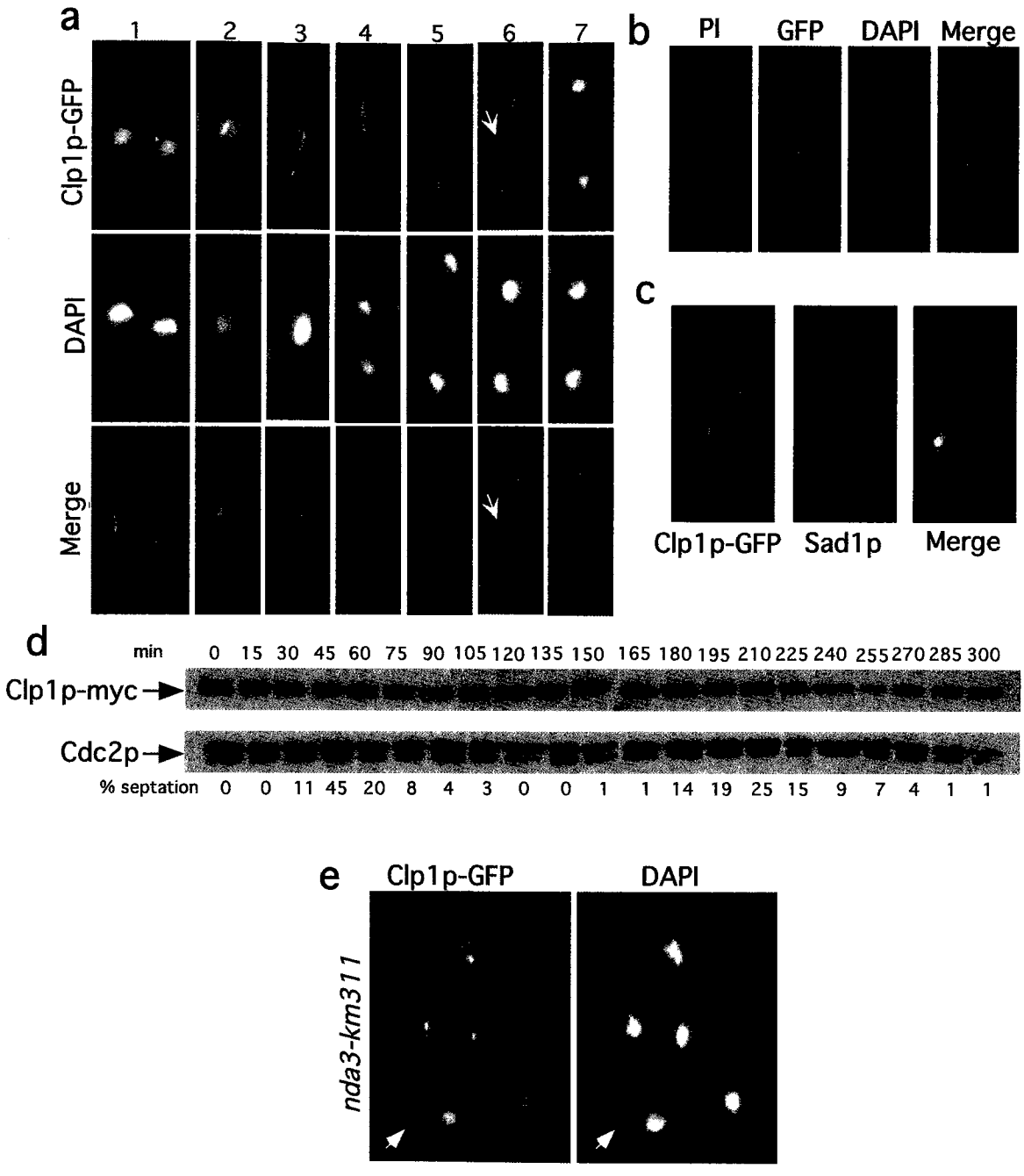

a
1
2
3
4
5
6
7
Clp1p-GFP
DAPI
Merge
b
PI
GFP
DAPI
Merge
c
Clp1p-GFP
Sad1p
Merge
d
min
0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240 255 270 285 300
Clp1p-myc
Cdc2p
% septation
0 0 11 45 20 8 4 3 0 0 1 1 14 19 25 15 9 7 4 1 1
e
Clp1p-GFP
DAPI
nda3-km311

Figure 2-3 Clp1p-GFP localization during the cell cycle. **a,** Clp1-GFP was expressed from the endogenous *clp1* promoter. Cells were fixed as previously described in methanol and stained with DAPI (Balasubramanian et al., 1997). Numbers indicate cell cycle stages described in the text. Merged images (Merge) showing DAPI in blue and Clp1p-GFP in green are also shown. Arrow in column 6 indicates constricted actomyosin ring. **b,** Live Clp1p-GFP cells were stained with propidium iodide (PI) for the nucleolus and DAPI. **c,** Clp1p-GFP expressing cells were stained for Sad1p. Clp1p-GFP (green), Sad1p (red), and the merged image are shown. **d,** Cells expressing Clp1p-13myc were synchronized by centrifugal elutriation and the levels of Clp1p-13myc, Cdc2p, and percent septation were monitored with time. **e,** Clp1p-GFP localization in *nda3-km311* cells. *nda3-km311 clp1*-GFP cells were blocked for 6 hrs at 19°C. Arrowhead indicates a cell that has not yet blocked that still displays nucleolar Clp1p-GFP. Figure 2-4

Figure 2-4

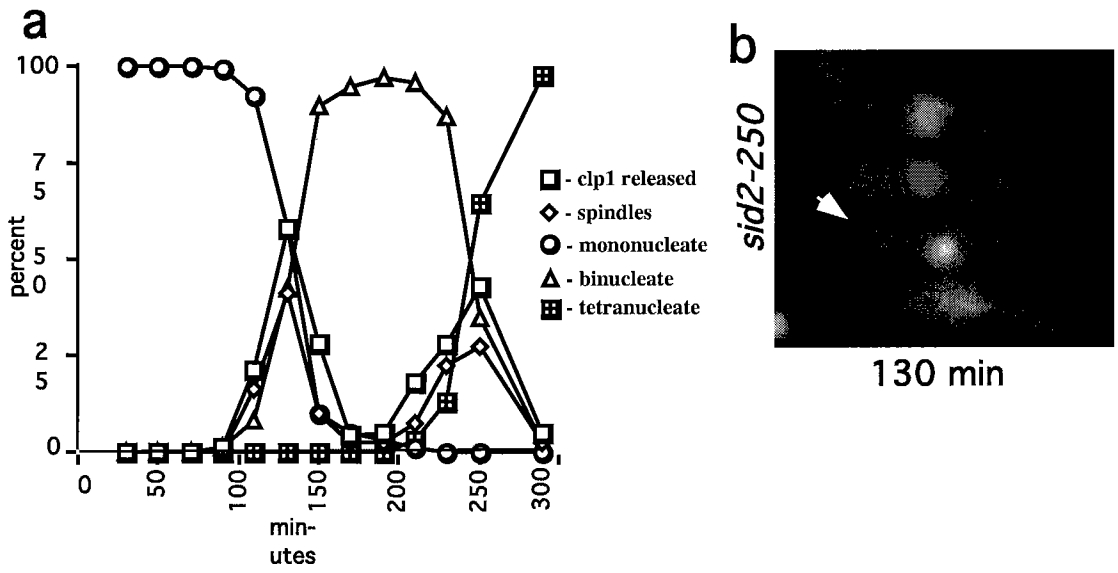

a
100
75
50
25
0
percent
0
50
100
150
200
250
300
minutes
clp1 released
spindles
mononucleate
binucleate
tetranucleate
b
sid2-250
130 min

Figure 4 a, *sid2-250* cells expressing Clp1p-GFP growing at 25°C were synchronized by centrifugal elutriation then shifted to 36°C. Cells were monitored by fluorescence microscopy to determine the percent of cells with Clp1p-GFP released from the nucleolus (clp1 released), mitotic spindles (spindles), 1 nuclei (mononucleate), 2 nuclei (binucleate), and 4 nuclei (tetranucleate). **b,** Clp1p-GFP localization in *sid2-250* cells at the 130 min time point. Arrowhead indicates a cell that has not yet entered mitosis that still displays nucleolar Clp1p-GFP.

Clp1p is essential for the cytokinesis checkpoint

Recent characterization of the *cps1-191* mutant has shown that in *S. pombe* a checkpoint exists that prevents further progression of the nuclear division cycle if cytokinesis is delayed or blocked (Le Goff et al., 1999a; Liu et al., 2000; Liu et al., 1999). Unlike SIN mutants, which undergo multiple nuclear division cycles, *cps1-191* mutants do not initiate actomyosin ring constriction, and arrest with a stable actomyosin ring and two nuclei in G2 phase of the cell cycle (Liu et al., 1999) (Figure 2- 5a). The nuclear division block in *cps1* mutants depends on the SIN and the Cdc2p inhibiting kinase Wee1p (Liu et al., 2000). Since Clp1p delays G2/M transition in a Wee1p dependent manner and deletion of *clp1* displays weak cytokinesis defects, we tested whether Clp1p was required for this checkpoint arrest. We examined the phenotype of a *clp1Δ cps1-191* double mutant and found that, unlike the *cps1-191* single mutant, nuclear division continued in these cells (Figure 2- 5a). The cytokinesis checkpoint also appears to be activated in the cytokinesis mutants *cdc3-124, cdc8-110,* and *cdc12-112*, which do not properly assemble actomyosin rings (Figure 2- 5b). After 4 hours at restrictive temperature these mutants were primarily binucleate. However, double mutants between the *cdc3, cdc8*, and *cdc12* mutants and either the SIN mutant *cdc11-123*, or *clp1Δ* became tetranucleate after 4 hours at restrictive temperature. Thus, as in the *cps1-191* mutant, the cytokinesis checkpoint appears to be active in the *cdc3, cdc8,* and *cdc12* mutants and requires both the SIN and Clp1p to maintain the nuclear division block.

Interestingly, in *cps1-191* blocked cells, Clp1p localized only faintly to the nucleolus and displayed a strong cytoplasmic signal (see uninucleate unblocked cell (arrowhead) for comparison (Figure 2-5c, 6c)), even though the nuclei in these cells were blocked in G2, a stage where Clp1p is normally in the nucleolus. Similarly Clp1p-GFP was maintained out of the

nucleus in a high percent of the *cdc3, cdc8*, and *cdc12* blocked cells (Figure 2-5c, and data not shown). Since *S. cerevisiae* Cdc14 is thought to be active in the cytoplasm, these data suggested that Clp1p is kept in the cytoplasm to inhibit Cdk activity to maintain the cytokinesis checkpoint.

To test how Clp1p might function to inhibit Cdk activity in order to maintain the nuclear division block in *cps1-191* cells, we examined the *cps1-191* phenotype more closely. Work in *S. cerevisiae* has shown that Cdc14p antagonizes Cdk activity at the end of mitosis by promoting B-type cyclin destruction and stabilizing the Cdk inhibitor Sic1p (Visintin et al., 1998). To determine if Clp1p functions in a similar manner to maintain the *cps1-191* block, we examined the levels of the B-type cyclin Cdc13p in *cps1-191* cells blocked at the restrictive temperature, and found that Cdc13p remained stable at the block point (Figure 2-5d). In addition, the nuclear division block in *cps1-191* mutants does not depend on the Cdk inhibitor Rum1p (Figure 2-5d). Together, these results suggest that Clp1p does not maintain the nuclei in interphase in *cps1-191* cells by promoting cyclin destruction or accumulation of Rum1p. Another possibility could be that Clp1p maintains the block in *cps1-191* cells not by affecting Cdc2p activity directly, but by dephosphorylating Cdc2p mitotic substrates faster than Cdc2p can phosphorylate them. If this were the case, then one would expect Cdc2p to become activated (tyrosine dephosphorylated) in *cps1-191* blocked cells, but unable to phosphorylate its substrates faster than Clp1p can dephosphorylate them. However, we found that Cdc2p tyrosine phosphorylation levels did not go down in a *cps1-191* block, as they do in a mitotic block (Figure 2-5d). Cdc2p kinase activity was low, similar to G2 blocked *cdc25* mutant cells and much lower than metaphase blocked *nda3* cells (Figure 2-5e). These data combined with the $clp1^{+}$ overexpression experiments and the fact that Wee1p is also required to maintain the *cps1-191* mutant block suggest that Clp1p

acts to inhibit Cdc2p kinase activity indirectly by promoting maintenance of interphase levels of tyrosine phosphorylated Cdc2p.

Figure 2-5

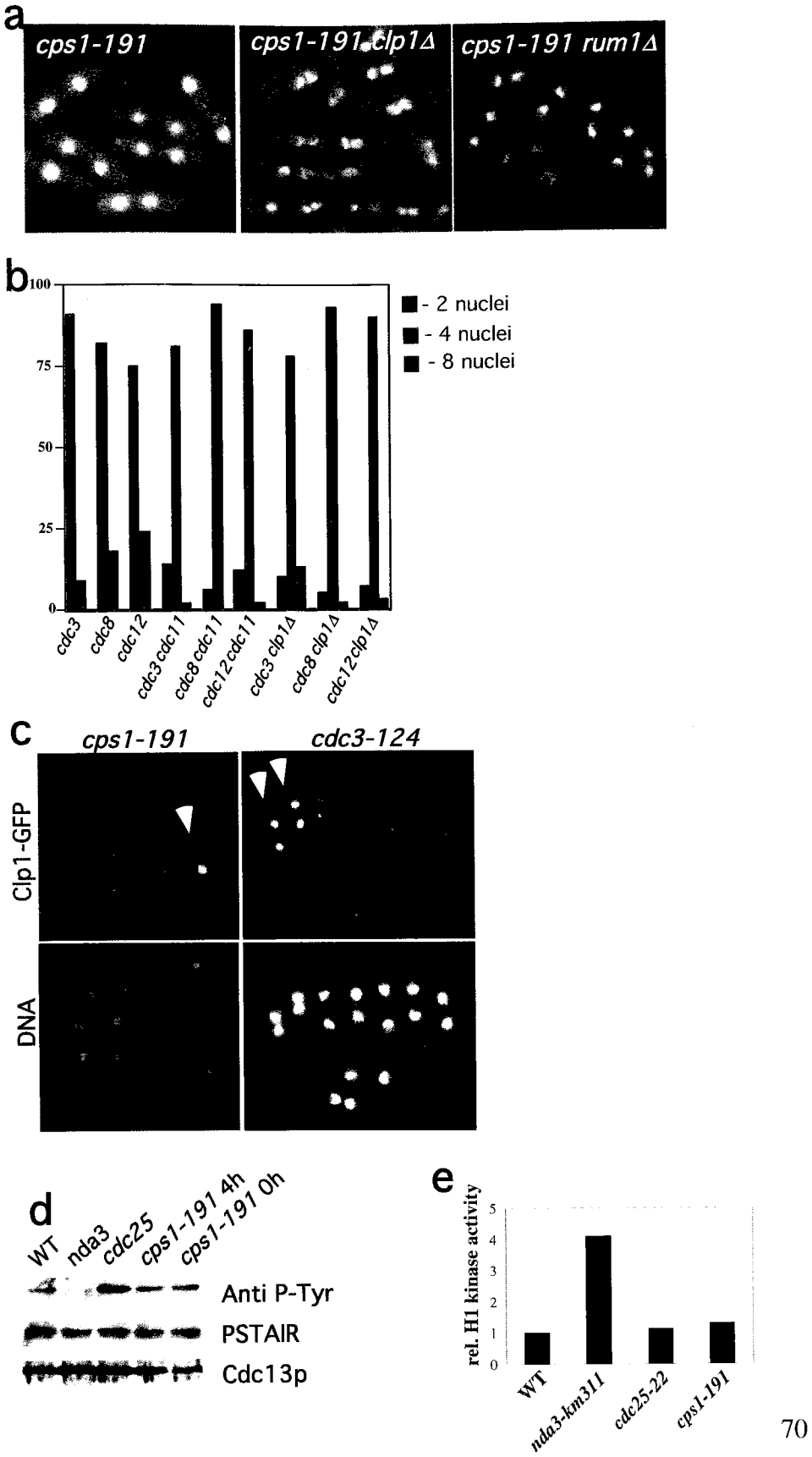

a
cps1-191
cps1-191 clp1Δ
cps1-191 rum1Δ
b
100
75
50
25
0
- 2 nuclei
- 4 nuclei
- 8 nuclei
cdc3
cdc8
cdc12
cdc3 cdc11
cdc8 cdc11
cdc12 cdc11
cdc3 clp1Δ
cdc8 clp1Δ
cdc12 clp1Δ
c
cps1-191
cdc3-124
Clp1-GFP
DNA
d
WT
nda3
cdc25
cps1-191 4h
cps1-191 0h
Anti P-Tyr
PSTAIR
Cdc13p
e
rel. H1 kinase activity
5
4
3
2
1
0
WT
nda3-km311
cdc25-22
cps1-191

Figure 2-5 *clp1*$^{+}$ is essential for the cytokinesis checkpoint in *cps1-191* cells. **a,** *cps1-191*, *clp1Δ cps1-191* cells, or *rum1Δ cps1-191* were incubated for four hours at 36°C and stained with DAPI. **b,** *cdc3-124, cdc8-110, cdc12-112* single mutants or in combination with either *cdc11-123* or *clp1Δ* mutations were incubated at 36°C for 4 hrs and their numbers of nuclei determined by DAPI staining. **c,** Clp1p-GFP fails to re-enter the nucleolus in *cps1-191* and *cdc3-124* cells blocked at 36°C for 4 hrs. Arrowheads indicate cells with Clp1p-GFP in the nucleolus. **d,** The levels of tyrosine phosphorylated Cdc2p (p-tyr), Cdc2p, and Cdc13p in *cps1-191* cells grown at 25°C, wild-type, *cps1-191*, and *cdc25-22* cells incubated at 36°C for four hrs, and *nda3-km311* cells incubated at 19°C for 6 hrs, were determined by western blotting. **e,** Histone H1 kinase assays were performed on extracts prepared from *cps1-191, cdc25-22,* and wild-type cells after 4 hours at 36°C and on extracts from *nda3-km311* cells after 6 hrs at 19°C as previously described (Guertin et al., 2000) and relative kinase activity was by normalizing the kinase activity to the level Cdc2p protein in each sample.

SIN and Clp1p function together in the cytokinesis checkpoint

Since both Clp1p and the SIN are essential for the *cps1-191* block, we investigated how Clp1p and the SIN may function together to maintain the *cps1-191* block. A previous study showed that the Sid1p kinase localizes to one SPB during anaphase, when the SIN is turned on, and leaves the SPB once cytokinesis is complete (Guertin et al., 2000). Interestingly, in the *cps1-191* block, Sid1p localized to one SPB (data not shown), showing that the SIN is active in the cytokinesis checkpoint. This is consistent with previous observations that the SIN pathway is required for the *cps1-191* block (Liu et al., 1999). We next examined whether Clp1p was required for Sid1p to localize to the SPB in the *cps1-191* block. Because Clp1p is required for the *cps1-191* nuclear division block, *cps1-191 clp1*Δ and *cps1-191* cells expressing GFP-Sid1p were shifted to restrictive temperature in the presence of the DNA synthesis inhibitor hydroxyurea (HU) in order to compare GFP-Sid1p localization in cells at the same stage of the cell cycle. Since most asynchronous *S. pombe* cells are in G2 phase, the *cps1-191* cells proceeded through mitosis and arrested as binucleate cells (Figure 2-6 a-d) without initiating the next round of DNA synthesis (data not shown). GFP-Sid1p localized to the SPB in *cps1-191*/HU blocked cells but did not localize in *cps1-191 clp1*Δ/HU blocked cells showing that GFP-Sid1p localization depended on Clp1p (Figure 2-6 a,b). Examination of synchronous cell populations showed that when G2 phase *cps1-191* cells were incubated at restrictive temperature Sid1p localized normally to the SPB in anaphase, but stayed at the SPB after exit from mitosis due to the cytokinesis failure. However when synchronous *cps1-191 clp1*Δ cells were shifted to restrictive temperature, Sid1p localized to the SPB in anaphase but was not maintained at the SPB despite the cytokinesis failure (Figure 2-6.g). Since Sid1p localization to the SPB is antagonized by Cdc2p activity (Guertin et al., 2000), Clp1p may function to maintain the SIN in

an on state in the *cps1-191* block by keeping Cdc2p kinase activity low and/or dephosphorylating a key Cdc2p substrate to allow Sid1p localization. Consistent with the later possibility, GFP-Sid1p localizes poorly in *clp1*Δ mutants, displaying 1/5 the signal at the SPB observed in wild-type cells (Figure 2-6 e). Localization of Cdc7p, which is not antagonized by Cdk activity (Guertin et al., 2000), was not effected by *clp1*Δ (Figure 2-6 e). Because maintenance of the *cps1-191* block also requires the SIN, we tested whether Clp1p exclusion from the nucleolus depended on the SIN. Examination of *sid1-239 cps1-191*/HU blocked cells showed that Clp1p-GFP returned to the nucleolus (Figure 2-6 f), unlike in *cps1-191*/HU blocked cells where it remained out of the nucleolus (Figure 2-6 c). Thus, the SIN is essential to keep Clp1p out of the nucleolus in the *cps1-191* block.

To test the effect of hyper-activation of the SIN on Clp1p localization, the Spg1p GTPase was over expressed in asynchronously growing cells. As previously observed (Balasubramanian et al., 1998; Schmidt et al., 1997), Spg1p overexpression caused most cells to initiate septation in interphase, but some cells completed mitosis and then formed multiple septa between the two separated nuclei. In cells which septated during interphase, Clp1p-GFP remained in the nucleolus (Figure 2-6 f). In contrast, in the cells that became blocked after completion of mitosis, Clp1p-GFP remained out of the nucleolus. Similar results were obtained when the SIN was activated by inactivating Cdc16p, a component of the Spg1p GTPase activating protein (Furge et al., 1998) (Figure 2-6 f). Thus, activation of the SIN pathway will not drive Clp1p out of the nucleolus in interphase, but once Clp1p has exited the nucleolus in mitosis, activation of the SIN will keep Clp1p from returning to the nucleolus. Together these results suggest a possible

feedback loop where SIN activity keeps Clp1p outside the nucleolus and Clp1p in turn antagonizes Cdk function allowing the SIN to remain active until cytokinesis is complete.

Figure 2-6

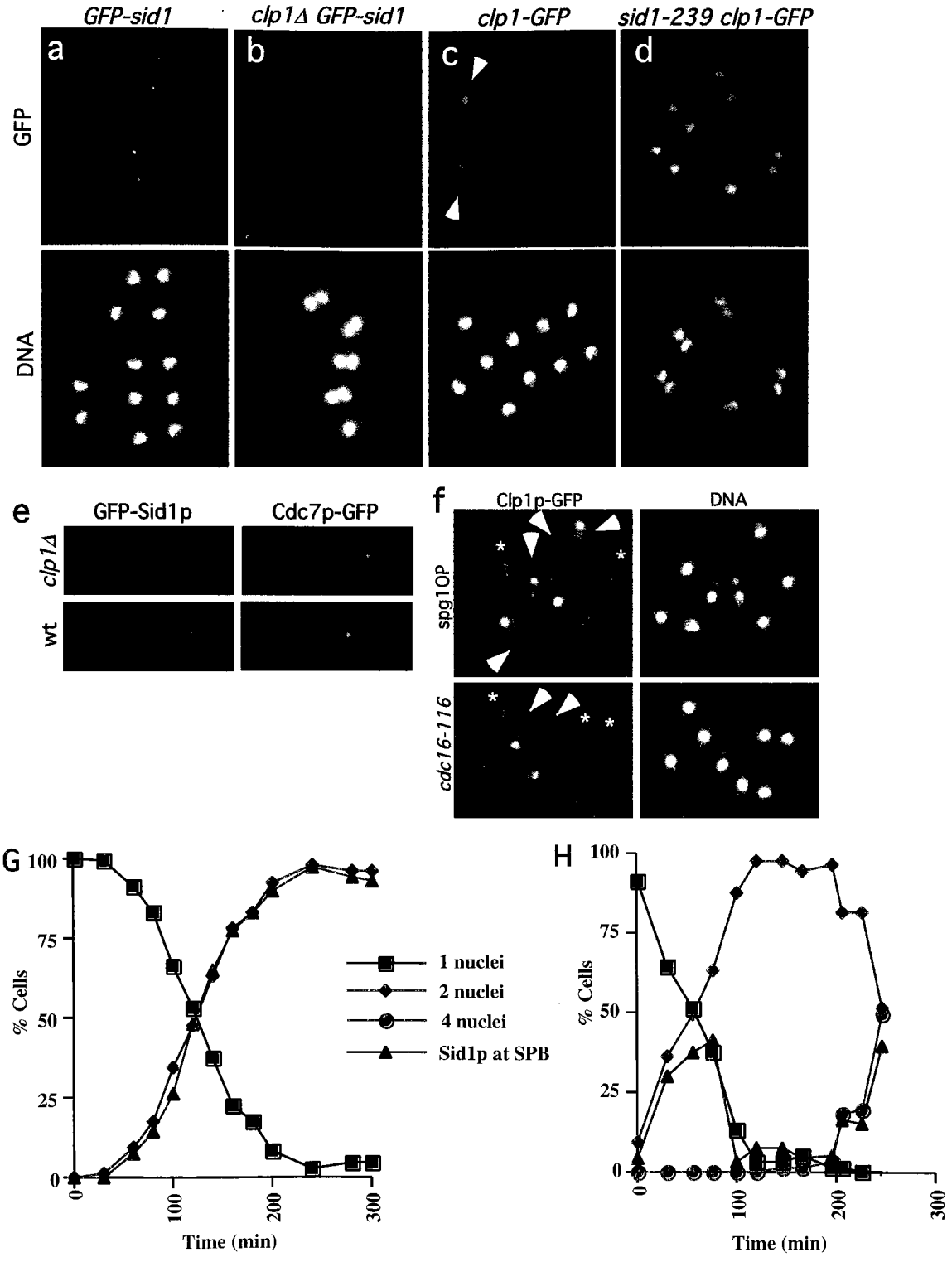

GFP-sid1
clp1Δ GFP-sid1
clp1-GFP
sid1-239 clp1-GFP
a
b
c
d
GFP
DNA
e
GFP-Sid1p
Cdc7p-GFP
clp1Δ
wt
f
Clp1p-GFP
DNA
spg1OP
cdc16-116
G
H
% Cells
Time (min)
1 nuclei
2 nuclei
4 nuclei
Sid1p at SPB

Figure 2-6 Interaction between *clp1* and the SIN pathway. *cps1-191 GFP-sid1* (**a**), *cps1-191 clp1Δ GFP-sid1* (**b**), *cps1-191 clp1-GFP* (**c**), and *cps1-191 sid1-239 clp1-GFP* (**d**) were grown at 25°C then shifted to 36°C in the presence of hydroxy urea for 4hrs and stained with DAPI. **e,** Localization of GFP-Sid1p and Cdc7p-GFP in wild-type or *clp1Δ* cells is shown. **f,** The SIN pathway was activated either by inducing expression of *spg1* from the *nmt1* promoter as previously describe (Balasubramanian et al., 1998) or by shifting *cdc16-116* mutant cells to 36°C for 2 hrs. Arrowheads indicate cells that septated out of interphase. Cells that became blocked after completion of septation are also indicated (*). **(g and h)** Analysis of the dependencies of localization of Sid1p and Clp1p in synchronous *cps1-191* cells. Either **(g)** *cps1-191 GFP-Sid1* or **(h)** *cps1-191 GFP-Sid1 clp1Δ* cells growing at 25°C were synchronized by centrifugal elutriation then shifted to 36°C. Cells were monitored by fluorescence microscopy to determine the percent of cells with 1 nuclei , 2 nuclei , 4 nuclei, and GFP-Sid1p at the SPB.

If Clp1p functions in late mitosis to keep the SIN active until cytokinesis is complete by antagonizing Cdk activity, then cytokinesis should be very sensitive to increases in Cdk activity in the absence of Clp1p. To test this, we constructed double mutants between *clp1Δ* and *wee1-50* or the activated alleles of *cdc2*, *cdc2-1w, cdc2-3w* and *cdc2-F15* which has the site of inhibitory tyrosine mutated to phenylalanine. Interestingly, all double mutants displayed pronounced defects in cytokinesis (Figure 2-7. a-d), suggesting that in the absence of Clp1p, the process of cytokinesis is particularly sensitive to increased Cdk activity. Consistent with this, SPB localized Sid1p was virtually undetectable in *clp1Δ wee1-50* cells (data not shown).

Figure 2-7

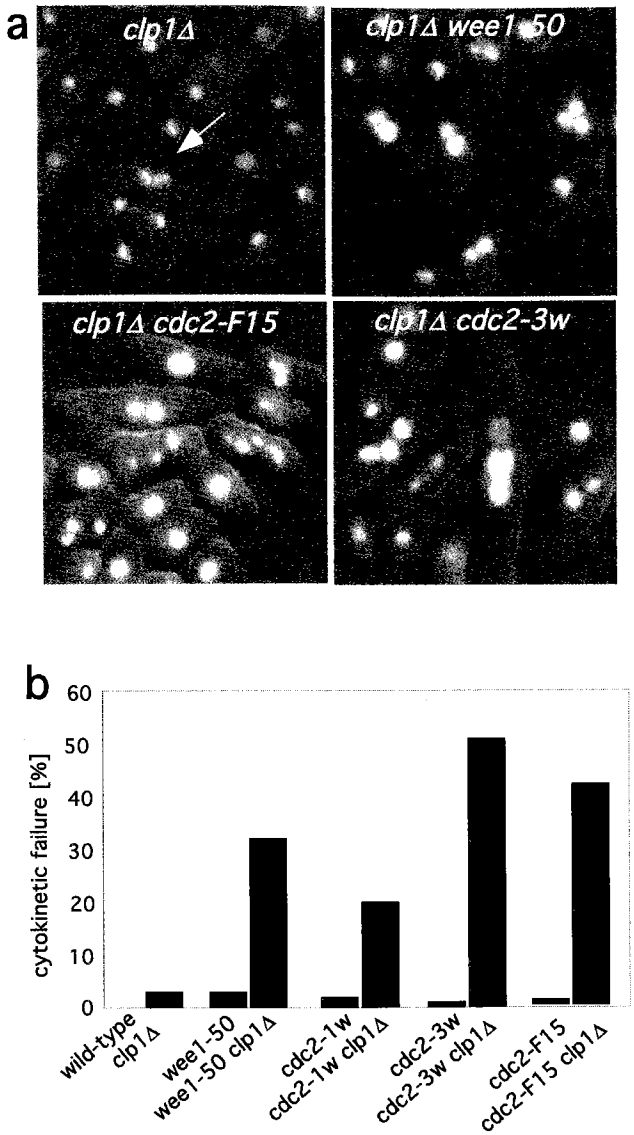

a
clp1Δ
clp1Δ wee1-50
clp1Δ cdc2-F15
clp1Δ cdc2-3w
b
cytokinetic failure [%]
60
50
40
30
20
10
0
wild-type
clp1Δ
wee1-50
wee1-50 clp1Δ
cdc2-1w
cdc2-1w clp1Δ
cdc2-3w
cdc2-3w clp1Δ
cdc2-F15
cdc2-F15 clp1Δ

Figure 2-7 **a,** Cytokinetic failure in *clp1Δ wee* mutants. Single and double mutant cells of the indicated genotypes were incubated at 36°C for 4 hrs then fixed, stained with DAPI and photographed using combined phase/fluorescence imaging. **b,** Cells that contained 2 or more closely spaced interphase nuclei without an intervening septum were scored as having failed to undergo cytokinesis.

Discussion and Unpublished Results Chapter II

Our data support a model where Clp1p may regulate G2/M transition and coordinate cytokinesis with initiation of the next cell cycle by antagonizing Cdk-dependent phosphorylation events (Figure 2-8.). A previous study has shown that *S. cerevisiae* Cdc14p dephosphorylates Cdk substrates (Visintin et al., 1998). In our study we find that *S. pombe Clp1p* appears to antagonize Cdk activity by promoting inhibitory tyrosine phosphorylation of the fission yeast Cdk, Cdc2p, which is carried out by its inhibitor Wee1p and relieved by its activator Cdc25p. Cdc2p kinase has been shown to promote its own activation by phosphorylating both Cdc25p and Wee1p, which activates Cdc25p and inhibits Wee1p (Hoffmann et al., 1993; Izumi and Maller, 1993; Mueller et al., 1995; Patra et al., 1999). Thus, Clp1p could be functioning by antagonizing autocatalytic activation of Cdc2p by dephosphorylating Cdc25p and Wee1p.

Although both Clp1p and Cdc14p function to antagonize Cdk activity, their mechanism of action appears quite different. Clp1p promotes inhibitory tyrosine phosphorylation of Cdc2p, whereas Cdc14p promotes cyclin destruction by dephosphorylating Hct1p/Cdh1p as well as accumulation of the Cdk inhibitor Sic1p (Visintin et al., 1998). In *S. cerevisiae* these two functions of Cdc14p are required for mitotic exit. The homolog of Cdh1 in *S. pombe*, Ste9p, is not essential for Cdc13p degradation to exit mitosis. Instead, Ste9p is required for Cdc13p degradation and maintenance of low CDK activity in G1 phase to allow cell cycle arrest for mating or in response to starvation (Kitamura et al., 1998; Yamaguchi et al., 1997). Like Ste9p, the CDK inhibitor Rum1p, the homolog of *S. cerevisiae* Sic1 is also required for G1 arrest (Labib and Moreno, 1996; Moreno and Nurse, 1994). Based on our study, it is possible, though unlikely, that Clp1p also acts on Ste9p and Rum1p. *clp1*Δ mutants are not sterile, as are *ste9*Δ

and *rum1*Δ, and because Clp1p does not seem to carry out its key functions by promoting cyclin B proteolysis, this possibility seems unlikely. Supporting our findings, a study by Cueille et al (2001) addressed in more detail whether Clp1p, named Flp1p in their publication, regulates Rum1p or Ste9p and came to the same conclusion. Deletion of *clp1/flp1* neither affected Rum1p levels, nor was the timing of Ste9p phosphorylation, which regulates its activity, changed. Further, Cdc13p was degraded with normal dynamics (Cueille et al., 2001).

Our previous studies have shown that Cdc2p inhibits cytokinesis by antagonizing the localization of Sid1p (Guertin et al., 2000). Release of Clp1p from the nucleolus may allow it to maintain activation of the SIN until cytokinesis is complete by antagonizing Cdc2 activity. We speculate based on our data that Clp1p inhibits Cdk activity by promoting tyrosine phosphorylation of Cdc2p. However, Clp1p may also activate the SIN by dephosphorylating inhibitory phosphorylation of SIN components by Cdc2p since expression of Cdc2p-F15 only causes cytokinesis defects in the presence of the *clp1*Δ mutation. In turn, the SIN inhibits return of Clp1p to the nucleolus. This would explain why the SIN, Wee1p and Clp1p are all required for the cytokinesis checkpoint. It is unclear what terminates this feedback loop once cytokinesis is complete.

It was previously shown that transient depolymerization of F-actin by treatment with high concentrations of Latrunculin A (100μM) caused the checkpoint block in *cps1-191* mutants to be partially overcome (Liu et al., 2000). The fact that the checkpoint is active in the actomyosin ring assembly mutants suggests that it may not be the presence of the actin ring that is monitored. However this cannot be ruled out, since these mutants do make medial assemblages of disorganized ring components (Motegi et al., 2000) (Dan McCollum unpublished obs.).

Alternatively the cytokinesis checkpoint might monitor changes in actin structures. In contrast to normal interphases, actin patches do not localize to the cell tips when the cytokinesis checkpoint is activated with the nuclei in interphase. Failure in the cytokinesis checkpoint as judged by nuclei clustering coincides with the relocalization of actin patches to the tips of the cell (Mishra et al., 2004). Whether this change in actin localization is cause or consequence of cytokinesis checkpoint failure is not clear, but it is possible that the checkpoint monitors actin relocalization from the cell middle to the cell ends. Clearly further studies will be required to elucidate what aspect of cytokinesis failure is monitored by the checkpoint.

We also observed that cells with an activated cytokinesis checkpoint positioned the nuclei to opposite cell ends, apart from each other. In cells that leaked beyond the checkpoint or SIN mutants that lack a checkpoint, the nuclei collapsed (Figure 2-5 c, arrowheads, 2-6 d). When nuclei collapse, the actomyosin ring might not be able to constrict between the closely packed nuclei. The collapsed nuclei localize their associated SPBs close to each other, suggesting that a microtubular force pulls the nuclei together. One factor that might be involved in maintaining nuclei apart from each other might be molecular motors. When testing several kinesins, we found that SIN mutants, which usually collapse their nuclei quickly, had evenly distributed nuclei at restrictive temperature when the kinesin *klp2* was deleted. This provided us with a tool to investigate whether cells with separated nuclei might prolonged G2 phase when cytokinesis was impaired. This was however, not the case, arguing that the spacing of the nuclei is not what is monitored by the cytokinesis checkpoint.

Coordinating cytokinesis with the next cell cycle is important for maintaining proper ploidy and genomic stability. We have shown that Clp1p plays a crucial role in this process.

Intriguingly, it has been shown recently that animal cells blocked in late cytokinesis delay initiation of the next cell cycle (Emoto and Umeda, 2000).

Given the conservation of key mechanisms of cell cycle control between *S. pombe* and animal cells, we speculate that Cdc14/Clp1 phosphatases may play a role in both regulating G2/M transition and coordinating completion of cytokinesis with the initiation of the next cell cycle in animal cells.

Figure 2-8

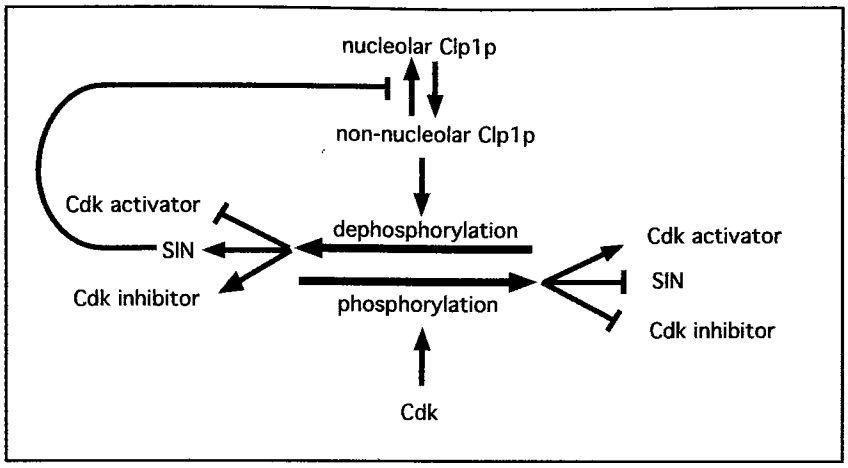

nucleolar Clp1p
non-nucleolar Clp1p
Cdk activator
SIN
Cdk inhibitor
dephosphorylation
phosphorylation
Cdk activator
SIN
Cdk inhibitor
Cdk

Figure 2-8 Model for Clp1p function. Cdk activates its activator and inhibits its inhibitor. This Cdk autoactivation loop is inhibited by Clp1p. Cdk activity also antagonizes the SIN. By keeping Clp1p out of the nucleolus, the SIN antagonizes Cdk activity.

CHAPTER III

Clp1p regulates chromosome biorientation and interacts with Aurora kinase

Figure 3-2 B was contributed by Dr Dannel McCollum

Most of this work was published in

Trautmann, S., Rajagopalan, S., and McCollum, D. (2004). The S. pombe Cdc14-like phosphatase Clp1p regulates chromosome biorientation and interacts with Aurora kinase. Dev Cell *7*, 755-762.

Summary

As shown in CHAPTER II, Clp1p regulates G2/M transition by antagonizing CDK activity and is essential for coordinating the nuclear division cycle with cytokinesis through the cytokinesis checkpoint. At the G2/M transition, Clp1p is released from the nucleolus and SPB and distributes throughout the nucleus, to the spindle and the contractile ring. This early relocalization is analogous to vertebrate Cdc14 homologs and stands in contrast to *S. cerevisiae* Cdc14p, which is not released from the nucleolus until metaphase/anaphase transition.

Here, we report that Clp1p localizes to kinetochores in prometaphase and functions in chromosome segregation, since deletion of *clp1* causes frequent co-segregation of sister chromatids, when sister kinetochores are prone to mono-orientation. Genetic, cytological and biochemical experiments suggest that Clp1p functions together with the chromosomal passenger protein Aurora kinase at kinetochores. Chromosomal passenger proteins have been shown to regulate sister chromatid bi-orientation in a variety of organisms. Together these results imply that Clp1p has a role in repairing mono-orientation of sister kinetochores together with the chromosomal passenger proteins.

Introduction Chapter III

It is crucial that cells faithfully segregate equal amounts of genetic material to daughter cells since failure to do so leads to aneuploidy, which is often associated with cancer. During mitosis, the sister chromatids attach to spindle microtubules at the kinetochores and move along the microtubules to opposite cell ends. The kinetochores are not only the site of chromosome attachment to the spindle, but also provide the detection and repair machinery when the attachment is absent or defective. The spindle checkpoint is a mechanism that monitors kinetochore attachment to microtubules and causes delay in chromosome segregation when kinetochores are not captured by microtubules. Attachment of kinetochores to microtubules per se is not sufficient to ensure equal chromosome segregation. For equal segregation the sister kinetochores need to be attached to microtubules from opposing poles in a bi-oriented fashion. This attachment to opposing poles in the presence of sister chromatid cohesion generates tension between the two chromatids. It is thought that presence of this tension is required to allow chromosome segregation. Recent discoveries show that the chromosomal passenger proteins aurora B and INCENP, which localize to the kinetochores in early mitosis, play a vital role in the repair of mono-oriented attachment in budding yeast (Biggins and Murray, 2001; Tanaka et al., 2002) as well as in vertebrate cells (Carmena and Earnshaw, 2003; Lampson et al., 2004). Chromosomal passenger proteins are a conserved family of proteins characterized by their localization at kinetochores in early mitosis and to the spindle midzone in anaphase (Adams et al., 2001; Morishita et al., 2001; Petersen et al., 2001; Rajagopalan and Balasubramanian, 2002).

As described in Chapter II, Clp1p shuttles between the nucleolus and the cytoplasm as does budding yeast Cdc14 (Trautmann and McCollum, 2002). In contrast to Cdc14p, which is released from the nucleolus at metaphase/anaphase transition, Clp1p is released from the nucleolus upon entry into mitosis, when it distributes throughout the nucleus, the cytoplasm and the contractile ring (Cueille et al., 2001; Trautmann et al., 2001). In late mitosis, Clp1p, like its *C. elegans* orthologue CeCdc14, concentrates at the spindle midzone, reminiscent of the passenger proteins (Grueneberg et al., 2002). The functional significance of the release of Clp1p in early mitosis as well as whether Clp1p, like the passenger proteins, localizes to the kinetochores in early mitosis had been unknown. Chapter III of this thesis describes that Clp1p localizes to kinetochores in prometaphase and regulates chromosome segregation.

Results Chapter III

Clp1p localizes to kinetochores in early mitosis and deletion of *clp1* causes chromosome loss

After analyzing Clp1p localization in early mitosis more closely, we observed Clp1p-GFP not just at the actomyosin ring as expected, but also at foci in the nucleus of cells arrested in metaphase using the cold sensitive β–tubulin mutant *nda3-km311*. Often three distinct spots, presumably associated with the three chromosome pairs, were observed (Figure 3-1. A). A similar localization was observed in *dis1* mutants at restrictive temperature. *dis1* cold sensitive mutants block in metaphase with elongated spindles but missegregated chromosome pairs at 19°C (Ohkura et al., 1988), sometimes allowing the three *S. pombe* chromosomes to be easily distinguishable. In these cells, Clp1p-GFP was found as a spot at each of the three *S. pombe* chromosomes (Figure 3-1. B). These spots correspond to kinetochores as judged by co-localization with the centromeric histone CenpA (*cnp1-3HA*) (Takahashi et al., 2000) (Figure 3-1 C). To investigate whether Clp1p-GFP localizes to kinetochores in non-arrested cells, we followed Clp1p-GFP in time-lapse movies. In addition to *clp1-GFP*, the cells also expressed the spindle pole body marker *sid4-GFP* to enable observation of SPB separation and entry into mitosis. Coinciding with SPB separation, rapidly moving Clp1p-GFP foci were observed between the SPBs (Figure 3-1 E (1), Figure 3-1 D, 15'-35', video1.mov (Clp1p-GFP Sid4p-GFP)). The Clp1p-GFP spots were maintained until the SPBs moved apart in anaphase. These foci were absent in cells expressing *sid4-GFP* alone (Figure 3-1 E (2), video2.mov (Sid4p-GFP)).

Figure 3-1

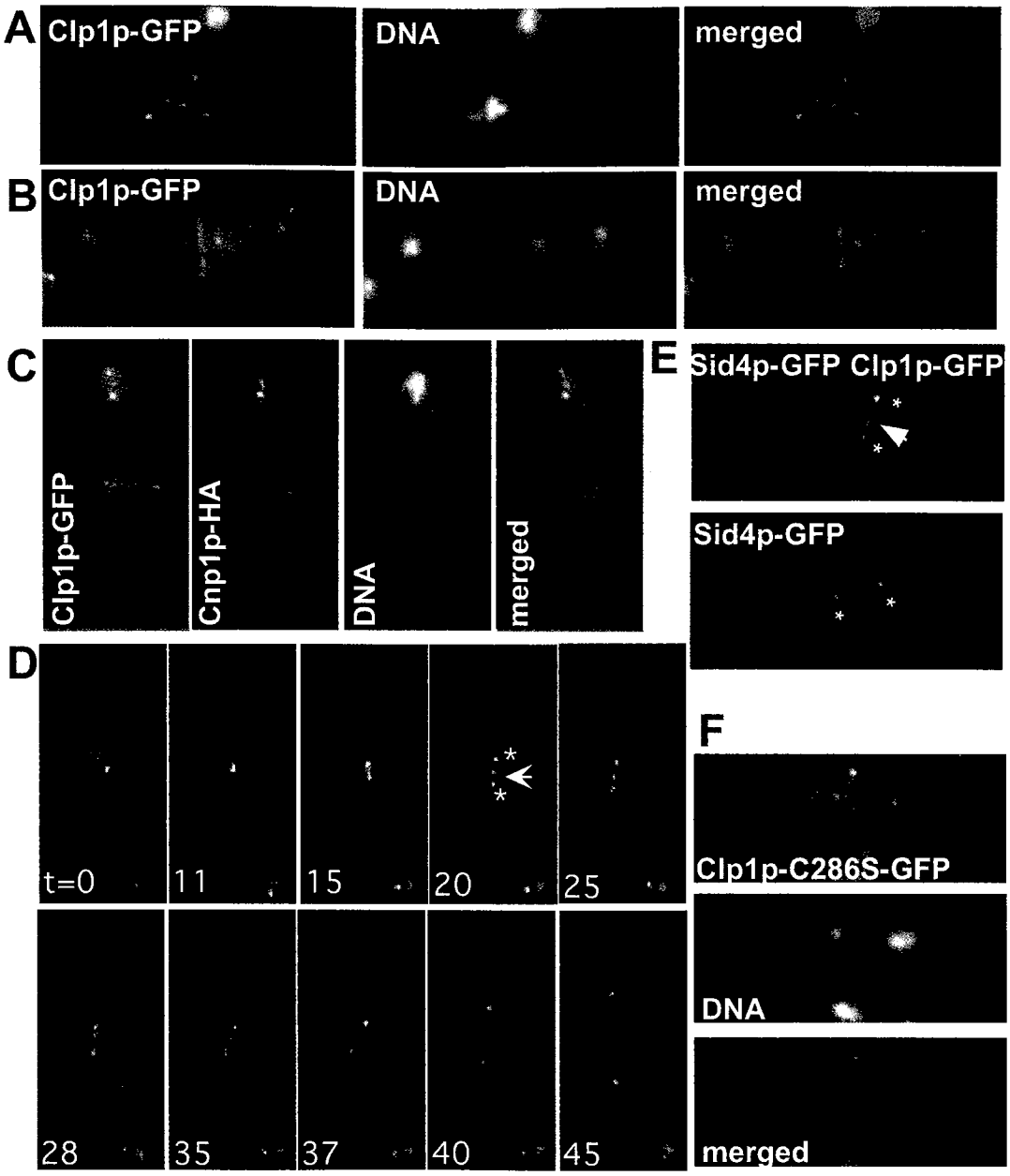

A
Clp1p-GFP
DNA
merged
B
Clp1p-GFP
DNA
merged
C
Clp1p-GFP
Cnp1p-HA
DNA
merged
D
t=0
11
15
20
25
28
35
37
40
45
E
Sid4p-GFP Clp1p-GFP
Sid4p-GFP
F
Clp1p-C286S-GFP
DNA
merged

Figure 3-1. Clp1p-GFP localizes to kinetochores during early mitosis. Cells in (A) (*clp1-GFP nda3-KM311*), (B) (*clp1-GFP dis1*), (C) (*clp1-GFP dis1 cnp1-3HA*) and (F) (*clp1-C286S-GFP dis1*) were grown at 30°C then shifted to 19∞C for 6h prior to fixation, and visualized for GFP fluorescence (A, B, F), or (C) immunostained for Clp1p-GFP and Cnp1p-HA with GFP and HA antibodies respectively. (D) Individual images from time-lapse analysis of *clp1-GFP sid4-GFP* expressing cells. Numbers indicate minutes after acquisition of the first image. Kinetochore Clp1p-GFP (arrowhead) and SPB marking Sid4p-GFP (asterisks) are indicated. The movie and further information are in the Supplemental material. (E) Images of live cells expressing *sid4-GFP clp1-GFP* or *sid4-GFP* in early mitosis. Asterisks indicate SPBs marked with Sid4p-GFP, the arrowhead points to Clp1p-GFP signal between SPBs.

Videos 1 and 2: Clp1p-GFP on kinetochores in early mitosis.

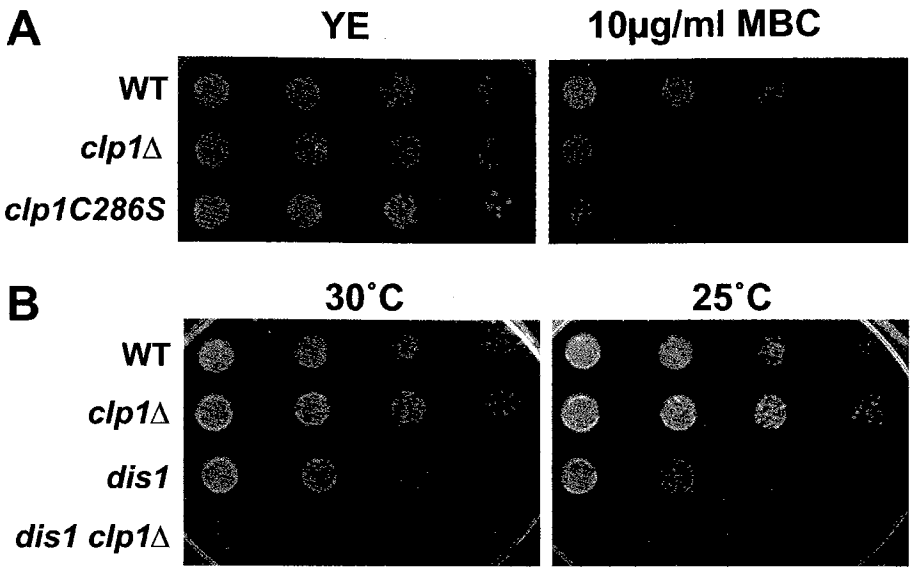
GFP signal from cells expressing Clp1p-GFP and Sid4p-GFP or Sid4p-GFP alone as viewed with a 50x Objective. Time-lapse movies are produced from frames taken once each minute and displayed at a rate of 15 frames/sec.

Video1: Mitotic *clp1-GFP sid4-GFP* cells show separating SPBs (Sid4p-GFP), the appearance of the contractile ring clp1-GFP signal and GFP foci at kinetochores rapidly moving between the SPBs before the SPBs move towards opposite poles in anaphase.

Video 2 was produced identically as movie 1, and shows cells expressing only *sid4-GFP* as a control to show absence of rapidly moving foci between the duplicated SPBs.

Because Clp1p-GFP localizes to kinetochores, we tested whether lack of *clp1* results in chromosome segregation defects. Similar to mutants defective in kinetochore proteins, *clp1*Δ cells showed hypersensitivity to the microtubule depolymerizing drug Carbendazim (MBC, Sigma-Aldrich) (Figure 3- 2. A). Comparison of the chromosome loss rate in *clp1*Δ mutants and wild type using an assay which monitors loss of a non-essential mini-chromosome (Niwa, 1986) revealed that *clp1*Δ mutants had a 28 fold increase in chromosome loss compared to wild type (Figure 3- 2. C), consistent with a role for Clp1p in chromosome segregation. To test whether Clp1p phosphatase activity is required for its role in chromosome segregation, we utilized a mutation in the conserved Cysteine of the phosphatase domain (C286) to Serine which results in a phosphatase inactive allele of Clp1p (Wolfe and Gould, 2004). The *clp1-C286S-GFP* allele was integrated into the genome and expressed from the native *clp1* promotor. Despite its correct localization (Figure 3-1. F), *clp1-C286S-GFP* was hypersensitive to MBC (Figure 3- 2 A) and lost the mini-chromosome at a rate 97.2 times higher than wild type (Figure 3- 2 C). The increased chromosome loss rate of *clp1-C286S-GFP* compared to the deletion mutation might be explained if, as with other Cdc14 proteins, the mutant protein acts in a dominant negative manner by binding but not releasing its substrates (Figure 3-3 F) (Xu et al., 2000).

Figure 3-2



C

	chromosome loss rate	fold increase
wild type	4.23×10^{-4} (4659)	
clp1Δ	1.12×10^{-2} (1811)	28.2
clp1C286S-GFP	4.11×10^{-2} (1509)	97.2

Figure 3-2. *clp1Δ* cells are sensitive to perturbations in the chromosome segregation machinery. (A) Wild-type and *clp1Δ* cultures were plated in serial dilutions on YE and YE plates containing 10μg/ml Carbendazim (MBC) and grown at 36°C. (B) Growth of single and double mutants in drop tests at different temperatures. Serial dilutions were spotted on YE plates and shifted to permissive (30°C), semi-permissive (25°C) temperatures. (C) The rate of mini-chromosome loss per cell division was determined as described in Materials and Methods. The numbers in parentheses indicate the total number of colonies screened.

Clp1p interacts with aurora kinase

Like chromosomal passenger proteins, Clp1p localizes to kinetochores in prometaphase, the spindle midzone in anaphase, and regulates chromosome segregation (Adams et al., 2001; Morishita et al., 2001; Petersen et al., 2001; Rajagopalan and Balasubramanian, 2002). We therefore tested for a genetic interaction between the only available thermo sensitive chromosomal passenger mutant *cut17-275*, an allele of the survivin homolog *bir1/cut17,* and *clp1Δ*. Tetrad dissection of 36 asci did not result in any viable *clp1Δ cut17-275* double mutants at the *cut17-275* permissive temperature of 25°C. The predicted double mutant cells divided once or twice and lysed (Figure 3- 3 A). The inactive *clp1-C286S-GFP* allele showed the same synthetic lethal interaction as the *clp1Δ* deletion mutant (data not shown). Interestingly, double mutants in *clp1Δ* and the cohesin mutant *rad21-k1*, which abolishes localization of aurora kinase Ark1p to kinetochores, grew poorly at 25°C and did not form colonies at 30°C, the *rad21-k1* permissive and semipermissive temperatures respectively (Figure 3- 3 B). Since both the cohesin Rad21p and the chromosomal passenger protein Bir1p are essential for localization of Ark1p, and thermo sensitive alleles of each gene are synthetically lethal with each other (Toyoda et al., 2002), the genetic interactions with *clp1Δ* support a model where Clp1p regulates chromosome segregation together with the chromosomal passenger proteins.

This led us to compare the localization of GFP tagged aurora kinase (Ark1p-GFP) in wild-type and *clp1Δ* cells. Cultures were synchronized with hydroxy urea in S phase and Ark1p-GFP localization was followed after release from the block. Live cells were analyzed every twenty minutes for Ark1p-GFP signal on the kinetochores and septation. In wild-type cells localization of Ark1p-GFP at the kinetochores peaked prior to

septation. The peak was drastically reduced in *clp1Δ* cells (Figure 3- 3 C). This behavior was consistent in 3 independent experiments. Examples of Ark1-GFP localization are shown in Figure 3- 3 D, where, in contrast to the above experiment, cells also express *sid4-GFP* to confirm the early mitotic stage. Ark1p-GFP at kinetochores of metaphase *clp1Δ* cells is either much fainter than in wild type cells or not detectable. The overall level of Ark1p-GFP was not reduced in *clp1Δ* compared to wild-type cells (Figure 3- 3 E, Lane2 and 3). Ark1p-GFP localization to the kinetochores was also reduced in the phosphatase inactive Clp1p-C286S allele. The effect of *clp1Δ* on Ark1p-GFP localization was specific, as we could not find a difference in localization of the kinetochore proteins Ndc80p-GFP, Mis6p-GFP, Mis12p-GFP and Bub1-GFP in *clp1Δ* deletion mutants compared to wild type (data not shown). The percentage of cells localizing Ark1p-GFP to kinetochores was also reduced in the absence of Clp1p in cells arrested in metaphase due to absence of microtubules and an activated spindle checkpoint in the *nda3-km311* mutant (Figure 3-3 H). The same was true for survivin Bir1p-GFP localization to the kinetochores, while Mad2p-GFP localization was not affected by deletion of *clp1* (Supplemental Figure S1). Interestingly, localization of the INCENP homolog Pic1p, which localizes like Bir1p-GFP and Ark1p-GFP (S. Trautmann unpublished observation), was the same whether Clp1p was present or not (Figure 3-3 H). Additionally, Clp1p-C286S-13myc and Ark1p-GFP co-immunoprecipitate in extracts from metaphase blocked cells and to a reduced level in lysates from asynchronous cultures (Figure 3-3 F). Interestingly, wild-type Clp1p and Ark1p only weakly co-immunoprecipitate (Figure 3-3 F). This is consistent with the enhanced phosphatase-substrate interaction of the phosphatase inactive point mutant Clp1p-C286S

(Xu et al., 2000). These results show that Clp1p exists in a complex with Ark1p and is required for efficient localization of Ark1p and Bir1p to the kinetochores. *S. cerevisiae* Cdc14p is essential for the localization of aurora kinase Ipl1p to the spindle (Pereira and Schiebel, 2003), however, *clp1*Δ mutants were not defective in Ark1p localization to the spindle mid-zone (Figure 3-3 G).

Figure 3-3 A-E

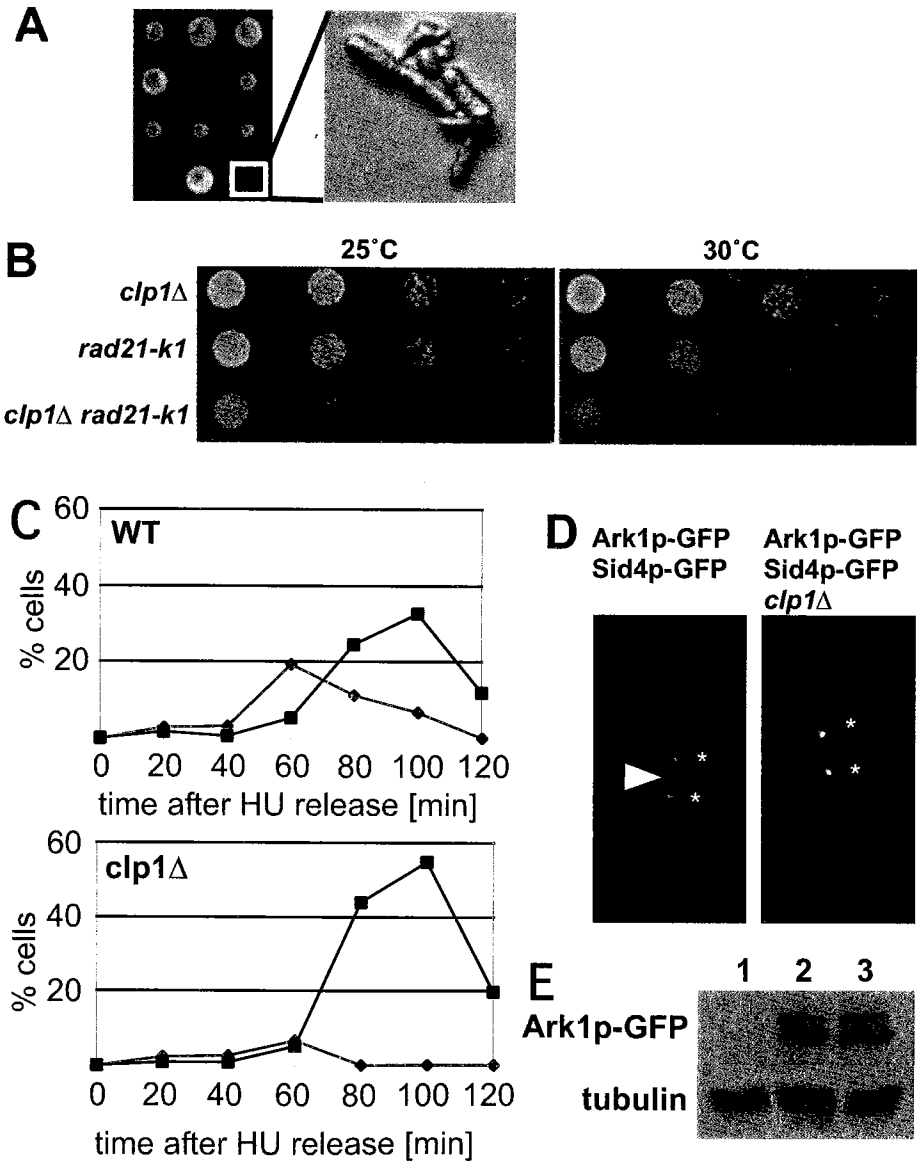

A
B
25°C
30°C
clp1Δ
rad21-k1
clp1Δ rad21-k1
C
WT
% cells
60
40
20
0 20 40 60 80 100 120
time after HU release [min]
clp1Δ
% cells
60
40
20
0 20 40 60 80 100 120
time after HU release [min]
D
Ark1p-GFP
Sid4p-GFP
Ark1p-GFP
Sid4p-GFP
clp1Δ
E
1 2 3
Ark1p-GFP
tubulin

Figure 3-3 F-H

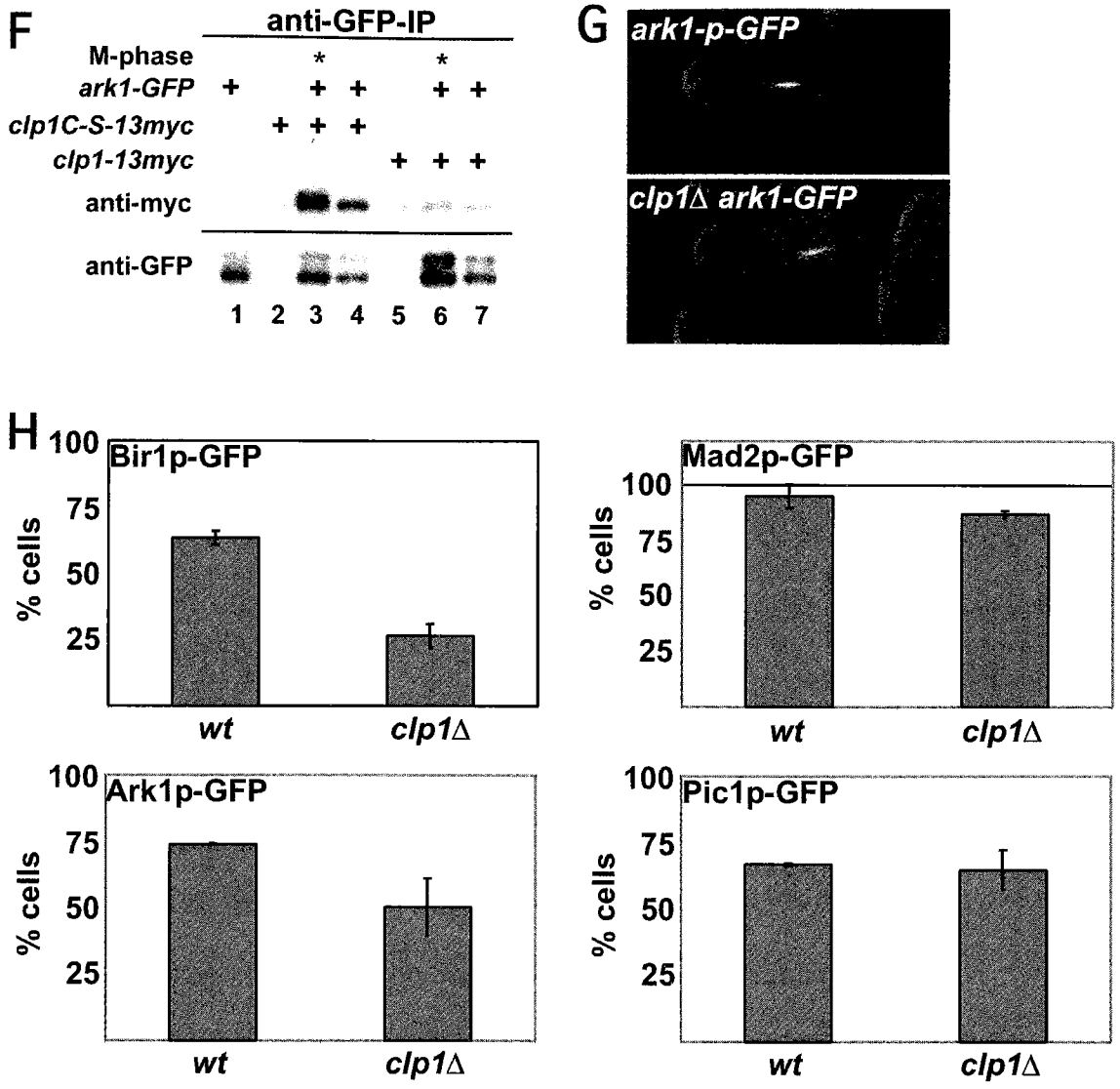

F
anti-GFP-IP
M-phase
ark1-GFP
clp1C-S-13myc
clp1-13myc
anti-myc
anti-GFP
1 2 3 4 5 6 7
G
ark1-p-GFP
clp1Δ ark1-GFP
H
Bir1p-GFP
Mad2p-GFP
Ark1p-GFP
Pic1p-GFP
% cells
100
75
50
25
wt
clp1Δ

Figure 3-3. Interactions between Clp1p and aurora kinase. **(A)** Three representative tetrad types from the cross of *cut17-275* with *clp1Δ* and an example of the *cut17-275 clp1Δ* phenotype are shown. **(B)** Growth of single and double mutants in serial dilution drop tests at permissive (25°C) and semi permissive (30°C) temperature **(C)** *ark1-GFP* and *ark1-GFP clp1Δ* cells grown at 30°C, were blocked in S-phase using 11mM HU for 5 hours, then released and monitored for Ark1p-GFP localization to kinetochores (diamonds) in live cells, as well as septation index (squares). **(D)** Live pictures of early mitotic *ark1-GFP* and *ark1-GFP c1pΔ* cells carrying *sid4-GFP* (marked by asterisks) to visualize the SPBs are shown. The arrowhead indicates Ark1p-GFP localization. **(E)** Ark1p-GFP levels are compared between wild type (lane 2) and *clp1Δ* (lane 3) cells, or from an untagged strain (lane 1) after immunoprecipitation and western blotting with GFP antibodies. The lower lane shows the tubulin loading control from whole extracts prior to immuno precipitation. **(F)** Anti-myc and anti-GFP western blots of anti-GFP-immunoprecipitates from cell lysates of the following cultures are shown: *ark1-GFP* (1), *clp1C286S-13myc* (2), *nda3-km311 ark1-GFP clp1C286S-13myc* synchronized in metaphase (3) and asynchronous (4), *clp1-13myc* (5), *nda3-km311 ark1-GFP clp1-13myc* blocked in metaphase (6) and asynchronous (7). **(G)** Live pictures of Ark1p-GFP anaphase localization merged with the dic-image in *clp1*$^{+}$ and *clp1Δ* strain are shown. **(H)** Quantification of kinetochore localizations in *nda3-km311* and *nda3-km311 clp1Δ* background after arrest in metaphase by shift to19°C for 6h.

Deletion of *clp1* reduces viability of *dis1* mutants

Since Clp1p localizes to kinetochores in *dis1* mutants, we tested whether its absence would affect the *dis1* mutant phenotype. Dis1p, is a *S. pombe* XMAP215/TOG/Stu2-family homolog, which binds microtubules and localizes to kinetochores (Nakaseko et al., 2001). Mutants in *dis1* lack the chromosome alignment phase in metaphase and arrest with elongated spindles and segregated, but unseparated chromosome pairs (Nabeshima et al., 1998; Ohkura et al., 1988). We constructed double mutants between *dis1* and *clp1*Δ and analyzed their growth rates at the *dis1* permissive (30°C), semi-permissive (25°C) and restrictive (19°C) temperature. While *dis1* mutants grew fine at 30°C, *dis1 clp1Δ* double mutants showed very poor growth at 30°C (Figure 3- 2 B). Double and single mutants containing *dis1* mutations were dead at 19°C (data not shown). In addition, *dis1 clp1Δ* double mutants were dead at 25°C, whereas *dis1* single mutants were able to grow, albeit slowly, at this temperature (Figure 3- 2 B). To exclude that deletion of *clp1* enhances all mutants defective in kinetochore proteins and chromosome segregation, we tested whether the *clp1Δ* mutation perturbed growth in kinetochore structure mutants *mis6-302, mis12-537* and *mal2-1* (Goshima et al., 1999; Jin et al., 2002; Saitoh et al., 1997). However, double mutants between *clp1*Δ and the above mutants did not show genetic interactions (data not shown).

clp1Δ causes aneuploidy in *dis1* cells

To identify the cause for the reduced viability in *dis1* mutants due to deletion of *clp1*, we examined the phenotypes on a cellular level. Chromosome segregation was compared by

DNA staining of asynchronous *dis1* and *dis1 clp1Δ* mutant cultures grown at 30°C or after incubation at 25°C, the *dis1 clp1Δ* restrictive temperature, for the duration of one cell cycle (4h). At 25°C *dis1 clp1Δ* cultures showed an increase in cells with uneven DNA segregation (inset Figure 3 - 4A) as compared to *dis1* single mutants (Figure 3- 4 A). The same phenotype was observed in *dis1 clp1-C286S-GFP* double mutants (Figure 3- 4 A).

Next, we examined how the deletion of *clp1* caused chromosome segregation defects. *dis1* mutants arrest with an activated spindle checkpoint and appear to experience an increase in sister chromatid co-segregation (70%) at full restrictive temperature. Such co-segregation results when mono-oriented attachment of sister kinetochores to microtubules from the same SPB is not corrected. Since *dis1* mutants lack the phase of constant spindle length when chromosomes are properly aligned (Nabeshima et al., 1998), it is possible that the chromosomes do not have enough time to become bi-oriented before spindle elongation and anaphase B chromosome movement. Therefore, the chromosome segregation defect caused by deletion of *clp1* could be due to either failure in the spindle checkpoint or failure to establish bi-orientation. We first tested whether *dis1 clp1Δ* double mutants are defective in spindle checkpoint activation. Since Mad2p localizes to kinetochores when the spindle checkpoint is active, we compared Mad2p-GFP localization in *dis1* and *dis1 clp1Δ* double mutants grown at 30°C or after 4h at 25°C. At 25°C the percentage of cells with Mad2p-GFP at the kinetochores was the same with or without Clp1p (Figure 3- 4 B). As shown above, localization of Mad2p-GFP to the kinetochores was also unperturbed in absence of Clp1p in a *nda3-km311* induced metaphase block. Although checkpoint response seems to be intact in

*clp1*Δ cells, the possibility remains that Clp1p could be specifically involved in checkpoint response to mono-oriented chromosome pairs as was shown for Ipl1p in *S. cerevisiae*. However, it has not been demonstrated whether the spindle checkpoint monitors mono-orientation and lack of tension in *S. pombe*.

To examine whether *dis1 clp1*Δ double mutants would experience a higher frequency of sister chromatid co-segregation compared to *dis1* single mutants, we created *dis1* and *dis1 clp1*Δ strains containing the centromere I-linked LacO array and GFP-LacI-NLS and analyzed the GFP localization at 30°C, or after shift to 25°C (Nabeshima et al., 1998). Cells that had completed anaphase, as judged by septum formation were analyzed for the presence of one GFP dot in each daughter nucleus (equal segregation), or for GFP dot(s) in only one daughter nucleus (co-segregation). Examples are shown in Figure 3- 4 C. At 30°C, 20% of *dis1 clp1*Δ cells showed co-segregation of chromosome I, compared to only 1% of *dis1* single mutant cells (Figure 3- 4 C). Co-segregation of chromatids in the absence of Clp1p was even more severe at 25°C (Figure 3- 4 C). Chromosome I segregation was not affected in *clp1*Δ single mutants (Figure 3- 4 C). The absence of severe chromosome segregation defects in *clp1*Δ single mutants suggests that Clp1p is required for a mechanism to repair defects such as mono-orientation that occur in *dis1* mutants.

Clp1p is required to prevent sister chromatid co-segregation

Whether Clp1p is required to prevent sister chromatid co-segregation in more physiological circumstances was not clear. In *S. pombe*, kinetochores are clustered at one spot on the nuclear envelope next to the SPB prior to mitosis (Funabiki et al., 1993).

Upon mitotic entry, kinetochores are presumably rapidly captured by microtubules emanating from the duplicated, nearby SPBs. However, in situations when microtubules are depolymerized, such as exposure to cold temperatures or naturally occurring microtubules depolymerizing drugs, cells would block in prometaphase unable to form a mitotic spindle and the kinetochores would lose their attachment to the SPB. Such a situation is mimicked in the cold sensitive *nda3-km311* β-tubulin mutant, where shift to 19°C results in a prometaphase block, lacking a spindle (Funabiki et al., 1993; Hiraoka et al., 1984). The displaced kinetochores now need to be captured by microtubules from opposite poles by search and capture mechanisms similar to mammalian cells. This may often lead to a situation where a chromosome pair is closer to one SPB than the other and result in a mono-oriented attachment (Dr E. Grishchuck and Dr R McIntosh, personal communication). We tested whether Clp1p was important to promote bi-orientation in this situation. *nda3-km311* and *nda3-km311 clp1*Δ cells containing a GFP marked centromere II (Kitajima et al., 2003) were arrested in metaphase by incubation at 19°C and then released from the block at 30°C to allow microtubules to reassemble and attach to kinetochores in a mono- or bi-oriented manner. The success of the following chromosome segregation can be evaluated by the localization of GFP-marked centromeres after septation. If sister chromatids co-segregate, only one daughter nucleus will receive a GFP focus as seen in Figure 3- 4 F (arrowhead) and Video 4 (*clp1*Δ *nda3-km311* cenII-GFP). Centromere II localization was scored 60 min after the release when cells had completed anaphase and were septating (84% wild type, 87% *clp1*Δ). Due to misplacement of the nucleus relative to the established actomyosin ring, some cells segregate the DNA within one cell compartment, but were scored as equally segregating

if the GFP dots appeared in two separate nuclei (Figure 3- 4 E cell (#)). The culture lacking Clp1p showed a higher frequency of centromere II co-segregation than *clp1*$^{+}$ cells (Figure 3- 4 D). The co-segregation appearing in *clp1*$^{+}$ background after the release is partly due to septation prior to release from the block without chromosome segregation (16% wild type and 15 % *clp1*Δ). The *clp1*Δ phenotype quantified above was also observed using time-lapse analysis (Figure 3- 4 F and Videos 3 ((*nda3-km311* cenII-GFP) and 4 (*clp1*Δ *nda3-km311* cenII-GFP)). GFP-LacI-NLS localizes diffusely throughout the nucleus, which makes the nucleus segregating without chromosome II visible (Figure 3- 4 F arrowhead). To exclude that co-segregation is a randomization of segregation due to premature loss of cohesion when Clp1p is absent, we compared the clustering of sister chromatids of chromosome II prior to release from the restrictive temperature, but found no difference between *clp1*$^{+}$ and *clp1*Δ cells (Figure 3- 4 G). This result further supports a role for Clp1p in establishing chromosome bi-orientation.

Figure 3-4

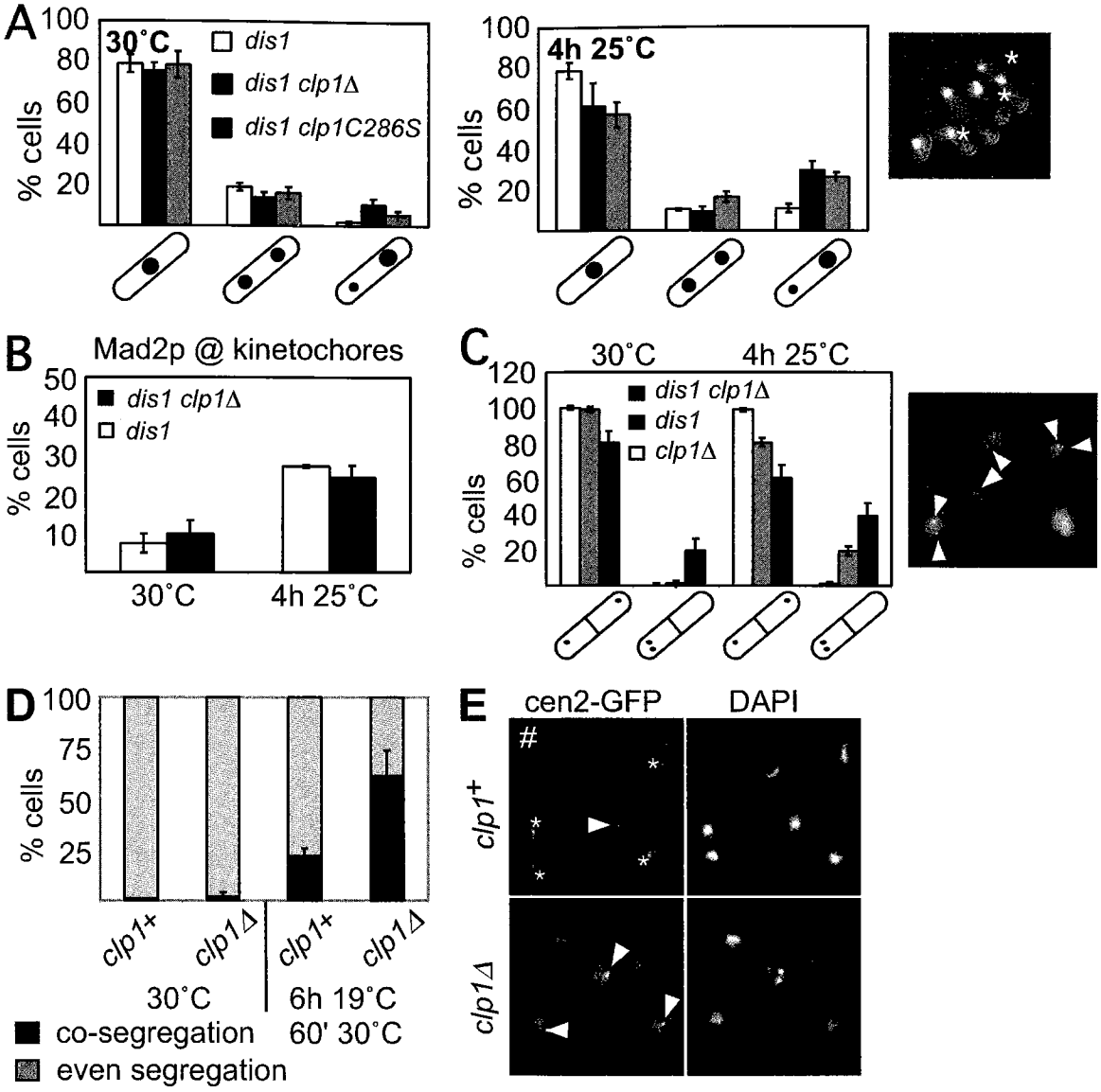

A
30°C
dis1
dis1 clp1Δ
dis1 clp1C286S
% cells
4h 25°C
B
Mad2p @ kinetochores
dis1 clp1Δ
dis1
30°C
4h 25°C
C
30°C
4h 25°C
dis1 clp1Δ
dis1
clp1Δ
D
clp1+
clp1Δ
6h 19°C
60' 30°C
co-segregation
even segregation
E
cen2-GFP
DAPI
clp1+
clp1Δ

Figure 3-4 F and G

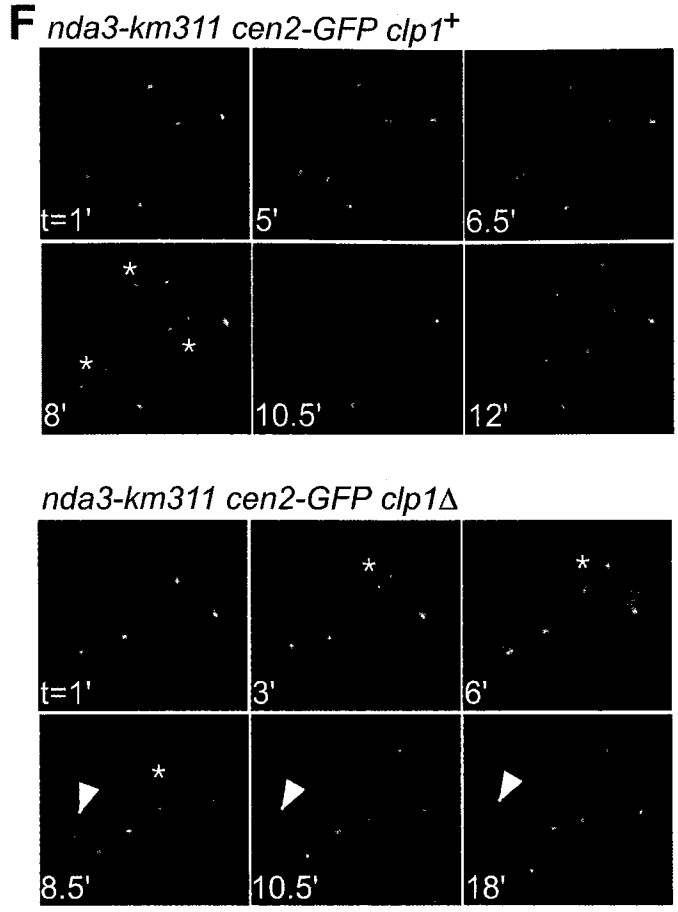


Figure 3-4. Deletion of *clp1* causes sister chromatid co-segregation. **(A)** Percentages of *dis1*, *dis1 clp1Δ* and *dis1 clp1-C286S-GFP* cells with even or unevenly segregated chromatin in interphase at 30°C and 25°C. Error bars indicate the standard deviation between different experiments. (Inset) Examples of *dis1 clp1Δ* cells at 25°C (asterisks mark septa). **(B)** Percentage *dis1* and *dis1 clp1Δ* cells with Mad2p-GFP at kinetochores grown at 30°C or after shift to 25°C. **(C)** Percentage of *clp1Δ*, *dis1* and *dis1 clp1Δ* cells segregating sister chromatids to the same or different nuclei at 30°C of 25°C as judged by centromeres I-GFP signal. Examples of *dis1 clp1*Δ cells after 4h at 25°C are shown in the picture. Arrowheads point to centromeres I dots. **(D)** Quantification of centromere II segregation in septating *clp1*$^{+}$ *nda3-km311* and *clp1*Δ *nda3-km311* cells after block and release to 30°C. Examples of centromere II segregation are shown in **(E)**. GFP-centromeres are marked with asterisks in cases of equal segregation and with arrows in cases scored as co-segregation. The # marked cell segregated DNA in one daughter cell due to nuclear mispositioning. **(F)** Still images of Videos 3 (*nda3-km311 cenII-GFP*) and 4 (*clp1*Δ *nda3-km311 cenII-GFP*), showing centromeres II-GFP from time lapse movies of *clp1*$^{+}$ and *clp1*Δ cells segregating DNA after *nda3* block and release (see Materials and Methods Chapter III). Asterisks indicate properly segregating cells. LacI-GFP-NLS localizes diffusely in the nucleus segregating without chromosome II (arrowhead). **(G)** Quantification of Centromere II clustering in *nda3-km311* blocked cells in wild type and *clp1*Δ background. Cells were grown at 19°C for 6h, fixed and prepared for microscopy. Percentage of cells displaying one or two centromere II-GFP dots is shown.

Discussion and Unpublished Results Chapter III

Chromosomal passenger proteins and Clp1p in bi-orientation in *S. pombe*

In *S. cerevisiae* as well as mammalian and frog tissue culture cells, aurora B kinase promotes bi-orientation by disrupting mono-oriented kinetochore– spindle attachments (Carmena and Earnshaw, 2003). Given that Clp1p is found in a complex with Ark1p and deletion of *clp1* reduces Ark1p localization to kinetochores, it seems likely that Clp1p regulates sister chromatid bi-orientation through aurora kinase Ark1p. However, it is not known whether chromosomal passenger proteins regulate chromosome bi-orientation in *S. pombe*. It is somewhat surprising that Mad2p localization is unaffected in *clp1*Δ mutants, given that Ark1p has been shown to be important for Mad2p localization (Petersen and Hagan, 2003). One explanation could be that there is a sufficient pool of Ark1p at kinetochores to recruit Mad2p in *clp1*Δ cells. How Clp1p regulates aurora kinase remains an interesting question. We expect that the other chromosomal passenger proteins, INCENP and survivin, may also be in the same complex with aurora and Clp1p, and could be direct targets of Clp1p. However, we have been unable to resolve this issue because these proteins are difficult to detect by western blotting (data not shown). Because Clp1p phosphatase activity is required for its role in chromosome segregation, Clp1p presumably dephosphorylates one or more components in the passenger protein complex. One possibility is that a localized reduction of CDK activity due to Clp1p is required for a stable passenger complex in metaphase. This requirement would be dispensable in the absence of high CDK activity as in late anaphase where the passengers localize normally in *clp1*Δ mutants. The fact that Pic1p-GFP localization is not affected

by *clp*Δ suggests that Pic1p localizes independently of Clp1p to the kinetochores. Dephosphosphorylation of Pic1p by Clp1p may then be required to recruit Ark1p and Cut17p/Bir1p to the kinetochores. How Pic1p functions with Ark1p and Cut17p/Bir1p and whether it is phosphorylated like its homolog in bussing yeast and humans is not known.

Cdc14-like phosphatases at the kinetochore

Our results also suggest that Clp1p functions quite differently from its *S. cerevisiae* ortholog Cdc14p. Similar to Clp1p, Cdc14p localizes to kinetochores and binds to the chromosomal passenger complex, however, it is required to dephosphorylate INCENP, which promotes localization of the chromosomal passenger complex to the spindle midzone (Pereira and Schiebel, 2003). We did not observe a defect in aurora localization to the spindle midzone in *clp1*Δ mutant cells. Similarly, knockdown of CeCdc14 expression using RNAi in *C. elegans* did not cause a total loss of AIR1p (aurora B) at the spindle midzone either (Grueneberg et al., 2002). A possible explanation for the difference between *S. cerevisiae* and *S. pombe* might be the timing of CDK downregulation. While budding yeast CDK activity stays high until mitotic exit after telophase, *S. pombe* efficiently degrades the mitotic cyclin Cdc13p already in early anaphase and lowers CDK activity earlier. Thus, dephosphorylation of INCENP might be required for stabilization and thus localization of the budding yeast chromosomal passenger complex in anaphase, while *S. pombe* only might require this dephosphorylation in metaphase. This model suggests that the budding yeast chromosomal passenger proteins are impaired in kinetochore localization in the absence

of Cdc14. The impact of Cdc14 on kinetochore localization of the chromosomal passengers in *S. cerevisiae* has not been addressed. Another reason for the difference between *S. pombe* and *S. cerevisiae* might be the different timing of Clp1p/Cdc14p release from the nucleolus. Cdc14p is not released from the nucleolus in early mitosis and therefore might not function in the exact same manner as Clp1p. To what extent Cdc14-related phosphatases regulate chromosome segregation in higher eukaryotes is not known.

Recently, hCdc14A was shown to localize to the spindle midzone in a kinesin mKlp2 dependent manner. Interestingly, localization of Aurora B kinase was also found to be MKLP2 dependent and hCdc14A can dephosphorylate human INCENP in vitro. Whether localization of Aurora B kinase depends on hCdc14A was not shown. Although CDC14 has not been localized to kinetochores in higher eukaryotes, human hCDC14B and *Xenopus* XCDC14α/β do localize to nucleoli during interphase (Kaiser et al., 2002) and are released upon nucleolar disassembly at entry into mitosis as in *S. pombe*. This is also the time when bi-orientation is established in culture cells (Tanaka, 2002), raising the possibility that the role of Clp1p in chromosome segregation may be a conserved function for CDC14 related phosphatases.

Chapter IV

Subcellular targeting of Clp1p reveals distinct nuclear and cytoplasmic functions and suggests a role for nuclear shuttling in Clp1p regulation

Most of these results will be published in:

Trautmann, S., and McCollum, D. (2005). Subcellular targeting of S. pombe Cdc14-like phosphatase Clp1p reveals distinct nuclear and cytoplasmic functions and suggests a role for nuclear shuttling in Clp1p regulation. Curr Biol, accepted.

Summary

As shown in the Results Chapters I and II of this thesis Clp1p, as other Cdc14-like phosphatases, displays cell cycle-dependent changes in localization that may be important to carry out distinct cellular functions (Trautmann and McCollum, 2002). Additionally, work on both budding and fission yeast suggested that Cdc14-like phosphatases are inhibited by nucleolar sequestration (Cueille et al., 2001; Stegmeier and Amon, 2004; Trautmann et al., 2001). In *S. cerevisiae*, Cdc14p is released from the nucleolus by the FEAR network and Cdk1 (Azzam et al., 2004; Stegmeier and Amon, 2004). The mechanism for the release from the nucleolus of Clp1p in *S. pombe* at mitotic entry is not known. The mitotic exit network (MEN) in *S.cerevisiae,* and its homologous network, the septation initiation network (SIN) in *S. pombe,* act through an unknown mechanism to keep the phosphatase out of the nucleolus in late mitosis (Stegmeier and Amon, 2004). In *S. pombe*, SIN-dependent cytoplasmic maintenance of Clp1p is thought to be essential for the cytokinesis checkpoint, which blocks further rounds of nuclear division until cytokinesis is completed. By targeting Clp1p to the cytoplasm or the nucleus, we demonstrate distinct functions for nuclear and cytoplasmic pools of Clp1p in chromosome segregation and cytokinesis respectively. In addition, our results suggest that the SIN does not keep Clp1p out of the nucleolus by regulating nucleolar affinity as proposed for *S. cerevisiae* Cdc14p, but instead, Clp1p may be regulated by nuclear import/export.

Introduction CHAPTER IV

Chapters II and III of this thesis show that Clp1p functions in the regulation of mitotic entry, the cytokinesis checkpoint and in chromosome segregation. When the cytokinesis checkpoint is activated, Clp1p localizes to the cytoplasm; failure in the cytokinesis checkpoint due to absence of SIN signaling allows Clp1p nucleolar accumulation. Further Clp1p localizes to the kinetochores, the site where chromosome segregation is mainly regulated. These results suggested that Clp1p localization to the cytoplasm is required for its function in the cytokinesis checkpoint, while localization to the kinetochores, which are inside the nucleus because of the closed mitosis in fission yeast, is required for its role in chromosome segregation.

How the localization of Clp1p is regulated is not understood. Budding yeast Cdc14 is released from the nucleolus and its inhibitor Net1/Cfi1 by a network of proteins, which together build the Cdc-Fourteen-Early-Anaphase-Release network as well as through Cdc28 and the MEN in anaphase (Stegmeier and Amon, 2004). Clp1p localizes to the nucleolus in interphase and is released from the nucleolus upon entry into mitosis. Which nucleolar proteins Clp1p binds to and how Clp1p is released is not understood. While release of Clp1p is SIN independent, relocalization of Clp1p to the nucleolus is opposed by SIN activity (Cueille et al., 2001; Trautmann et al., 2001), similar to *S. cerevisiae*, where Cdc14 cytoplasmic maintenance is MEN dependent (Stegmeier and Amon, 2004). How the SIN and the MEN prevent nucleolar return of Clp1p and Cdc14 respectively is not understood. One hypothesis put forward is that the affinity between the nucleolar binding partner and the phosphatase is regulated through phosphorylation by the SIN/MEN. Another hypothesis proposes that the SIN/MEN disallow the nuclear

accumulation of the phosphatase through regulation of nuclear import or export mechanisms. Substrates for the most downstream kinases Sid2p/Dbf2p/Dbf20p of the SIN/MEN have not been identified as of yet.

In this study, we wished to address whether Clp1p function in the cytokinesis checkpoint is dependent on its localization to the cytoplasm. Targeting Clp1p inside or outside the nucleus by fusion to nuclear import signals (NLS) or nuclear export signals (NES) showed that cytoplasmic localization of Clp1p is necessary for its cytokinesis checkpoint function. Further, Clp1p nuclear localization is required for its role in the regulation of chromosome segregation.

Fusion of Clp1p with an NLS localizes Clp1p to the nucleus independent of the normal regulatory mechanisms. This allowed us to examine whether Clp1p shows affinity for the nucleolus in the presence of SIN signaling since the ectopic NLS uncoupled a possible regulation of nuclear import and SIN. Surprisingly, Clp1p-NLS shows affinity for the nucleolus, even in the presence of SIN signaling. This result suggests that Clp1p cytoplasmic retention by the SIN is not regulated through nucleolar affinity, but through nuclear import/export mechanisms. Interestingly, Rad24p, a 14-3-3 protein, which has previously been described to facilitate nuclear export of Cdc25p (Lopez-Girona et al., 1999), is required for Clp1p cytoplasmic retention and for the cytokinesis checkpoint (Mitilesh Mishra and Mohan Balasubramanian, personal communication). Targeting Clp1p to the cytoplasm through fusion to the nuclear export signal partially rescues the cytokinesis checkpoint defect of *rad24Δ* mutants, suggesting that Clp1p is maintained in the cytoplasm through nuclear export by Rad24p.

Results Chapter IV

Localization of Clp1p-NLS-GFP and Clp1p-NES-GFP

In order to distinguish between Clp1p nuclear and cytoplasmic functions, we sought to target Clp1p either inside or outside the nucleus, by tagging *clp1-GFP* with nuclear export signals (NES) or with nuclear localization signals (NLS). In detail, the *clp1* chromosomal locus was tagged with either GFP as a control, or GFP followed by two SV40 nuclear localization signals, or two nuclear export signals from the c-AMP protein kinase inhibitor PKI (Edgington and Futcher, 2001). The resulting strains carry a single tagged copy of *clp1*, expressed from its own promoter (see Appendix: Materials and Methods of Chapter IV). The control Clp1p-GFP localized as expected to the spindle pole body (SPB) and nucleolus in interphase and to the actin ring, the mitotic spindle and kinetochores during mitosis (Figure 4-1 A, D) (Cueille et al., 2001; Trautmann et al., 2004; Trautmann et al., 2001). Clp1p-GFP-NES and Clp1p-GFP-NLS localized as predicted: Clp1p-GFP-NES was found at cytoplasmic sites: the SPB during interphase and the contractile actin ring in mitosis (Figure 4-1 B). Moreover, Clp1p-GFP-NES was absent form the nuclear sites, the nucleolus in interphase and the spindle and kinetochores during mitosis (Figure 4-1 B, E). Conversely, Clp1p-GFP-NLS was only detected at nuclear sites, the nucleolus in interphase and the spindle during anaphase (Figure 4-1 C) as well as the kinetochores in metaphase (Figure 4-1 F). Clp1p-GFP-NLS did not localize to the cytoplasmic sites, the SPB during interphase or the actin ring during mitosis (Figure 4-1 C). Both Clp1p-GFP-NES and Clp1p-GFP-NLS presumably still shuttle across the nuclear envelope, but their steady state levels are shifted to either the

cytoplasm or the nucleus. Clp1p-GFP-NES is absent from the nucleolus, the site where Clp1p is thought to be sequestered and inactive. Interestingly, relief from the nucleolus does not affect cell cycle progression as was reported for Clp1p over expression. Overproduction of Clp1p leads to a delay in interphase, which causes cells to grow longer and septate at an increased size. Conversely, *clp1*Δ mutants enter mitosis precociously and divide as smaller cells than wild type (Trautmann et al., 2001). Neither forcing Clp1p into or out of the nucleus had a significant effect on the cell cycle during normal growth, as judged by the cell size at cell division (Figure 4-1 G).

Figure 4-1

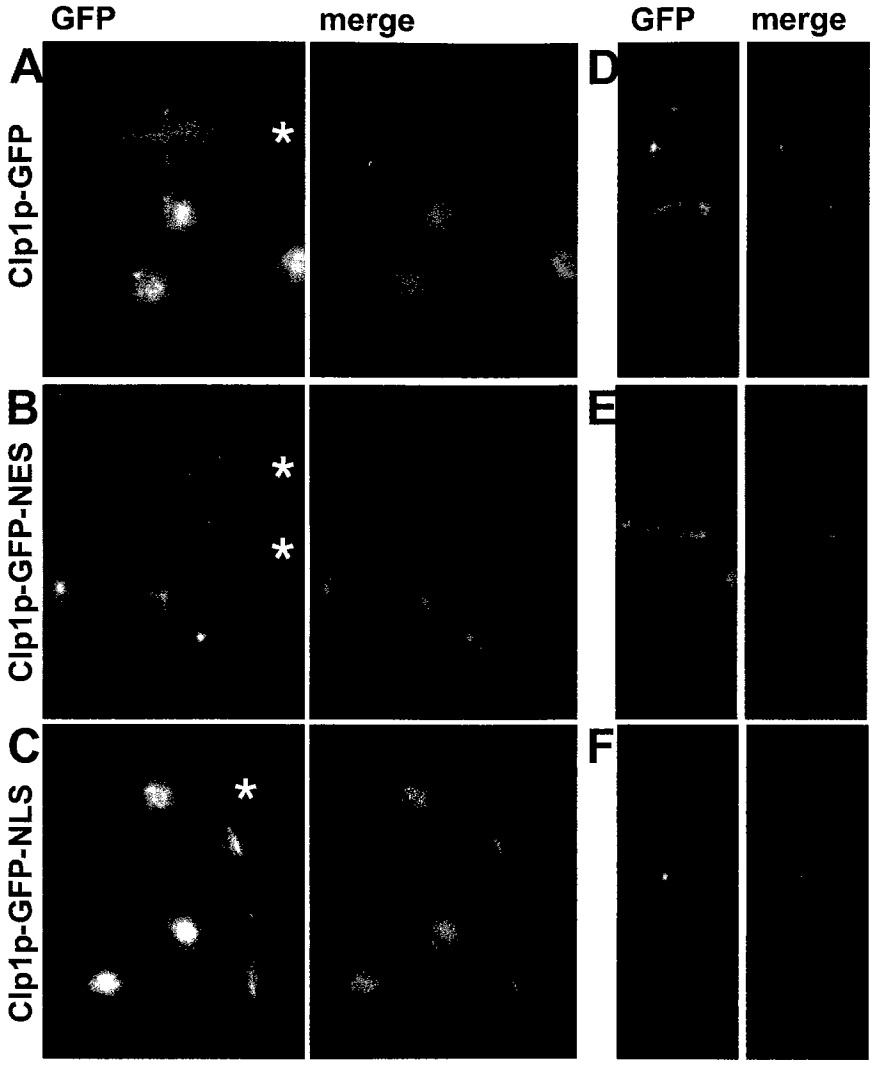

GFP
merge
GFP
merge
A
D
Clp1p-GFP
B
E
Clp1p-GFP-NES
C
F
Clp1p-GFP-NLS

Figure 4-1: Clp1p localization in the cytoplasm and nucleus

Clp1p-GFP (**A, D**), Clp1p-GFP-NES (**B, E**) and Clp1p-GFP-NLS (**C, F**) localization and merged with the DAPI signal are shown in fixed cells. **A-C** show interphase and anaphase (*) stages. Cells shown in **D-F** carry a mutation in the *dis1* gene and were blocked in metaphase by shift to 19°C for 6h prior to fixation. **(G)** The cell length of septating live cells of the indicated genotypes is shown in μm.

In order to characterize the dynamics of Clp1p-GFP-NES and Clp1p-GFP-NLS, localization, we analyzed the GFP signals in synchronized cells. Cultures were synchronized in G2 phase through inactivation of the temperature sensitive *cdc25-22* allele by shift to 36°C for 4 hours. *clp1-GFP-NES cdc25-22* and *clp1-GFP-NLS cdc25-22* cultures were then released from the block by return to the permissive temperature of 25°C and the localization pattern analyzed in 10 minute intervals in fixed cells. Clp1p-GFP-NES localized at the SPB in cells arrested in G2-phase (Figure 4-2 B (1)). 20 minutes after release of the culture from the G2 arrest, Clp1p-GFP-NES left the SPB and appeared in punctate dots at the medial cell cortex proximal to the nucleus, where the actin ring forms. As cells progressed, the punctate Clp1p-GFP-NES signal assembled into a concise ring (Figure 4-2 B (2)). This concise signal appeared around anaphase, as previously reported for other ring components (Figure 4-2 A, B (2)) (Wu et al., 2003a). After completion of ring constriction, Clp1p disappeared from the cell middle and reappeared at the SPBs (Figure 4-2 A, B (3)). Clp1p-GFP-NLS was exclusively nucleolar in cells arrested in G2 phase (Figure 4-2 D (1)) and appeared at additional nuclear spots, presumably the kinetochores, shortly after release into mitosis. This coincided with release of Clp1p-GFP-NLS throughout the nucleus in early mitosis (Figure 4-2 D (2)). As cells entered anaphase, Clp1p-GFP-NLS localized to the mitotic spindle until spindle break down (Figure 4-2 D (3)). Thereafter, Clp1p-GFP-NLS relocalized to the nucleolus (Figure 4-2 D (4)).

In *S. pombe*, kinetochores are positioned in close proximity to the SPBs in interphase and anaphase (Funabiki et al., 1993). Therefore, SPB and kinetochore localization cannot be distinguished easily when proteins such as Clp1p, localize to both

sites in interphase and at the end of anaphase (Ding et al., 1997). However, the kinetochores are within the nucleus and the SPBs outside the nucleus. Therefore, the nuclear *clp1-GFP-NLS* and cytoplasmic *clp1-GFP-NES* alleles allowed us to differentiate between Clp1p SPB and kinetochore localization. Since Clp1p-GFP-NLS did not localize at a spot on the nuclear periphery during interphase, we concluded that it is absent from the kinetochores during interphase. Further, Clp1p-NLS-GFP did not localize to the ends of the mitotic spindle in anaphase. Therefor, Clp1p localization to the kinetochores is restricted to early mitosis.

Conversely, Clp1p-GFP-NES did not localize in spots at the spindle ends during mitosis, showing that Clp1p does not localize to the cytoplasmic side of the SPB during anaphase. This localization is very similar to human CDC14A, which localizes to the centrosome, the functional homolog of the SPB, only during interphase.

Figure 4-2

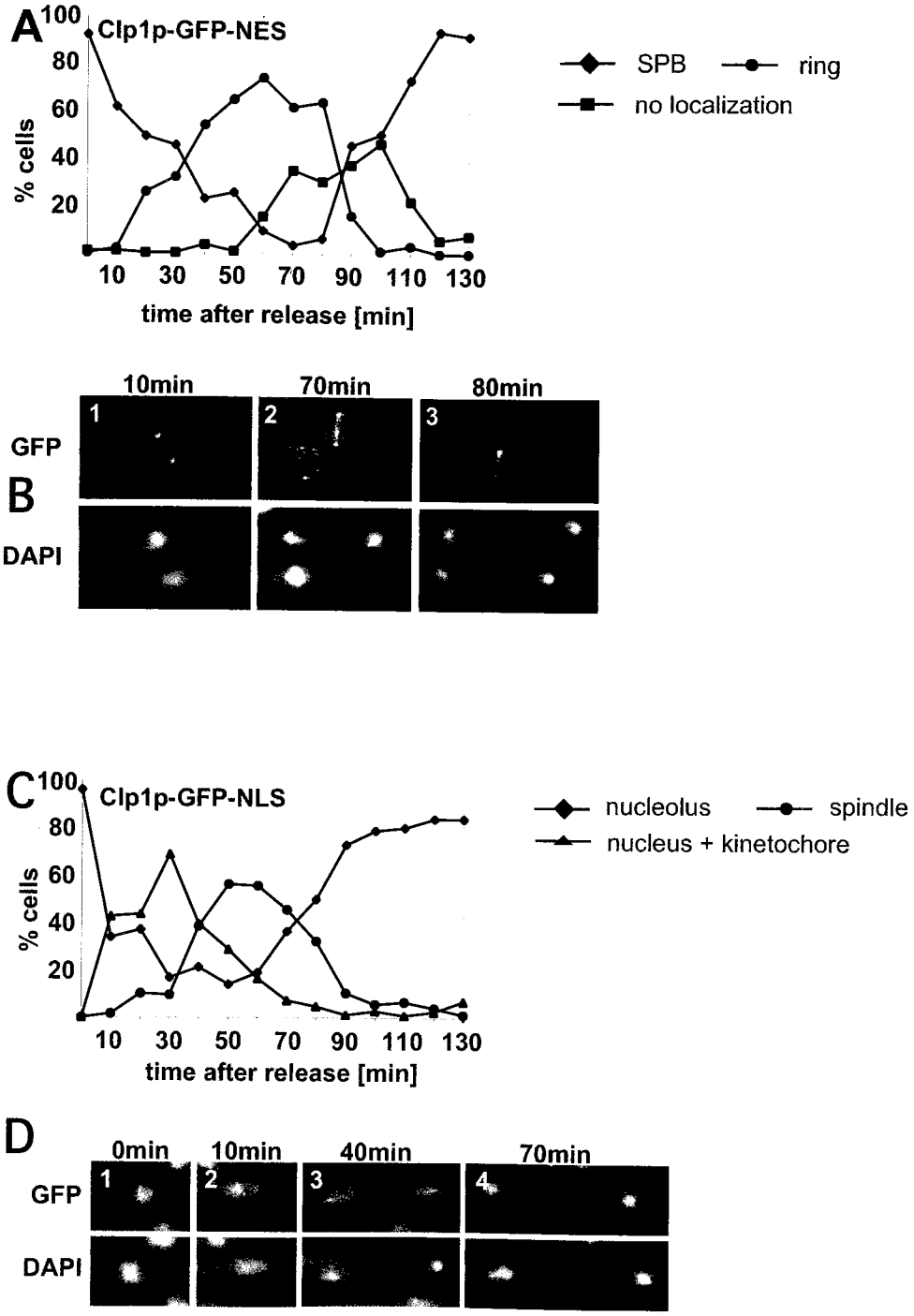

A
Clp1p-GFP-NES
% cells
100
80
60
40
20
10 30 50 70 90 110 130
time after release [min]
SPB
ring
no localization
10min
70min
80min
GFP
B
DAPI
C
Clp1p-GFP-NLS
% cells
100
80
60
40
20
10 30 50 70 90 110 130
time after release [min]
nucleolus
spindle
nucleus + kinetochore
D
0min
10min
40min
70min
GFP
DAPI

Figure 2: Clp1p-GFP-NES and Clp1p-GFP-NLS localization throughout the cell cycle

cdc25-22 clp1-GFP-NES **(A, B)** and *cdc25-22 clp1-GFP-NLS* **(C, D)** cells were shifted to 36°C for 4h and released into mitosis by return to 25°C. Samples were fixed every 10min in MeOH and GFP localization analyzed. **B** and **D** show examples of cells scored in **A** and **C** respectively at the indicated time points.

Absence of Clp1p from the nucleus causes defects in chromosome segregation

Clp1p is required for faithful chromosome segregation and localizes to the kinetochores and the mitotic spindle during mitosis (Trautmann et al., 2004). Clp1p-GFP-NES localizes to neither the kinetochores, nor to the mitotic spindle (Figures 4-1B, E and 4-2 B(2)). This allowed us to test whether Clp1p localization to the nucleus is required for its function in chromosome segregation.

*clp1*Δ has negative genetic interactions with mutations in the *dis1* gene, which is required for chromosome segregation (Trautmann et al., 2004). We therefore constructed double mutants between *clp1-GFP-NES*, *clp1-GFP-NLS*, and the cold sensitive *dis1* mutant and compared their growth rate at different temperatures. *dis1* mutants grow at a permissive temperature of 30°C as well as at a semipermissive temperature of 25°C. *dis1 clp1*Δ double mutants grow very slowly at 30°C, and are dead at 25°C (Figure 4-3A) (Trautmann et al., 2004). While the *clp1-GFP-NLS* mutant as well as the control *clp1-GFP* did not display any negative genetic interactions with *dis1*, *dis1 clp1-GFP-NES* double mutants grew to wild type levels at 30°C, but showed little growth at 25°C, indicating that Clp1p is required in the nucleus for its function in chromosome segregation (Figure 4-3A).

Clp1p was previously shown to function in the regulation of the chromosomal passenger proteins. Double mutants between *clp1*Δ and *cut17-275*, a temperature sensitive allele of *S. pombe* survivin *cut17/bir1* (Morishita et al., 2001), were not viable (Trautmann et al., 2004). Consistent with the genetic interaction with the *dis1* mutant, double mutants between *cut17-275* and *clp1-GFP-NES* were dead, whereas double

mutants between *cut17-275* and *clp1-GFP* or *clp1-GFP-NLS* were viable (data not shown).

To analyze whether the withdrawal of Clp1p from the nucleus resulted in genetic instability, we determined the chromosome loss rate of *clp1-GFP-NES* through a minichromosome loss assay (Appendix: Material and Methods of Chapter IV). *clp1-GFP-NES* showed a 7.8 fold higher rate of chromosome loss than wild type. While this is a moderate chromosome loss rate compared to *clp1*Δ (28 fold) (Trautmann et al., 2004) (Table 4-I), it is noteworthy that *clp1-GFP-NES* shifts the equilibrium of Clp1p to the cytoplasm, but does not exclude Clp1p from the nucleus. *clp1-GFP-NLS* lost the minichromosome at a rate only slightly (2.9 fold) higher than wild type. Indeed, when nuclear export signal dependent export was inhibited with the help of a cold sensitive exportin mutant *crm1-AC1*, Clp1p-GFP-NES accumulated in the nucleus (Trautmann and McCollum, 2005).

Combined, these data show that Clp1p function in chromosome segregation during mitosis is dependent on its presence in the nucleus, where it localizes to the kinetochores and the spindle during mitosis. It was not possible to distinguish whether the effect on chromosome segregation was due to Clp1p absence from the spindle or the kinetochores. *clp1*Δ mutants, however, do not show significant defects in spindle stability or chromosomal passenger localization to the spindle mid zone (Trautmann et al., 2004), supporting the view that the chromosome segregation defects in *clp1-GFP-NES* are not due to loss of Clp1p from the spindle but rather the kinetochores.

Figure 4-3

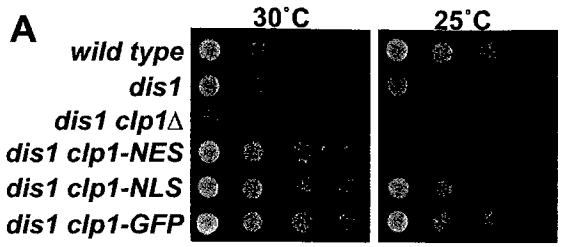


Table I: minichromosome loss rate of *clp1* alleles

	chromosome loss	fold increase
wild type	4.23×10^{-4} (4659)*	
clp1Δ	1.12×10^{-2} (1811)*	28.2
clp1-NES-GFP	3.33×10^{-3} (3308)*	7.87
clp1-NLS-GFP	1.24×10^{-3} (4416)*	2.9

* number of colonies screened

Figure 4-3: Clp1p localization and function

(A) *dis1* mutants in different *clp1* mutant backgrounds were spotted on YE plates in serial dillutions and kept at 25˚C or 30˚C.

Clp1p functions in the cytoplasm to maintain the cytokinesis checkpoint

Clp1p is released from the nucleolus and localizes throughout the cytoplasm and nucleus in binucleate cells, which delay nuclear division in the event of cytokinesis defects (Cueille et al., 2001; Trautmann et al., 2001). Clp1p functions as part of the cytokinesis checkpoint which ensures this delay when the contractile ring is perturbed (Cueille et al., 2001; Mishra et al., 2004; Trautmann et al., 2001). This suggests that Clp1p needs to be released from the nucleolus to maintain the cytokinesis checkpoint. Whether Clp1p functions in the nucleus or the cytoplasm and whether Clp1p release from the nucleolus is indeed required for the cytokinesis checkpoint, remained to be shown. The cytoplasmic and nuclear alleles of Clp1p allowed us to address those questions.

We first activated the cytokinesis checkpoint by treatment with low concentrations of the actin depolymerizing drug Latrunculin B (Lat B, Molecular Probes, Portland/OR). As with low doses of Latrunculin A, treatment with low concentrations of Lat B slows cytokinesis, causing cells to delay in a binucleate stage with an activated cytokinesis checkpoint (Mishra et al., 2004). We compared the activity of the cytokinesis checkpoint in wild type (*clp1-GFP*), *clp1*Δ, *clp1-GFP-NLS* and *clp1-GFP-NES* genetic background by the accumulation of nuclei per cell over time. As previously shown, the cytokinesis checkpoint was activated in *clp1-GFP* background and deletion of *clp1* resulted in loss of the checkpoint and rapid accumulation of nuclei (Figure 4-3 B (1)) (Mishra et al., 2004). While cytoplasmic targeting of Clp1p in *clp1-GFP-NES* cells did not affect cytokinesis checkpoint maintenance, *clp1-GFP-NLS* cells were unable to maintain the cytokinesis checkpoint and accumulated nuclei at a rate similar to *clp1*Δ mutants (Figure 4-3 B (1)).

To rule out that this result was specific to cytokinesis checkpoint activation by Lat B treatment, we triggered the cytokinesis checkpoint through a temperature sensitive mutation in profilin *cdc3*, which disrupts formation of the contractile ring (Trautmann et al., 2001). This experiment yielded similar results to the Lat B experiment, with *cdc3-124 clp1-GFP-NLS* cells accumulating nuclei earlier than *cdc3-124 clp1-GFP* or *cdc3-124 clp1-GFP-NES* cells at *cdc3-124* restrictive temperature 36°C (Figure 4-3 B (2)). Therefore, Clp1p functions in the cytoplasm to maintain the cytokinesis checkpoint.

We also assessed whether the cytokinesis checkpoint is dependent on Clp1p phosphatase activity. The cytokinesis checkpoint was activated either by Lat B treatment or with the *cdc3-124* allele in the Clp1p phosphatase inactive mutant *clp1C286S-GFP*. In Lat B treated *clp1C286S-GFP* cells as well as in *cdc3-124 clp1C286S-GFP* mutants, nuclei accumulated at a rate similar to the *clp1*Δ background, showing that Clp1p phosphatase activity is required for maintenance of the cytokinesis checkpoint (Figure 4-3 B (1), (2)).

Cytoplasmic Clp1p does not rescue the cytokinesis checkpoint in SIN mutants

The cytokinesis checkpoint is not only regulated through Clp1p. The septation initiation network (SIN) is a signaling cascade essential for cytokinesis. The SIN acts in late mitosis to promote contractile ring stability, ring constriction and septum formation, and is also required for the cytokinesis checkpoint. As shown in Chapter II, the SIN and Clp1p function together in cytokinesis checkpoint maintenance, with the SIN keeping Clp1p out of the nucleolus and Clp1p promoting SIN activity (Cueille et al., 2001; Trautmann et al., 2001). Therefore, maintenance of Clp1p in the cytoplasm might rescue

the cytokinesis checkpoint defect of SIN mutants. We tested for presence of the cytokinesis checkpoint by comparison of nuclei accumulation per cell in the SIN mutant *sid2-250*, expressing either wild type *clp1-GFP* or *clp1-GFP-NES*. *sid2-250 clp1-GFP* cells showed no delay in nuclear division following cytokinesis failure, even though the ring disassembles in anaphase in *sid2*-250 mutants (Figure 4-3 B (3)) (Mishra et al., 2004; Wu et al., 2003a). *sid2-250 clp1-GFP-NES* cells did not differ in the timing of mitotic entry and accumulated nuclei at a similar rate (Figure 4-3 B (3)). Thus, Clp1p-GFP-NES cannot rescue the cytokinesis checkpoint in SIN mutants. This indicates that Clp1p acts on the cytokinesis checkpoint upstream of the SIN. This was previously suggested based on the fact that deletion of *clp1* does not increase the speed of the nuclear cycle when the SIN is hyperactivated due to the *cdc16-116* mutation (Mishra et al., 2004).

Figure 4-3

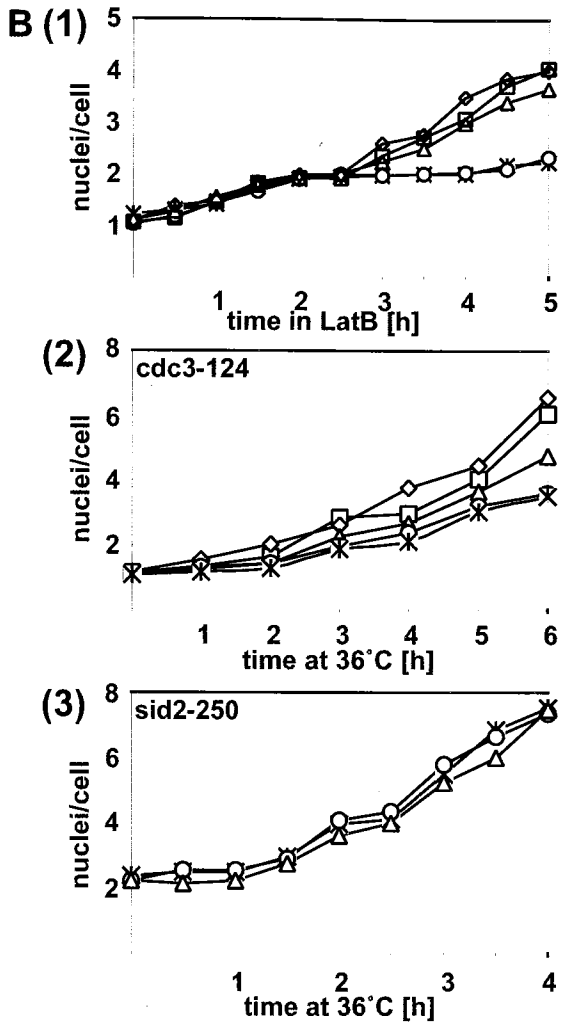

B (1)
nuclei/cell
time in LatB [h]
(2)
cdc3-124
nuclei/cell
time at 36˚C [h]
(3)
sid2-250
nuclei/cell
time at 36˚C [h]

Figure 4-3: Clp1p localization and function

(B) The number of nuclei per cell in wild type *clp1-GFP* (asterisk), *clp1Δ* (square), *clp1-NES* (circle), *clp1-GFP-NLS* (triangle) and *clp1C286S-GFP* (diamond) were scored over time. Culture were either treated with 3μM Latrunculin B (1), or shifted to 36°C in a *cdc3-124* (2) or in a *sid2-250* mutant background (3).

Cytoplasmic localization of Clp1p by the SIN is maintained by mechanisms other than loss of nucleolar binding affinity

To examine whether the cytokinesis checkpoint defect in *clp1-GFP-NLS* cells could be due to premature return of Clp1p to not just the nucleus but the possibly sequestering and inhibiting nucleolus, we analyzed its localization in synchronized cells after cytokinesis checkpoint activation. *cdc3-124 clp1-GFP* or *cdc3-124 clp1-GFP-NLS* cultures were synchronized as small G2 cells by centrifugal elutriation, shifted to restrictive temperature 36°C to trigger the cytokinesis checkpoint. Cells were then analyzed every 10min for GFP localization. *cdc3-124 clp1-GFP* cells entered mitosis and delayed as binucleate cells. Clp1p-GFP was released from the nucleolus upon mitotic entry and maintained in the cytoplasm for as long as the cytokinesis checkpoint was active as judged by the percentage of binucleate cells (Figure 4-4 A, B). Similarly, Clp1p-GFP-NLS was released from the nucleolus to the nucleus in early mitosis. However, Clp1p-GFP-NLS returned to the nucleolus shortly after anaphase, even though cells were binucleate (Figure 4-4 A, C). Clp1p-GFP-NLS relocalization to the nucleolus in *cdc3-124* cells was similar to Clp1p-GFP relocalization when SIN was inactivated in *cdc3-124* cells (Figure 4-4 D). This implies that the SIN regulates cytoplasmic retention during the cytokinesis checkpoint as previously suggested (Cueille et al., 2001; Trautmann et al., 2001).

Figure 4-4

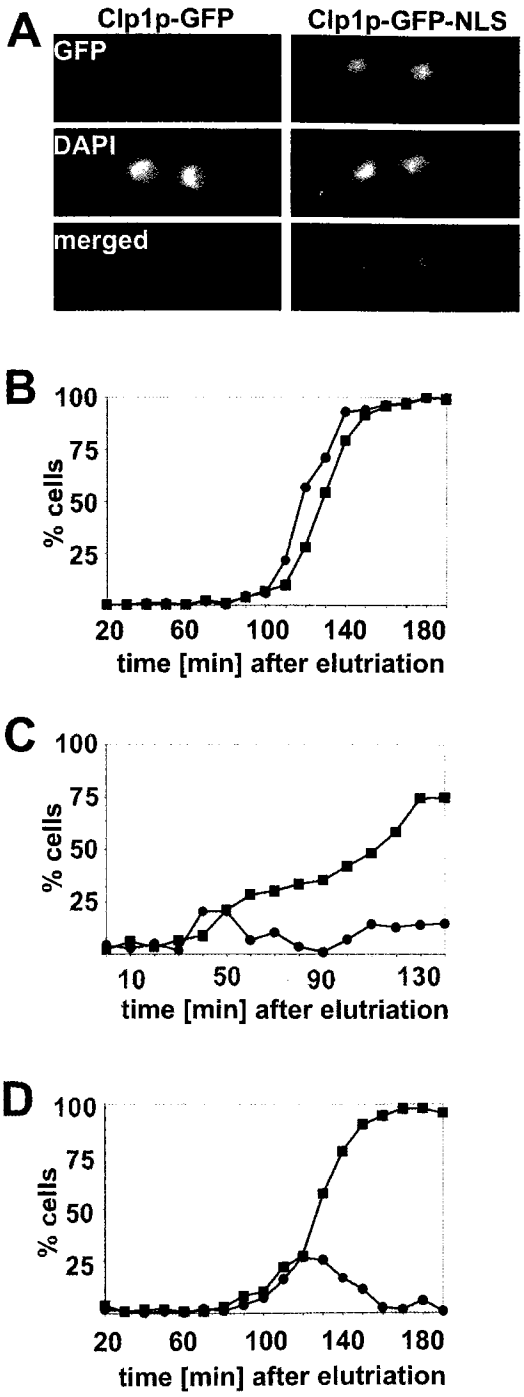

A
Clp1p-GFP
Clp1p-GFP-NLS
GFP
DAPI
merged
B
100
75
50
25
% cells
20
60
100
140
180
time [min] after elutriation
C
100
75
50
25
% cells
10
50
90
130
time [min] after elutriation
D
100
75
50
25
% cells
20
60
100
140
180
time [min] after elutriation

Figure 4-4: Clp1p cytoplasmic retention is not regulated through loss of nucleolar affinity

Clp1p-GFP and Clp1p-GFP-NLS in *cdc3-124*, 80 min after elutriation and shift to 36°C (A). (B-D) Nucleolar release of Clp1p-GFP (circles) and appearance of binucleate cells (squares) in *cdc3-124 clp1-GFP* (B), *cdc3-124 clp1-GFP-NLS* (C) and *sid2-250 cdc3-124 clp1-GFP* cells (D) after elutriation and shift to 36°C is shown.

The SIN could keep Clp1p out of the nucleolus through two different mechanisms. One possibility is that the SIN inhibits the affinity between the nucleolus and Clp1p. Alternatively the SIN could keep Clp1p in the cytoplasm by promoting its nuclear export or inhibiting its nuclear import in the presence of nucleolar affinity, thereby preventing nuclear and nucleolar accumulation. Targeting Clp1p to the nucleus is sufficient to allow nucleolar localization in cells triggered for the cytokinesis checkpoint. Since the SIN is activated during active cytokinesis checkpoint (Trautmann et al., 2001) and Clp1p-GFP-NLS relocalization to the nucleolus is similar to Clp1p-GFP in SIN mutants, the SIN might affect nuclear transport of Clp1p rather than nucleolar affinity for Clp1p. This conclusion is complicated by the fact that lack of Clp1p reduces SIN activity during the cytokinesis checkpoint (Trautmann et al., 2001). Since *clp1-GFP-NLS* cells are defective for the cytokinesis checkpoint, SIN activity might be decreased in *clp1-GFP-NLS* cells, which could potentially affect Clp1p localization through another mechanism.

We therefore sought a situation where SIN activity is independent of Clp1p and wild type Clp1p-GFP is cytoplasmic. Inhibition of the SIN inhibitor Cdc16p hyper activates the SIN (Marks et al., 1992), even in the absence of Clp1p, with some cells septating out of interphase and others completing mitosis and building multiple septa in binucleate cells. SIN activation in interphase cannot drive Clp1p out of the nucleolus. However, cells that proceeded through mitosis before becoming blocked by SIN activation, arrest as binucleate, multiseptated interphase cells and maintain Clp1p out of the nucleolus (Cueille et al., 2001; Minet et al., 1979; Trautmann et al., 2001).

To test whether nucleolar affinity for Clp1p is present when the SIN is hyperactivated, we analyzed Clp1p-GFP-NLS localization in *cdc16-116* mutant cells.

cdc16-116 clp1-GFP or *cdc16-116 clp1-GFP-NLS* cells were shifted to the restrictive temperature for 2 hours. As previously noted Clp1p-GFP was released from the nucleolus in 65% of binucleate multiseptated cells (Trautmann et al., 2001) (Figure 4-4 E, F). In contrast, Clp1p-GFP-NLS was released from the nucleolus in only 5% of the binucleate, multiseptated cells (Figure 4-4 E, F). This result shows that nucleolar affinity for Clp1p exists in the presence of SIN signaling. Therefore, the SIN may keep Clp1p out of the nucleolus by regulating nuclear transport and not by regulating nucleolar affinity for Clp1p.

Interestingly, protein purification of the Clp1p complex by tandem affinity purification and mass-spectrometry identified the 14-3-3 family protein Rad24p as a Clp1p interactor (Choi and McCollum, unpublished results). Rad24p regulates nuclear export of its interactor Cdc25p and might similarly regulate Clp1p nuclear export (Lopez-Girona et al., 1999). In the accompanying study, Mishra et al. independently identify Rad24p as a protein required for the cytokinesis checkpoint and a binding partner of Clp1p. They find that deletion of *rad24* results in nucleolar relocalization of Clp1p when the cytokinesis checkpoint is triggered. These results prompted us to test whether cytoplasmic Clp1p-GFP-NES can rescue the cytokinesis checkpoint defect of *rad24Δ*. *rad24Δ*, *rad24Δ clp1-GFP-NES* mutant and *clp1-GFP* control cells were treated with LatB to activate the cytokinesis checkpoint and monitored for the accumulation of nuclei. Wild type *clp1-GFP* cells delayed as binucleates while the *rad24Δ* mutant failed in the cytokinesis checkpoint. Targeting Clp1p to the cytoplasm slowed down accumulation of nuclei significantly in *rad24Δ* mutants (Figure 4 G). This shows that Clp1p maintenance

in the cytoplasm and Clp1p-dependent activation of the cytokinesis checkpoint are regulated through the 14-3-3 protein Rad24p.

Figure 4-4

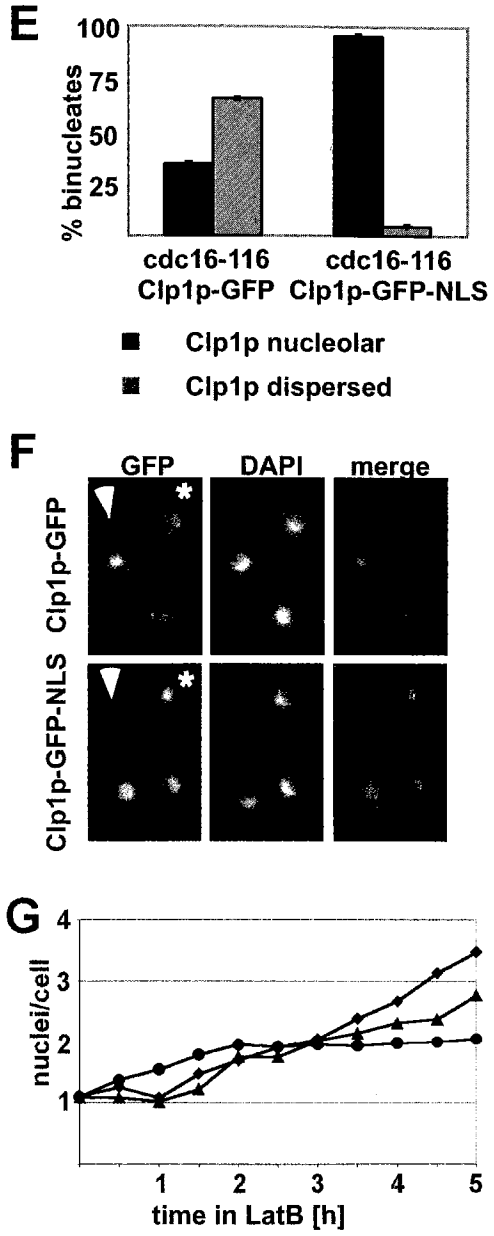

E
100
75
50
25
% binucleates
cdc16-116 Clp1p-GFP
cdc16-116 Clp1p-GFP-NLS
Clp1p nucleolar
Clp1p dispersed
F
GFP
DAPI
merge
Clp1p-GFP
Clp1p-GFP-NLS
G
4
3
2
1
nuclei/cell
1
2
3
4
5
time in LatB [h]

Figure 4-4: Nucleolar affinity of Clp1p is not affected by the SIN and nuclear localization of Clp1p is sufficient for nucleolar localization

(E) Quantification of nucleolar or dispersed localization of Clp1p-GFP and Clp1p-GFP-NLS in binucleate, septated *cdc16-116* mutants after incubation at 36°C for 2h was scored. (F) Examples of cells described in E are shown. Arrowhead indicates a mononucleate cell septated during interphase and binucleate cells with multiple septa are indicated by asterisks. (G) Accumulation of nuclei per cell in wild type *clp1-GFP* (circle), *rad24Δ clp1-GFP* (diamond) and *rad24Δ clp1-GFP-NES* (triangle) after treatment with 3μM LatrunculinB.

Discussion Chapter IV

How Cdc14–like phosphatases are released from the nucleolus and retained in the cytoplasm is an important yet not well understood subject. In *S. cerevisiae*, the FEAR network causes release of Cdc14p from the nucleolus by inhibiting binding of Cdc14p to its nucleolar binding partner Net1p/Cfi1p (Azzam et al., 2004; Stegmeier and Amon, 2004). The mechanism of release of Clp1p from the nucleolus in early mitosis in *S. pombe* is not known. However, the fact that Clp1p-GFP-NLS is still released from the nucleolus in early mitosis, suggests that loss of nucleolar affinity and not nuclear shuttling may drive its release. Once Clp1p and Cdc14p have been released from the nucleolus, the homologous networks, the SIN in *S. pombe* and the MEN in *S. cerevisiae*, are required to inhibit return of the phosphatases to the nucleolus and to maintain them in the cytoplasm (Bardin et al., 2000; Cueille et al., 2001; Trautmann et al., 2001). *S. pombe* Clp1p needs to be kept from nucleolar relocalization in order to fulfill its function in the cytokinesis checkpoint and premature nucleolar localization of *S. cerevisiae* Cdc14p correlates with defects in mitotic exit (Shou et al., 1999; Visintin et al., 1999). The main hypothesis proposed for how the MEN in *S. cerevisiae* promotes Cdc14p maintenance in the cytoplasm, is loss of a nucleolar affinity (Visintin and Amon, 2001). More specifically, the nucleolar protein Net1p is thought to loose its ability to bind Cdc14p in the presence of MEN activity. Here we show that Clp1p nucleolar affinity is not affected by SIN activity. Further, nuclear localization of Clp1p is sufficient for nucleolar relocalization even in the presence of SIN signaling, suggesting that Clp1p retention in the cytoplasm is regulated by nuclear shuttling, possibly through the 14-3-3 protein Rad24p. The FEAR network releases Cdc14p into the nucleus, but not the

cytoplasm (Stegmeier et al., 2002). The MEN might therefore be required for nuclear export of Cdc14p into the cytoplasm and MEN inactivation would lead to Cdc14p nuclear accumulation. Interestingly, mutations in nuclear cytoplasmic transporters have been identified as suppressors of mitotic exit network mutants in *S. cerevisiae* (Asakawa and Toh, 2002; Shou and Deshaies, 2002). Mutations in these karyopherins might uncouple MEN signaling from Cdc14p localization. How in detail the MEN and the SIN regulate Cdc14-like phosphatase localization remains elusive. Interestingly, 14-3-3 proteins like Rad24p, bind phosphoproteins. Therefore, phosphorylation of Clp1p/Cdc14p by SIN/MEN might activate 14-3-3-protein-dependent nuclear export of Clp1p/Cdc14p. In human cells, removal of the nuclear export signal from hCDC14A results in mislocalization from the centrosomes to the nucleoli during interphase (Mailand et al., 2002) and thus, Cdc14-like phosphatase regulation through nuclear shuttling might not be confined to lower eukaryotes.

Appendix A:

Cell cycle: New functions for cdc14-family phosphatases

(Current Biology, Vol 12 R733-735, October 29, 2002)

Cell Cycle: New functions for Cdc14-family phosphatases

The Cdc14 phosphatase was first identified as a gene required for mitotic exit in the budding yeast *S.cerevisiae*. Although Cdc14 homologs exist throughout the eukaryotic kingdom, it was unclear whether their function would also be conserved. In the last year, analysis in the fission yeast *S. pombe*, humans and now *C.elegans* suggests numerous other functions for this family of proteins.

While activation of cyclin dependent kinases (CDKs) drives cells into mitosis, mitotic exit depends on inhibition of CDK activity mainly through degradation of mitotic cyclins and accumulation of CDK inhibitors (Morgan, 1999; Ohi and Gould, 1999). Additionally, it is presumed that phosphatases must reverse CDK phosphorylation by dephosphorylation. The budding yeast Cdc14 phosphatase is a good candidate for this function. In budding yeast, the Cdc14 phosphatase is essential for antagonizing CDK activity in late mitosis, allowing cells to exit mitosis (Bardin and Amon, 2001; McCollum and Gould, 2001). The fission yeast homolog Clp1p/Flp1p also seems to function to antagonize CDK activity albeit in a different manner and for different purposes (Cueille et al., 2001; Trautmann et al., 2001). Also consistent with this model, Cdc14 in both *S.cerevisiae* and humans specifically dephosphorylates Cdk substrates in vitro (Jaspersen and Morgan, 2000; Kaiser et al., 2002; Li et al., 2000; Visintin et al., 1998; Xu et al., 2000). Despite these biochemical similarities, the function of Cdc14-like proteins seems to vary dramatically between species. In *S.cerevisiae* Cdc14 is absolutely required for

mitotic exit and plays a non-essential function in cytokinesis (Frenz et al., 2000; Luca et al., 2001; Menssen et al., 2001; Yoshida and Toh-e, 2001). In contrast, in *S. pombe*, Clp1/Flp1p is not required for mitotic exit but functions mainly in regulating cytokinesis and affects the timing of entry into mitosis (Cueille et al., 2001; Trautmann et al., 2001). Given the significant differences in Cdc14 function between the two yeast species, it has been of considerable interest whether Cdc14 homologs in animal cells function in mitotic exit, in cytokinesis or have other functions. Examination of Cdc14 loss of function in human cells has not definitively ascribed functions to the two human Cdc14-like proteins Cdc14A and Cdc14B (Kaiser et al., 2002; Mailand et al., 2002). However depletion of hCdc14A by RNAi was reported to cause a variety of mitotic defects, including cytokinesis failure (Mailand et al., 2002).

Now, a new study by Grueneberg et al. (Grueneberg et al., 2002) shows that in *C.elegans* embryos CeCdc14 is required for cytokinesis and not mitotic exit. Embryos depleted for CeCdc14 by RNAi do not undergo cytokinesis resulting in one multinucleate cell. However, mitotic exit and progression through the nuclear cycle does not appear to be perturbed. They find that CeCdc14 localizes to the spindle midzone, a structure known to be essential for cytokinesis (Grueneberg et al., 2002) and that depletion of Cdc14 results in a defective spindle midzone.

These findings nicely place CeCdc14 in a known framework of genes involved in cytokinesis. The spindle midzone is a microtubular array resulting from the former mitotic spindle microtubules that remains in the cell middle after chromosome segregation. Two complexes, which localize to the spindle midzone are crucial for the formation of the array. One complex is called centralspindilin and is composed of a

conserved kinesin (*C.elegans* ZEN-4, mammals CHO1/MKLP1, *D.melanogaster* Pavarotti) and a GTPase of the Rho family (CYK-4 in *C.elegans*). The other complex termed ABI, consists of Aurora kinase B, INCENP and survivin. In *C.elegans*, aurora kinase B is essential for the maintenance of the ZEN-4/Cyk-4 complex at the spindle midzone and thus for cytokinesis (Glotzer, 2001). Interestingly, CeCdc14 localization depends on the kinesin ZEN-4 and vice versa. In contrast, the aurora kinase B AIR-2 is able to localize to the residual spindle midzone present in CeCdc14 depleted embryos (Grueneberg et al., 2002). This is consistent with previous studies placing Air-2 upstream of ZEN-4 (Glotzer, 2001). Since work in other systems suggests that Cdc14 may function to dephosphorylate CDK substrates, it seems likely that CeCdc14 may function to dephosphorylate CDK substrates at the spindle midzone. Consistent with such a role is a study showing that loss of mitotic cyclin B, and thus CDK activity, in anaphase is essential for spindle midzone formation (Severson et al., 2000). It is interesting to note that *S. pombe* Clp1p/Flp1p also localizes to the spindle midzone in late anaphase suggesting this may be a conserved function (see below, and Figure 1).

A key mechanism by which Cdc14 is regulated in both budding and fission yeast is by regulated shuttling between the nucleolus and the cytoplasm. In interphase, the homologs are in the nucleolus where they are thought to be sequestered and inactive. The proteins are then released from the nucleolus in mitosis, Clp1p/Flp1p in prophase and Cdc14 in anaphase. When released, both proteins are free to carry out their respective functions and localize to the spindle pole bodies, and the *S. pombe* protein also localizes to the actomyosin ring and the central spindle. As in yeast, both human proteins, Cdc14A and B, localize at least partly to the nucleolus. HCdc14A localizes to the centrosome in

interphase, while hCdc14B is in the nucleolus. Both distribute in the cytoplasm during mitosis. Interestingly, both hCdc14A and B contain a nuclear export signal (NES), which is absent in yeasts (Mailand et al., 2002). Deletion of the NES in hCdc14A causes hCdc14A to accumulate in the nucleolus, while hCdc14B presumably stays in the nucleolus because affinity for the nucleolus opposes nuclear export (Kaiser et al., 2002; Mailand et al., 2002). Somewhat surprisingly, given the work in yeast and humans, CeCdc14 was not observed in the nucleolus or on the centrosome. This may be because CeCdc14 carries the same nuclear export signal, discovered in hCdc14A, which may cause it to only transiently localize to the nucleolus, like hCdc14A. Alternatively, the protein may not be sequestered in the nucleolus in the early embryo, which largely lacks G1 and G2 phases.

This leaves us with the question: What is the conserved function of Cdc14? At first glance, all homologs seem to function quite differently and in different cell cycle stages. This might be explained by the different challenges confronting each organism in addition to differences between developmental stages. A common process that is affected by Cdc14 in all investigated organisms is cytokinesis. *S. pombe* Clp1p/Flp1p and *C.elegans* CeCdc14 both function in cytokinesis. In addition the experiments on the human Cdc14 counterparts indicate a possible role for hCdc14A in cytokinesis, and recent studies in *S.cerevisiae* show that Cdc14 seems to function in cytokinesis as well as in mitotic exit (Frenz et al., 2000; Luca et al., 2001; Menssen et al., 2001; Yoshida and Toh-e, 2001). The absolute requirement for Cdc14 in mitotic exit in budding yeast may reflect the peculiar challenge budding yeast face to ensure that the spindle has extended

through the narrow bud neck before exiting mitosis (McCollum and Gould, 2001). Thus, regulating cytokinesis may be the one conserved function of Cdc14 phosphatases.

It remains possible that Cdc14 phosphatases may function in each species to inhibit CDK activity, and to regulate different cell cycle transitions, since CDK activity is important for many cell cycle processes. The studies from budding and fission yeast, as well as human cells, suggest that Cdc14-like proteins may antagonize CDK activity. Even in worms, where no evidence was reported for this, it remains a distinct possibility, since the *C.elegans* homolog has only been investigated in early embryos, which regulate their G1/S and G2/M transitions differently from somatic cells. Thus, its behavior and functions may be different at later stages of development. For future studies it will be important to analyze Cdc14 in more developmental stages as well as through genetic analysis in other eukaryotes, such as flies and mammals. Finding proteins involved in the regulation of Cdc14 homologs as well as the relevant substrates will surely bring more light into the gray box around Cdc14.

Figure A-1

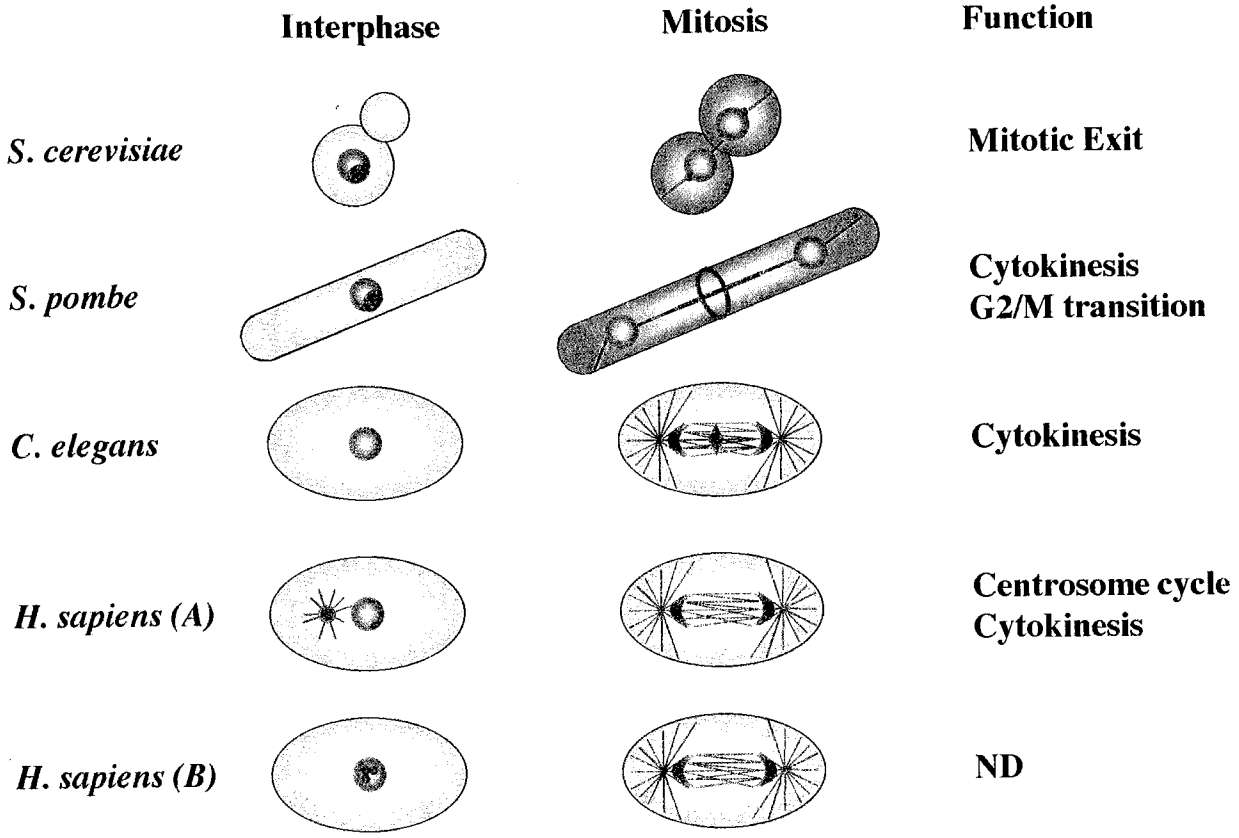

Interphase
Mitosis
Function
S. cerevisiae
Mitotic Exit
S. pombe
Cytokinesis
G2/M transition
C. elegans
Cytokinesis
H. sapiens (A)
Centrosome cycle
Cytokinesis
H. sapiens (B)
ND

Figure A-1. Comparison of Cdc14 orthologs.

Localization of Cdc14 orthologs during interphase and late mitosis is shown along with possible functions in each organism. (A) and (B) indicate the two *H. sapiens* isoforms Cdc14A and Cdc14B respectively. Nuclei (blue), microtubules (red), and Cdc14 (green) are shown.

Appendix B

Materials and Methods

Materials and Methods Chapter II

To delete *clp1*$^{+}$, 94% of the coding region was deleted using the *ura4*$^{+}$ gene as described (Bähler et al., 1998b). Fluorescence microscopy was performed as previously described (Balasubramanian et al., 1997). Antibody staining for Sad1p was done as previously described (Hagan and Yanagida, 1995). Propidium iodide staining was done as described (Drummond and Cross, 2000). Endogenous Clp1p was tagged with 13myc, GFP, and protein A as described previously (Bähler et al., 1998b; Knop et al., 1999).

Materials and Methods Chapter III

Strains and culture

Strains used in this study are listed in Supplemental Table I. Standard fission yeast techniques and media were employed (Moreno et al., 1991). For growth tests 5μl of 0.1 OD^{595} cultures and 1:10 dilutions were spotted onto plates. The entire coding region of the phosphatase inactive allele *clp1-C286S* was cloned into the pJK210 vector between the Sal1 and Xho1 sites and tagged at its C-terminus by inserting GFP at the XhoI site. The start codon of *clp1-C286S* was replaced with a stop codon. The resulting plasmid was linearized with SnaBI and integrated into the genome.

Microscopy

Unless otherwise noted, cells were fixed in MeOH as previously described (Balasubramanian et al., 1997). Indirect immunoflourescence was performed as previously described (Balasubramanian et al., 1997; Hagan and Hyams, 1988) with the following modifications. Cells were fixed for 30min in 4% Paraformaldehyde (Electron Microscopy Science), and treated according to (Balasubramanian et al., 1997) with PEM as buffer instead of PBS (Hagan and Hyams, 1988). Monoclonal mouse αHA (HA 11 MMS-101P,COVANCE, dil. 1:1000), and rabbit αGFP serum (A-6455, Molecular Probes, dil. 1:1000) were used as primary antibodies and detected by Alexa Flour 594 goatαmouse and 488 goatαrabbit 2° antibodies (Molecular Probes, dil. 1:300). Centromere I GFP was imaged by Z-series combined with dic-imaging on live cells. Centromere II-GFP was captured in methanol fixed cells. All images on fixed cells were taken with a 100X objective. Time-lapse movies of Clp1p-GFP and Sid4p-GFP were produced from frames taken once each minute with a 50X objective. For time-lapse movies of the segregating centromere II, cells were blocked at restrictive temperature 19°C for 6h to accumulate in prometaphase prior to filming and transferred onto a slide heated to 30°C on the microscope stage with an objective heater (Bioptechs objective controller). Z-series were taken every 30 seconds with a 100X objective and the projections combined into time-lapse movies.

Immunoprecipitation

Immunoprecipitation was done as described (Sparks et al., 1999). Mouse monoclonal αGFP (2μl per 1ml lysate, A-11120, Molecular Probes) antibody was used for immuno-precipitation and rabbit αGFP serum (dil. 1:1000, A-6455, Molecular Probes) and αmyc

mouse monoclonal antibody (dil. 1:1000, clone 9E10) were used for western blotting. For the immunoprecipitations of Ark1p-GFP, crude cell lysates were cleared by centrifugation at 3000g in the presence of phosphatase inhibitors (1mM $NaVO_4$ and 5mM NaF).

Minichromosome loss assay

Minichromosome loss assays were done as described (Allshire et al., 1995; Fleig et al., 1996). Briefly, loss of the nonessential minichromosome causes cells to accumulate a red pigment. The rate of chromosome loss can be determined by counting the frequency of half sectored colonies, which result from mini-chromosome loss in the first division. Here, the rate of chromosome loss equals the number of half red sectored colonies divided by the total number of colonies minus red colonies, which had lost the minichromosome prior to the assay.

Materials and Methods Chapter IV

Strains and culture

Strains used in this study are listed in Supplemental Table I. Standard fission yeast techniques and media were employed (Moreno et al., 1991). Elutriation was performed as in (Guertin et al., 2002b). For growth tests 5μl of 0.1 OD^{595} cultures and 1:10 dilutions were spotted onto plates. For *clp1* tagging, the entire *clp1* coding region was cloned into the pJK210 integrating vector between the Sal1 and Xho1 sites and tagged at its C-terminus by inserting GFP, GFP-NES or GFP-NLS at the XhoI site. The start codon of

clp1 was replaced with a stop codon. The resulting plasmid was linearized with SnaBI within *clp1* and integrated into the genome, generating a expressed, tagged copy of *clp1* and a silenced *clp1* copy, starting with a stop codon. Peter Pryciak generously provided GFP-NES and GFP-NLS. For LatrunculinB treatment, cells were grown to log-phase and 5ml cultures diluted to 0.15 OD^{595} treated with 3μM LatrunculinB (Molecular Probes, Oregon) final concentration.

Microscopy

Cells were fixed in MeOH as previously described (Balasubramanian et al., 1997). All images on fixed cells were taken with a 100X objective on a Nikon Eclipse 600 microscope and images were obtained and processed with a Hamamatsu ORCA-ER digital camera and IPlab Spectrum (Signal Analytics Corp.).

Minichromosome loss assay

Minichromosome loss assays were done as described (Allshire et al., 1995; Fleig et al., 1996). Briefly, loss of the nonessential minichromosome causes cells to accumulate a red pigment. The rate of chromosome loss can be determined by counting the frequency of half sectored colonies, which result from mini-chromosome loss in the first division. Here, the rate of chromosome loss equals the number of half red sectored colonies divided by the total number of colonies minus red colonies, which had lost the minichromosome prior to the assay.

References

Adams, R. R., Carmena, M., and Earnshaw, W. C. (2001). Chromosomal passengers and the (aurora) ABCs of mitosis. Trends Cell Biol *11*, 49-54.

Alexandru, G., Zachariae, W., Schleiffer, A., and Nasmyth, K. (1999). Sister chromatid separation and chromosome re-duplication are regulated by different mechanisms in response to spindle damage. Embo J *18*, 2707-2721.

Alfa, C. E., Booher, R., Beach, D., and Hyams, J. S. (1989). Fission yeast cyclin: subcellular localisation and cell cycle regulation. J Cell Sci Suppl *12*, 9-19.

Allshire, R. C., Nimmo, E. R., Ekwall, K., Javerzat, J. P., and Cranston, G. (1995). Mutations derepressing silent centromeric domains in fission yeast disrupt chromosome segregation. Genes Dev *9*, 218-233.

Andreassen, P. R., Lohez, O. D., Lacroix, F. B., and Margolis, R. L. (2001). Tetraploid state induces p53-dependent arrest of nontransformed mammalian cells in G1. Mol Biol Cell *12*, 1315-1328.

Angers-Loustau, A., Cote, J. F., Charest, A., Dowbenko, D., Spencer, S., Lasky, L. A., and Tremblay, M. L. (1999). Protein tyrosine phosphatase-PEST regulates focal adhesion disassembly, migration, and cytokinesis in fibroblasts. J Cell Biol *144*, 1019-1031.

Asakawa, K., and Toh, E. A. (2002). A Defect of Kap104 Alleviates the Requirement of Mitotic Exit Network Gene Functions in Saccharomyces cerevisiae. Genetics *162*, 1545-1556.

Audit, M., Barbier, M., Soyer-Gobillard, M. O., Albert, M., Geraud, M. L., Nicolas, G., and Lenaers, G. (1996). Cyclin B (p56cdc13) localization in the yeast Schizosaccharomyces pombe: an ultrastructural and immunocytochemical study. Biol Cell *86*, 1-10.

Azzam, R., Chen, S. L., Shou, W., Mah, A. S., Alexandru, G., Nasmyth, K., Annan, R. S., Carr, S. A., and Deshaies, R. J. (2004). Phosphorylation by cyclin B-Cdk underlies release of mitotic exit activator Cdc14 from the nucleolus. Science *305*, 516-519.

Bähler, J., Steever, A. B., Wheatley, S., Wang, Y., Pringle, J. R., Gould, K. L., and McCollum, D. (1998a). Role of Polo Kinase and Mid1p in Determining the Site of Cell Division in Fission Yeast. J Cell Biol *143*, 1603-1616.

Bähler, J., Wu, J. Q., Longtine, M. S., Shah, N. G., McKenzie, A. r., Steever, A. B., Wach, A., Philippsen, P., and Pringle, J. R. (1998b). Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe. Yeast *14*, 943-951.

Balasubramanian, M. K., Bi, E., and Glotzer, M. (2004). Comparative analysis of cytokinesis in budding yeast, fission yeast and animal cells. Curr Biol *14*, R806-818.

Balasubramanian, M. K., Helfman, D. M., and Hemmingsen, S. M. (1992). A new tropomyosin essential for cytokinesis in the fission yeast S. pombe. Nature *360*, 84-87.

Balasubramanian, M. K., Hirani, B. R., Burke, J. D., and Gould, K. L. (1994). The Schizosaccharomyces pombe cdc3+ gene encodes a profilin essential for cytokinesis. J Cell Biol *125*, 1289-1301.

Balasubramanian, M. K., McCollum, D., Chang, L., Wong, K. C., Naqvi, N. I., He, X., Sazer, S., and Gould, K. L. (1998). Isolation and characterization of new fission yeast cytokinesis mutants. Genetics *149*, 1265-1275.

Balasubramanian, M. K., McCollum, D., and Gould, K. L. (1997). Cytokinesis in fission yeast Schizosaccharomyces pombe. Methods Enzymol *283*, 494-506.

Bardin, A. J., and Amon, A. (2001). Men and sin: what's the difference? Nat Rev Mol Cell Biol *2*, 815-826.

Bardin, A. J., Visintin, R., and Amon, A. (2000). A mechanism for coupling exit from mitosis to partitioning of the nucleus. Cell *102*, 21-31.

Bembenek, J., and Yu, H. (2001). Regulation of the anaphase-promoting complex by the dual specificity phosphatase human Cdc14a. J Biol Chem *276*, 48237-48242.

Bembenek, J., and Yu, H. (2003). Regulation of CDC14: pathways and checkpoints of mitotic exit. Front Biosci *8*, d1275-1287.

Bezanilla, M., Forsburg, S. L., and Pollard, T. D. (1997). Identification of a second myosin-II in Schizosaccharomyces pombe: Myp2p is conditionally required for cytokinesis. Mol Biol Cell *8*, 2693-2705.

Bichsel, S. J., Tamaskovic, R., Stegert, M. R., and Hemmings, B. A. (2004). Mechanism of activation of NDR (nuclear Dbf2-related) protein kinase by the hMOB1 protein. J Biol Chem *279*, 35228-35235.

Biggins, S., and Murray, A. W. (2001). The budding yeast protein kinase Ipl1/Aurora allows the absence of tension to activate the spindle checkpoint. Genes Dev *15*, 3118-3129.

Buck, V., Russell, P., and Millar, J. B. (1995). Identification of a cdk-activating kinase in fission yeast. Embo J *14*, 6173-6183.

Buonomo, S. B., Rabitsch, K. P., Fuchs, J., Gruber, S., Sullivan, M., Uhlmann, F., Petronczki, M., Toth, A., and Nasmyth, K. (2003). Division of the nucleolus and its release of CDC14 during anaphase of meiosis I depends on separase, SPO12, and SLK19. Dev Cell *4*, 727-739.

Buvelot, S., Tatsutani, S. Y., Vermaak, D., and Biggins, S. (2003). The budding yeast Ipl1/Aurora protein kinase regulates mitotic spindle disassembly. J Cell Biol *160*, 329-339.

Canman, J. C., Hoffman, D. B., and Salmon, E. D. (2000). The role of pre- and post-anaphase microtubules in the cytokinesis phase of the cell cycle. Curr Biol *10*, 611-614.

Carmena, M., and Earnshaw, W. C. (2003). The cellular geography of aurora kinases. Nat Rev Mol Cell Biol *4*, 842-854.

Cenamor, R., Jimenez, J., Cid, V. J., Nombela, C., and Sanchez, M. (1999). The budding yeast Cdc15 localizes to the spindle pole body in a cell-cycle-dependent manner. Mol Cell Biol Res Commun *2*, 178-184.

Chan, E. H., Nousiainen, M., Chalamalasetty, R. B., Schafer, A., Nigg, E. A., and Sillje, H. H. (2005). The Ste20-like kinase Mst2 activates the human large tumor suppressor kinase Lats1. Oncogene *24*, 2076-2086.

Chang, F., Drubin, D., and Nurse, P. (1997). cdc12p, a protein required for cytokinesis in fission yeast, is a component of the cell division ring and interacts with profilin. J Cell Biol *137*, 169-182.

Chang, F., and Nurse, P. (1996). How fission yeast find the middle. Cell *84*, 191-194.

Chang, L., and Gould, K. L. (2000). Sid4p is required to localize components of the septation initiation pathway to the spindle pole body in fission yeast. Proc Natl Acad Sci U S A *97*, 5249-5254.

Chang, L., Morrell, J. L., Feoktistova, A., and Gould, K. L. (2001). Study of cyclin proteolysis in anaphase-promoting complex (apc) mutant cells reveals the requirement for

apc function in the final steps of the fission yeast septation initiation network. Mol Cell Biol *21*, 6681-6694.

Cohen-Fix, O., and Koshland, D. (1999). Pds1p of budding yeast has dual roles: inhibition of anaphase initiation and regulation of mitotic exit. Genes Dev *13*, 1950-1959.

Cohen-Fix, O., Peters, J. M., Kirschner, M. W., and Koshland, D. (1996). Anaphase initiation in Saccharomyces cerevisiae is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. Genes Dev *10*, 3081-3093.

Coleman, T. R., and Dunphy, W. G. (1994). Cdc2 regulatory factors. Curr Opin Cell Biol *6*, 877-882.

Creanor, J., and Mitchison, J. M. (1996). The kinetics of the B cyclin p56cdc13 and the phosphatase p80cdc25 during the cell cycle of the fission yeast Schizosaccharomyces pombe. J Cell Sci *109 (Pt 6)*, 1647-1653.

Cueille, N., Salimova, E., Esteban, V., Blanco, M., Moreno, S., Bueno, A., and Simanis, V. (2001). Flp1, a fission yeast orthologue of the s. cerevisiae CDC14 gene, is not required for cyclin degradation or rum1p stabilisation at the end of mitosis. J Cell Sci *114*, 2649-2664.

Cuif, M. H., Possmayer, F., Zander, H., Bordes, N., Jollivet, F., Couedel-Courteille, A., Janoueix-Lerosey, I., Langsley, G., Bornens, M., and Goud, B. (1999). Characterization of GAPCenA, a GTPase activating protein for Rab6, part of which associates with the centrosome. Embo J *18*, 1772-1782.

D'Amours, D., and Amon, A. (2004). At the interface between signaling and executing anaphase--Cdc14 and the FEAR network. Genes Dev *18*, 2581-2595.

D'Amours, D., Stegmeier, F., and Amon, A. (2004). Cdc14 and condensin control the dissolution of cohesin-independent chromosome linkages at repeated DNA. Cell *117*, 455-469.

Damagnez, V., Makela, T. P., and Cottarel, G. (1995). Schizosaccharomyces pombe Mop1-Mcs2 is related to mammalian CAK. Embo J *14*, 6164-6172.

Devroe, E., Erdjument-Bromage, H., Tempst, P., and Silver, P. A. (2004). Human Mob proteins regulate the NDR1 and NDR2 serine-threonine kinases. J Biol Chem *279*, 24444-24451.

Ding, R., West, R. R., Morphew, D. M., Oakley, B. R., and McIntosh, J. R. (1997). The spindle pole body of Schizosaccharomyces pombe enters and leaves the nuclear envelope as the cell cycle proceeds. Mol Biol Cell *8*, 1461-1479.

Drummond, D. R., and Cross, R. A. (2000). Dynamics of interphase microtubules in schizosaccharomyces pombe [In Process Citation]. Curr Biol *10*, 766-775.

Edgington, N. P., and Futcher, B. (2001). Relationship between the function and the location of G1 cyclins in S. cerevisiae. J Cell Sci *114*, 4599-4611.

Emoto, K., and Umeda, M. (2000). An essential role for a membrane lipid in cytokinesis. Regulation of contractile ring disassembly by redistribution of phosphatidylethanolamine. J Cell Biol *149*, 1215-1224.

Eng, K., Naqvi, N. I., Wong, K. C., and Balasubramanian, M. K. (1998). Rng2p, a protein required for cytokinesis in fission yeast, is a component of the actomyosin ring and the spindle pole body. Curr Biol *8*, 611-621.

Esteban, V., Blanco, M., Cueille, N., Simanis, V., Moreno, S., and Bueno, A. (2004). A role for the Cdc14-family phosphatase Flp1p at the end of the cell cycle in controlling the rapid degradation of the mitotic inducer Cdc25p in fission yeast. J Cell Sci *117*, 2461-2468.

Fankhauser, C., Marks, J., Reymond, A., and Simanis, V. (1993). The *S. pombe cdc16* gene is required both for maintenance of p34 Cdc2 kinase activity and regulation of septum formation: a link between mitosis and cytokinesis? EMBO J *12*, 2697-2704.

Fankhauser, C., Reymond, A., Cerutti, L., Utzig, S., Hofmann, K., and Simanis, V. (1995). The S. pombe cdc15 gene is a key element in the reorganization of F-actin at mitosis [published erratum appears in Cell 1997 Jun 27;89(7):1185]. Cell *82*, 435-444.

Fankhauser, C., and Simanis, V. (1993). The Schizosaccharomyces pombe cdc14 gene is required for septum formation and can also inhibit nuclear division. Mol Biol Cell *4*, 531-539.

Fleig, U., Sen-Gupta, M., and Hegemann, J. H. (1996). Fission yeast mal2+ is required for chromosome segregation. Mol Cell Biol *16*, 6169-6177.

Frenz, L. M., Lee, S. E., Fesquet, D., and Johnston, L. H. (2000). The budding yeast Dbf2 protein kinase localises to the centrosome and moves to the bud neck in late mitosis. J Cell Sci *113*, 3399-3408.

Funabiki, H., Hagan, I., Uzawa, S., and Yanagida, M. (1993). Cell cycle-dependent specific positioning and clustering of centromeres and telomeres in fission yeast. J Cell Biol *121*, 961-976.

Furge, K. A., Wong, K., Armstrong, J., Balasubramanian, M., and Albright, C. F. (1998). Byr4 and Cdc16 form a two-component GTPase-activating protein for the Spg1 GTPase that controls septation in fission yeast. Curr Biol *8*, 947-954.

Glotzer, M. (2001). Animal cell cytokinesis. Annu Rev Cell Dev Biol *17*, 351-386.

Goshima, G., Saitoh, S., and Yanagida, M. (1999). Proper metaphase spindle length is determined by centromere proteins Mis12 and Mis6 required for faithful chromosome segregation. Genes Dev *13*, 1664-1677.

Gould, K., and Simanis, V. (1997). The control of septum formation in fission yeast. Genes Dev *11*, 2939-2951.

Gould, K. L., Moreno, S., Owen, D. J., Sazer, S., and Nurse, P. (1991). Phosphorylation at Thr167 is required for *Schizosaccaromyces pombe* p34cdc2 function. EMBO J *10*, 3297-3309.

Gould, K. L., and Nurse, P. (1989). Tyrosine phosphorylation of the fission yeast cdc2+ protein kinase regulates entry into mitosis. Nature *342*, 39-45.

Grallert, A., Krapp, A., Bagley, S., Simanis, V., and Hagan, I. M. (2004). Recruitment of NIMA kinase shows that maturation of the S. pombe spindle-pole body occurs over consecutive cell cycles and reveals a role for NIMA in modulating SIN activity. Genes Dev *18*, 1007-1021.

Gray, C. H., and Barford, D. (2003). Getting in the ring: proline-directed substrate specificity in the cell cycle proteins Cdc14 and CDK2-cyclinA3. Cell Cycle *2*, 500-502.

Gray, C. H., Good, V. M., Tonks, N. K., and Barford, D. (2003). The structure of the cell cycle protein Cdc14 reveals a proline-directed protein phosphatase. Embo J *22*, 3524-3535.

Gromley, A., Jurczyk, A., Sillibourne, J., Halilovic, E., Mogensen, M., Groisman, I., Blomberg, M., and Doxsey, S. (2003). A novel human protein of the maternal centriole is required for the final stages of cytokinesis and entry into S phase. J Cell Biol *161*, 535-545.

Grueneberg, U., Campbell, K., Simpson, C., Grindlay, J., and Schiebel, E. (2000). Nud1p links astral microtubule organization and the control of exit from mitosis. Embo J *19*, 6475-6488.

Grueneberg, U., Glotzer, M., Gartner, A., and Nigg, E. A. (2002). The CeCDC-14 phosphatase is required for cytokinesis in the Caenorhabditis elegans embryo. J Cell Biol *158*, 901-914.

Gruneberg, U., Neef, R., Honda, R., Nigg, E. A., and Barr, F. A. (2004). Relocation of Aurora B from centromeres to the central spindle at the metaphase to anaphase transition requires MKlp2. J Cell Biol *166*, 167-172.

Guertin, D. A., Chang, L., Irshad, F., Gould, K. L., and McCollum, D. (2000). The role of the sid1p kinase and cdc14p in regulating the onset of cytokinesis in fission yeast. Embo J *19*, 1803-1815.

Guertin, D. A., Trautmann, S., and McCollum, D. (2002a). Cytokinesis in eukaryotes. Microbiol Mol Biol Rev *66*, 155-178.

Guertin, D. A., Venkatram, S., Gould, K. L., and McCollum, D. (2002b). Dma1 prevents mitotic exit and cytokinesis by inhibiting the septation initiation network (SIN). Dev Cell *3*, 779-790.

Hagan, I., and Yanagida, M. (1995). The product of the spindle formation gene sad1+ associates with the fission yeast spindle pole body and is essential for viability. J Cell Biol *129*, 1033-1047.

Hagan, I. M., and Hyams, J. S. (1988). The use of cell division cycle mutants to investigate the control of microtubule distribution in the fission yeast Schizosaccharomyces pombe. J Cell Sci *89*, 343-357.

Harvey, K. F., Pfleger, C. M., and Hariharan, I. K. (2003). The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. Cell *114*, 457-467.

He, X., Patterson, T. E., and Sazer, S. (1997). The Schizosaccharomyces pombe spindle checkpoint protein mad2p blocks anaphase and genetically interacts with the anaphase-promoting complex. Proc Natl Acad Sci U S A *94*, 7965-7970.

Hermand, D., Pihlak, A., Westerling, T., Damagnez, V., Vandenhaute, J., Cottarel, G., and Makela, T. P. (1998). Fission yeast Csk1 is a CAK-activating kinase (CAKAK). Embo J *17*, 7230-7238.

Higuchi, T., and Uhlmann, F. (2005). Stabilization of microtubule dynamics at anaphase onset promotes chromosome segregation. Nature *433*, 171-176.

Hinchcliffe, E. H., Li, C., Thompson, E. A., Maller, J. L., and Sluder, G. (1999). Requirement of Cdk2-cyclin E activity for repeated centrosome reproduction in Xenopus egg extracts. Science *283*, 851-854.

Hirano, A., and Kurimura, T. (1974). Virally transformed cells and cytochalasin B. I. The effect of cytochalasin B on cytokinesis, karyokinesis and DNA synthesis in cells. Exp Cell Res *89*, 111-120.

Hiraoka, Y., Toda, T., and Yanagida, M. (1984). The NDA3 gene of fission yeast encodes beta-tubulin: a cold-sensitive nda3 mutation reversibly blocks spindle formation and chromosome movement in mitosis. Cell *39*, 349-358.

Hirota, T., Morisaki, T., Nishiyama, Y., Marumoto, T., Tada, K., Hara, T., Masuko, N., Inagaki, M., Hatakeyama, K., and Saya, H. (2000). Zyxin, a regulator of actin filament assembly, targets the mitotic apparatus by interacting with h-warts/LATS1 tumor suppressor. J Cell Biol *149*, 1073-1086.

Hisaoka, M., Tanaka, A., and Hashimoto, H. (2002). Molecular alterations of h-warts/LATS1 tumor suppressor in human soft tissue sarcoma. Lab Invest *82*, 1427-1435.

Hoffmann, I., Clarke, P. R., Marcote, M. J., Karsenti, E., and Draetta, G. (1993). Phosphorylation and activation of human cdc25-C by cdc2--cyclin B and its involvement in the self-amplification of MPF at mitosis. Embo J *12*, 53-63.

Hou, M. C., Guertin, D. A., and McCollum, D. (2004). Initiation of cytokinesis is controlled through multiple modes of regulation of the Sid2p-Mob1p kinase complex. Mol Cell Biol *24*, 3262-3276.

Hou, M. C., Salek, J., and McCollum, D. (2000). Mob1p interacts with the Sid2p kinase and is required for cytokinesis in fission yeast. Curr Biol *10*, 619-622.

Hoyt, M. A., Trotis, L., and Roberts, B. T. (1991). *S. cerevisiae* genes required for cell cycle arrest in response to loss of microtubule function. Cell *66*, 507-517.

Hu, F., Wang, Y., Liu, D., Li, Y., Qin, J., and Elledge, S. J. (2001). Regulation of the Bub2/Bfa1 GAP complex by Cdc5 and cell cycle checkpoints. Cell *107*, 655-665.

Iida, S., Hirota, T., Morisaki, T., Marumoto, T., Hara, T., Kuninaka, S., Honda, S., Kosai, K., Kawasuji, M., Pallas, D. C., and Saya, H. (2004). Tumor suppressor WARTS ensures

genomic integrity by regulating both mitotic progression and G1 tetraploidy checkpoint function. Oncogene *23*, 5266-5274.

Irniger, S., Piatti, S., Michaelis, C., and Nasmyth, K. (1995). Genes involved in sister chromatid separation are needed for B-type cyclin proteolysis in budding yeast [published erratum appears in Cell 1998 May 1;93(3):487]. Cell *81*, 269-278.

Izumi, T., and Maller, J. L. (1993). Elimination of cdc2 phosphorylation sites in the cdc25 phosphatase blocks initiation of M-phase. Mol Biol Cell *4*, 1337-1350.

Jallepalli, P. V., and Lengauer, C. (2001). Chromosome segregation and cancer: cutting through the mystery. Nat Rev Cancer *1*, 109-117.

Jaspersen, S. L., Charles, J. F., and Morgan, D. O. (1999). Inhibitory phosphorylation of the APC regulator Hct1 is controlled by the kinase Cdc28 and the phosphatase Cdc14. Curr Biol *9*, 227-236.

Jaspersen, S. L., Charles, J. F., Tinker-Kulberg, R. L., and Morgan, D. O. (1998). A late mitotic regulatory network controlling cyclin destruction in Saccharomyces cerevisiae. Mol Biol Cell *9*, 2803-2817.

Jaspersen, S. L., and Morgan, D. O. (2000). Cdc14 activates cdc15 to promote mitotic exit in budding yeast. Curr Biol *10*, 615-618.

Jia, J., Zhang, W., Wang, B., Trinko, R., and Jiang, J. (2003). The Drosophila Ste20 family kinase dMST functions as a tumor suppressor by restricting cell proliferation and promoting apoptosis. Genes Dev *17*, 2514-2519.

Jin, Q. W., Pidoux, A. L., Decker, C., Allshire, R. C., and Fleig, U. (2002). The mal2p protein is an essential component of the fission yeast centromere. Mol Cell Biol *22*, 7168-7183.

Justice, R. W., Zilian, O., Woods, D. F., Noll, M., and Bryant, P. J. (1995). The Drosophila tumor suppressor gene warts encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation. Genes Dev *9*, 534-546.

Kaiser, B. K., Nachury, M. V., Gardner, B. E., and Jackson, P. K. (2004). Xenopus Cdc14 alpha/beta are localized to the nucleolus and centrosome and are required for embryonic cell division. BMC Cell Biol *5*, 27.

Kaiser, B. K., Zimmerman, Z. A., Charbonneau, H., and Jackson, P. K. (2002). Disruption of Centrosome Structure, Chromosome Segregation, and Cytokinesis by Misexpression of Human Cdc14A Phosphatase. Mol Biol Cell *13*, 2289-2300.

Kitajima, T. S., Yokobayashi, S., Yamamoto, M., and Watanabe, Y. (2003). Distinct cohesin complexes organize meiotic chromosome domains. Science *300*, 1152-1155.

Kitamura, K., Maekawa, H., and Shimoda, C. (1998). Fission yeast Ste9, a homolog of Hct1/Cdh1 and Fizzy-related, is a novel negative regulator of cell cycle progression during G1-phase. Mol Biol Cell *9*, 1065-1080.

Kitayama, C., Sugimoto, A., and Yamamoto, M. (1997). Type II myosin heavy chain encoded by the myo2 gene composes the contractile ring during cytokinesis in Schizosaccharomyces pombe. J Cell Biol *137*, 1309-1319.

Knapp, D., Bhoite, L., Stillman, D. J., and Nasmyth, K. (1996). The transcription factor Swi5 regulates expression of the cyclin kinase inhibitor p40SIC1. Mol Cell Biol *16*, 5701-5707.

Knop, M., Siegers, K., Pereira, G., Zachariae, W., Winsor, B., Nasmyth, K., and Schiebel, E. (1999). Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. Yeast *15*, 963-972.

Komarnitsky, S. I., Chiang, Y. C., Luca, F. C., Chen, J., Toyn, J. H., Winey, M., Johnston, L. H., and Denis, C. L. (1998). DBF2 protein kinase binds to and acts through the cell cycle-regulated MOB1 protein. Mol Cell Biol *18*, 2100-2107.

Kovelman, R., and Russell, P. (1996). Stockpiling of Cdc25 during a DNA replication checkpoint arrest in Schizosaccharomyces pombe. Mol Cell Biol *16*, 86-93.

Krapp, A., Cano, E., and Simanis, V. (2004a). Analysis of the S. pombe signalling scaffold protein Cdc11p reveals an essential role for the N-terminal domain in SIN signalling. FEBS Lett *565*, 176-180.

Krapp, A., Gulli, M. P., and Simanis, V. (2004b). SIN and the art of splitting the fission yeast cell. Curr Biol *14*, R722-730.

Krapp, A., Schmidt, S., Cano, E., and Simanis, V. (2001). S. pombe cdc11p, together with sid4p, provides an anchor for septation initiation network proteins on the spindle pole body. Curr Biol *11*, 1559-1568.

Krishnan, R., Pangilinan, F., Lee, C., and Spencer, F. (2000). Saccharomyces cerevisiae BUB2 prevents mitotic exit in response to both spindle and kinetochore damage [In Process Citation]. Genetics *156*, 489-500.

Labib, K., and Moreno, S. (1996). rum1: a CDK inhibitor regulating G1 progression in fission yeast. Trends Cell Biol *6*, 62-66.

Lacey, K. R., Jackson, P. K., and Stearns, T. (1999). Cyclin-dependent kinase control of centrosome duplication. Proc Natl Acad Sci U S A *96*, 2817-2822.

Lai, Z. C., Wei, X., Shimizu, T., Ramos, E., Rohrbaugh, M., Nikolaidis, N., Ho, L. L., and Li, Y. (2005). Control of cell proliferation and apoptosis by mob as tumor suppressor, mats. Cell *120*, 675-685.

Lampson, M. A., Renduchitala, K., Khodjakov, A., and Kapoor, T. M. (2004). Correcting improper chromosome-spindle attachments during cell division. Nat Cell Biol *6*, 232-237.

Le Goff, X., Motegi, F., Salimova, E., Mabuchi, I., and Simanis, V. (2000). The S. pombe rlc1 gene encodes a putative myosin regulatory light chain that binds the type II myosins myo3p and myo2p [In Process Citation]. J Cell Sci *113*, 4157-4163.

Le Goff, X., Utzig, S., and Simanis, V. (1999a). Controlling septation in fission yeast: finding the middle, and timing it right [In Process Citation]. Curr Genet *35*, 571-584.

Le Goff, X., Woollard, A., and Simanis, V. (1999b). Analysis of the cps1 gene provides evidence for a septation checkpoint in Schizosaccharomyces pombe. Mol Gen Genet *262*, 163-172.

Lee, J., Hwang, H. S., Kim, J., and Song, K. (1999a). Ibd1p, a possible spindle pole body associated protein, regulates nuclear division and bud separation in Saccharomyces cerevisiae. Biochim Biophys Acta *1449*, 239-253.

Lee, K. M., Saiz, J. E., Barton, W. A., and Fisher, R. P. (1999b). Cdc2 activation in fission yeast depends on Mcs6 and Csk1, two partially redundant Cdk-activating kinases (CAKs). Curr Biol *9*, 441-444.

Lee, S. E., Frenz, L. M., Wells, N. J., Johnson, A. L., and Johnston, L. H. (2001). Order of function of the budding-yeast mitotic exit-network proteins Tem1, Cdc15, Mob1, Dbf2, and Cdc5. Curr Biol *11*, 784-788.

Li, L., Ljungman, M., and Dixon, J. E. (2000). The human Cdc14 phosphatases interact with and dephosphorylate the tumor suppressor protein p53. J Biol Chem *275*, 2410-2414.

Li, Y., Pei, J., Xia, H., Ke, H., Wang, H., and Tao, W. (2003). Lats2, a putative tumor suppressor, inhibits G1/S transition. Oncogene *22*, 4398-4405.

Lippincott, J., Shannon, K. B., Shou, W., Deshaies, R. J., and Li, R. (2001). The Tem1 small GTPase controls actomyosin and septin dynamics during cytokinesis. J Cell Sci *114*, 1379-1386.

Liu, J., Wang, H., and Balasubramanian, M. K. (2000). A checkpoint that monitors cytokinesis in Schizosaccharomyces pombe. J Cell Sci *113*, 1223-1230.

Liu, J., Wang, H., McCollum, D., and Balasubramanian, M. K. (1999). Drc1p/Cps1p, a 1,3-beta-glucan synthase subunit, is essential for division septum assembly in Schizosaccharomyces pombe. Genetics *153*, 1193-1203.

Lopez-Girona, A., Furnari, B., Mondesert, O., and Russell, P. (1999). Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein. Nature *397*, 172-175.

Luca, F. C., Mody, M., Kurischko, C., Roof, D. M., Giddings, T. H., and Winey, M. (2001). Saccharomyces cerevisiae Mob1p Is Required for Cytokinesis and Mitotic Exit. Mol Cell Biol *21*, 6972-6983.

Luca, F. C., and Winey, M. (1998). MOB1, an essential yeast gene required for completion of mitosis and maintenance of ploidy. Mol Biol Cell *9*, 29-46.

Mah, A. S., Jang, J., and Deshaies, R. J. (2001). Protein kinase Cdc15 activates the Dbf2-Mob1 kinase complex. Proc Natl Acad Sci U S A *98*, 7325-7330.

Mailand, N., Lukas, C., Kaiser, B. K., Jackson, P. K., Bartek, J., and Lukas, J. (2002). Deregulated human Cdc14A phosphatase disrupts centrosome separation and chromosome segregation. Nat Cell Biol *4*, 318-322.

Margolis, R. L., Lohez, O. D., and Andreassen, P. R. (2003). G1 tetraploidy checkpoint and the suppression of tumorigenesis. J Cell Biochem *88*, 673-683.

Marks, J., Fankhauser, C., and Simanis, V. (1992). Genetic interactions in the control of septation in Schizosaccharomyces pombe. J Cell Sci *101*, 801-808.

Marston, A. L., Lee, B. H., and Amon, A. (2003). The Cdc14 phosphatase and the FEAR network control meiotic spindle disassembly and chromosome segregation. Dev Cell *4*, 711-726.

Martin-Castellanos, C., Labib, K., and Moreno, S. (1996). B-type cyclins regulate G1 progression in fission yeast in opposition to the p25rum1 cdk inhibitor. Embo J *15*, 839-849.

Martineau, S. N., Andreassen, P. R., and Margolis, R. L. (1995). Delay of HeLa cell cleavage into interphase using dihydrocytochalasin B: retention of a postmitotic spindle and telophase disc correlates with synchronous cleavage recovery. J Cell Biol *131*, 191-205.

May, K. M., Watts, F. Z., Jones, N., and Hyams, J. S. (1997). Type II myosin involved in cytokinesis in the fission yeast, Schizosaccharomyces pombe. Cell Motil Cytoskeleton *38*, 385-396.

McCollum, D., Balasubramanian, M. K., Pelcher, L. E., Hemmingsen, S. M., and Gould, K. L. (1995). Schizosaccharomyces pombe cdc4+ gene encodes a novel EF-hand protein essential for cytokinesis. J Cell Biol *130*, 651-660.

McCollum, D., and Gould, K. L. (2001). Timing is everything: regulation of mitotic exit and cytokinesis by the MEN and SIN. Trends Cell Biol *11*, 89-95.

Menssen, R., Neutzner, A., and Seufert, W. (2001). Asymmetric spindle pole localization of yeast Cdc15 kinase links mitotic exit and cytokinesis. Curr Biol *11*, 345-350.

Meraldi, P., Honda, R., and Nigg, E. A. (2002). Aurora-A overexpression reveals tetraploidization as a major route to centrosome amplification in p53-/- cells. Embo J *21*, 483-492.

Minet, M., Nurse, P., Thuriaux, P., and Mitchison, J. M. (1979). Uncontrolled septation in a cell division cycle mutant of the fission yeast Schizosaccharomyces pombe. J Bacteriol *137*, 440-446.

Mishra, M., Karagiannis, J., Trautmann, S., Wang, H., McCollum, D., and Balasubramanian, M. K. (2004). The Clp1p/Flp1p phosphatase ensures completion of cytokinesis in response to minor perturbation of the cell division machinery in Schizosaccharomyces pombe. J Cell Sci *117*, 3897-3910.

Mishra, M., Sevugan, M., Karagiannis, J., P., S., and Balasubramanian, M. K. (2005). The 14-3-3 protein, Rad24p, modulates function of the Cdc14p family phosphatase

Clp1p/Flp1p and increases the efficiency of Septation Initiation Network signaling in Fission Yeast. Curr Biol accepted.

Moll, T., Tebb, G., Surana, U., Robitsch, H., and Nasmyth, K. (1991). The role of phosphorylation and the CDC28 protein kinase in cell cycle-regulated nuclear import of the S. cerevisiae transcription factor SWI5. Cell *66*, 743-758.

Moreno, S., Klar, A., and Nurse, P. (1991). Molecular genetic analysis of fission yeast, *Schizosaccharomyces pombe*. Methods Enzymol *194*, 795-823.

Moreno, S., and Nurse, P. (1994). Regulation of progression through the G1 phase of the cell cycle by the rum1+ gene [see comments]. Nature *367*, 236-242.

Moreno, S., Nurse, P., and Russell, P. (1990). Regulation of mitosis by cyclic accumulation of p80cdc25 mitotic inducer in fission yeast. Nature *344*, 549-552.

Morgan, D. O. (1997). Cyclin-dependent kinases: engines, clocks, and microprocessors. Annu Rev Cell Dev Biol *13*, 261-291.

Morgan, D. O. (1999). Regulation of the APC and the exit from mitosis. Nat Cell Biol *1*, E47-53.

Morisaki, T., Hirota, T., Iida, S., Marumoto, T., Hara, T., Nishiyama, Y., Kawasuzi, M., Hiraoka, T., Mimori, T., Araki, N., *et al.* (2002). WARTS tumor suppressor is phosphorylated by Cdc2/cyclin B at spindle poles during mitosis. FEBS Lett *529*, 319-324.

Morishita, J., Matsusaka, T., Goshima, G., Nakamura, T., Tatebe, H., and Yanagida, M. (2001). Bir1/Cut17 moving from chromosome to spindle upon the loss of cohesion is required for condensation, spindle elongation and repair. Genes Cells *6*, 743-763.

Morrell, J. L., Tomlin, G. C., Rajagopalan, S., Venkatram, S., Feoktistova, A. S., Tasto, J. J., Mehta, S., Jennings, J. L., Link, A., Balasubramanian, M. K., and Gould, K. L. (2004). Sid4p-Cdc11p assembles the septation initiation network and its regulators at the S. pombe SPB. Curr Biol *14*, 579-584.

Motegi, F., Mishra, M., Balasubramanian, M. K., and Mabuchi, I. (2004). Myosin-II reorganization during mitosis is controlled temporally by its dephosphorylation and spatially by Mid1 in fission yeast. J Cell Biol *165*, 685-695.

Motegi, F., Nakano, K., and Mabuchi, I. (2000). Molecular mechanism of myosin-II assembly at the division site in Schizosaccharomyces pombe. J Cell Sci *113*, 1813-1825.

Mueller, P. R., Coleman, T. R., and Dunphy, W. G. (1995). Cell cycle regulation of a Xenopus Wee1-like kinase. Mol Biol Cell *6*, 119-134.

Mulvihill, D. P., Petersen, J., Ohkura, H., Glover, D. M., and Hagan, I. M. (1999). Plo1 kinase recruitment to the spindle pole body and its role in cell division in Schizosaccharomyces pombe. Mol Biol Cell *10*, 2771-2785.

Nabeshima, K., Nakagawa, K., Straight, A. F., Murray, A. W., Chikashige, Y., Ymamshita, Y. M., Yasushi, H., and Yanagida, M. (1998). Dynamics of Centromeres during Metaphase-Anaphase Transition in Fission Yeast: Dis1 Is Implicated in Force Balance in Metaphase Bipolar Spindle. Molecular Biology of the Cell *9*, 3211-3225.

Nakaseko, Y., Goshima, G., Morishita, J., and Yanagida, M. (2001). M phase-specific kinetochore proteins in fission yeast: microtubule-associating Dis1 and Mtc1 display rapid separation and segregation during anaphase. Curr Biol *11*, 537-549.

Nasmyth, K. (2002). Segregating sister genomes: the molecular biology of chromosome separation. Science *297*, 559-565.

Nilsson, I., and Hoffmann, I. (2000). Cell cycle regulation by the Cdc25 phosphatase family. Prog Cell Cycle Res *4*, 107-114.

Niwa, O., T. Matsumoto, and M.Yanagida (1986). Construction of a minichromosome by deletion and its mitotic and meiotic behaviour in fission yeast. Molecular and General Genetics *203*, 397-405.

Ohi, R., and Gould, K. L. (1999). Regulating the onset of mitosis. Curr Opin Cell Biol *11*, 267-273.

Ohkura, H., Adachi, Y., Kinoshita, N., Niwa, O., Toda, T., and Yanagida, M. (1988). Cold-sensitive and caffeine supersensitive mutants of the Schizosaccharomyces pombe dis genes implicated in sister chromatid separation during mitosis. EMBO J *7*, 1465-1473.

Ohkura, H., Hagan, I. M., and Glover, D. M. (1995). The conserved Schizosaccharomyces pombe kinase plo1, required to form a bipolar spindle, the actin ring, and septum, can drive septum formation in G1 and G2 cells. Genes Dev *9*, 1059-1073.

Pantalacci, S., Tapon, N., and Leopold, P. (2003). The Salvador partner Hippo promotes apoptosis and cell-cycle exit in Drosophila. Nat Cell Biol *5*, 921-927.

Patra, D., Wang, S. X., Kumagai, A., and Dunphy, W. G. (1999). The xenopus Suc1/Cks protein promotes the phosphorylation of G(2)/M regulators. J Biol Chem *274*, 36839-36842.

Pereira, G., Hofken, T., Grindlay, J., Manson, C., and Schiebel, E. (2000). The Bub2p spindle checkpoint links nuclear migration with mitotic exit. Mol Cell *6*, 1-10.

Pereira, G., Manson, C., Grindlay, J., and Schiebel, E. (2002). Regulation of the Bfa1p-Bub2p complex at spindle pole bodies by the cell cycle phosphatase Cdc14p. J Cell Biol *157*, 367-379.

Pereira, G., and Schiebel, E. (2003). Separase regulates INCENP-Aurora B anaphase spindle function through Cdc14. Science *302*, 2120-2124.

Peters, J. M. (2002). The anaphase-promoting complex: proteolysis in mitosis and beyond. Mol Cell *9*, 931-943.

Petersen, J., and Hagan, I. M. (2003). S. pombe Aurora Kinase/Survivin Is Required for Chromosome Condensation and the Spindle Checkpoint Attachment Response. Curr Biol *13*, 590-597.

Petersen, J., Paris, J., Willer, M., Philippe, M., and Hagan, I. M. (2001). The S. pombe aurora-related kinase Ark1 associates with mitotic structures in a stage dependent manner and is required for chromosome segregation. J Cell Sci *114*, 4371-4384.

Pidoux, A., and Allshire, R. (2003). Chromosome segregation: clamping down on deviant orientations. Curr Biol *13*, R385-387.

Rabitsch, K. P., Petronczki, M., Javerzat, J. P., Genier, S., Chwalla, B., Schleiffer, A., Tanaka, T. U., and Nasmyth, K. (2003). Kinetochore recruitment of two nucleolar proteins is required for homolog segregation in meiosis I. Dev Cell *4*, 535-548.

Rajagopalan, S., and Balasubramanian, M. K. (2002). Schizosaccharomyces pombe Bir1p, a nuclear protein that localizes to kinetochores and the spindle midzone, is essential for chromosome condensation and spindle elongation during mitosis. Genetics *160*, 445-456.

Ross, K. E., and Cohen-Fix, O. (2004). A role for the FEAR pathway in nuclear positioning during anaphase. Dev Cell *6*, 729-735.

Russell, P., and Nurse, P. (1986). Schizosaccharomyces pombe and Saccharomyces cerevisiae: a look at yeasts divided. Cell *45*, 781-782.

Russell, P., and Nurse, P. (1987). Negativ regulation of mitosis by *wee1+*, a gene encoding a protein kinase homolog. Cell, 559-567.

Saito, R. M., Perreault, A., Peach, B., Satterlee, J. S., and van den Heuvel, S. (2004). The CDC-14 phosphatase controls developmental cell-cycle arrest in C. elegans. Nat Cell Biol *6*, 777-783.

Saitoh, S., Takahashi, K., and Yanagida, M. (1997). Mis6, a fission yeast inner centromere protein, acts during G1/S and forms specialized chromatin required for equal segregation. Cell *90*, 131-143.

Schmidt, S., Sohrmann, M., Hofmann, K., Woollard, A., and Simanis, V. (1997). The Spg1p GTPase is an essential, dosage-dependent inducer of septum formation in Schizosaccharomyces pombe. Genes Dev *11*, 1519-1534.

Schwab, M., Lutum, A. S., and Seufert, W. (1997). Yeast Hct1 is a regulator of Clb2 cyclin proteolysis. Cell *90*, 683-693.

Severson, A. F., Hamill, D. R., Carter, J. C., Schumacher, J., and Bowerman, B. (2000). The aurora-related kinase AIR-2 recruits ZEN-4/CeMKLP1 to the mitotic spindle at metaphase and is required for cytokinesis. Curr Biol *10*, 1162-1171.

Shirayama, M., Matsui, Y., Tanaka, K., and Toh-e, A. (1994a). Isolation of a CDC25 family gene, MSI2/LTE1, as a multicopy suppressor of ira1. Yeast *10*, 451-461.

Shirayama, M., Matsui, Y., and Toh, E. A. (1994b). The yeast TEM1 gene, which encodes a GTP-binding protein, is involved in termination of M phase. Mol Cell Biol *14*, 7476-7482.

Shou, W., and Deshaies, R. J. (2002). Multiple telophase arrest bypassed (tab) mutants alleviate the essential requirement for Cdc15 in exit from mitosis in S. cerevisiae. BMC Genet *3*, 4.

Shou, W., Seol, J. H., Shevchenko, A., Baskerville, C., Moazed, D., Chen, Z. W., Jang, J., Charbonneau, H., and Deshaies, R. J. (1999). Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex. Cell *97*, 233-244.

Sohrmann, M., Fankhauser, C., Brodbeck, C., and Simanis, V. (1996). The dmf1/mid1 gene is essential for correct positioning of the division septum in fission yeast. Genes Dev *10*, 2707-2719.

Sohrmann, M., Schmidt, S., Hagan, I., and Simanis, V. (1998). Asymmetric segregation on spindle poles of the *Schizosaccharomyces pombe* septum-inducing protein kinase Cdc7p. Genes Dev *12*, 84-94.

Song, K., Mach, K. E., Chen, C. Y., Reynolds, T., and Albright, C. F. (1996). A novel suppressor of ras1 in fission yeast, byr4, is a dosage-dependent inhibitor of cytokinesis. J Cell Biol *133*, 1307-1319.

Song, S., Grenfell, T. Z., Garfield, S., Erikson, R. L., and Lee, K. S. (2000). Essential function of the polo box of Cdc5 in subcellular localization and induction of cytokinetic structures. Mol Cell Biol *20*, 286-298.

Song, S., and Lee, K. S. (2001). A novel function of Saccharomyces cerevisiae CDC5 in cytokinesis. J Cell Biol *152*, 451-469.

Sparks, C. A., Morphew, M., and McCollum, D. (1999). Sid2p, a spindle pole body kinase that regulates the onset of cytokinesis. J Cell Biol *146*, 777-790.

Spencer, S., Dowbenko, D., Cheng, J., Li, W., Brush, J., Utzig, S., Simanis, V., and Lasky, L. A. (1997). PSTPIP: a tyrosine phosphorylated cleavage furrow-associated protein that is a substrate for a PEST tyrosine phosphatase. J Cell Biol *138*, 845-860.

St John, M. A., Tao, W., Fei, X., Fukumoto, R., Carcangiu, M. L., Brownstein, D. G., Parlow, A. F., McGrath, J., and Xu, T. (1999). Mice deficient of Lats1 develop soft-tissue sarcomas, ovarian tumours and pituitary dysfunction. Nat Genet *21*, 182-186.

Stegmeier, F., and Amon, A. (2004). Closing mitosis: the functions of the Cdc14 phosphatase and its regulation. Annu Rev Genet *38*, 203-232.

Stegmeier, F., Huang, J., Rahal, R., Zmolik, J., Moazed, D., and Amon, A. (2004). The replication fork block protein Fob1 functions as a negative regulator of the FEAR network. Curr Biol *14*, 467-480.

Stegmeier, F., Visintin, R., and Amon, A. (2002). Separase, polo kinase, the kinetochore protein Slk19, and Spo12 function in a network that controls Cdc14 localization during early anaphase. Cell *108*, 207-220.

Straight, A. F., Cheung, A., Limouze, J., Chen, I., Westwood, N. J., Sellers, J. R., and Mitchison, T. J. (2003). Dissecting temporal and spatial control of cytokinesis with a myosin II Inhibitor. Science *299*, 1743-1747.

Stukenberg, P. T. (2004). Triggering p53 after cytokinesis failure. J Cell Biol *165*, 607-608.

Sullivan, M., Higuchi, T., Katis, V. L., and Uhlmann, F. (2004). Cdc14 phosphatase induces rDNA condensation and resolves cohesin-independent cohesion during budding yeast anaphase. Cell *117*, 471-482.

Sullivan, M., and Uhlmann, F. (2003). A non-proteolytic function of separase links the onset of anaphase to mitotic exit. Nat Cell Biol *5*, 249-254.

Surana, U., Amon, A., Dowzer, C., McGrew, J., Byers, B., and Nasmyth, K. (1993). Destruction of the CDC28/CLB mitotic kinase is not required for the metaphase to anaphase transition in budding yeast. Embo J *12*, 1969-1978.

Takahashi, K., Chen, E. S., and Yanagida, M. (2000). Requirement of Mis6 centromere connector for localizing a CENP-A-like protein in fission yeast. Science *288*, 2215-2219.

Tanaka, K., Petersen, J., MacIver, F., Mulvihill, D. P., Glover, D. M., and Hagan, I. M. (2001). The role of Plo1 kinase in mitotic commitment and septation in Schizosaccharomyces pombe. Embo J *20*, 1259-1270.

Tanaka, T. U. (2002). Bi-orienting chromosomes on the mitotic spindle. Curr Opin Cell Biol *14*, 365-371.

Tanaka, T. U., Rachidi, N., Janke, C., Pereira, G., Galova, M., Schiebel, E., Stark, M. J., and Nasmyth, K. (2002). Evidence that the Ipl1-Sli15 (Aurora kinase-INCENP) complex promotes chromosome bi-orientation by altering kinetochore-spindle pole connections. Cell *108*, 317-329.

Tao, W., Zhang, S., Turenchalk, G. S., Stewart, R. A., St John, M. A., Chen, W., and Xu, T. (1999). Human homologue of the Drosophila melanogaster lats tumour suppressor modulates CDC2 activity. Nat Genet *21*, 177-181.

Tarapore, P., Tokuyama, Y., Horn, H. F., and Fukasawa, K. (2001). Difference in the centrosome duplication regulatory activity among p53 'hot spot' mutants: potential role of Ser 315 phosphorylation-dependent centrosome binding of p53. Oncogene *20*, 6851-6863.

Tinker-Kulberg, R. L., and Morgan, D. O. (1999). Pds1 and Esp1 control both anaphase and mitotic exit in normal cells and after DNA damage. Genes Dev *13*, 1936-1949.

Tomlin, G. C., Morrell, J. L., and Gould, K. L. (2002). The spindle pole body protein cdc11p links sid4p to the fission yeast septation initiation network. Mol Biol Cell *13*, 1203-1214.

Toyn, J. H., Johnson, A. L., Donovan, J. D., Toone, W. M., and Johnston, L. H. (1997). The Swi5 transcription factor of Saccharomyces cerevisiae has a role in exit from mitosis through induction of the cdk-inhibitor Sic1 in telophase. Genetics *145*, 85-96.

Toyoda, Y., Furuya, K., Goshima, G., Nagao, K., Takahashi, K., and Yanagida, M. (2002). Requirement of chromatid cohesion proteins rad21/scc1 and mis4/scc2 for normal spindle-kinetochore interaction in fission yeast. Curr Biol *12*, 347-358.

Trautmann, S., and McCollum, D. (2002). Cell cycle: new functions for Cdc14 family phosphatases. Curr Biol *12*, R733-735.

Trautmann, S., and McCollum, D. (2005). Subcellular targeting of S. pombe Cdc14-like phosphatase Clp1p reveals distinct nuclear and cytoplasmic functions and suggests a role for nuclear shuttling in Clp1p regulation. Curr Biol, accepted.

Trautmann, S., Rajagopalan, S., and McCollum, D. (2004). The S. pombe Cdc14-like phosphatase Clp1p regulates chromosome biorientation and interacts with Aurora kinase. Dev Cell *7*, 755-762.

Trautmann, S., Wolfe, B. A., Jorgensen, P., Tyers, M., Gould, K. L., and McCollum, D. (2001). Fission yeast Clp1p phosphatase regulates G2/M transition and coordination of cytokinesis with cell cycle progression. Curr Biol *11*, 931-940.

Udan, R. S., Kango-Singh, M., Nolo, R., Tao, C., and Halder, G. (2003). Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. Nat Cell Biol *5*, 914-920.

Uetake, Y., and Sluder, G. (2004). Cell cycle progression after cleavage failure: mammalian somatic cells do not possess a "tetraploidy checkpoint". J Cell Biol *165*, 609-615.

Visintin, R., and Amon, A. (2001). Regulation of the mitotic exit protein kinases Cdc15 and Dbf2. Mol Biol Cell *12*, 2961-2974.

Visintin, R., Craig, K., Hwang, E. S., Prinz, S., Tyers, M., and Amon, A. (1998). The phosphatase Cdc14 triggers mitotic exit by reversal of Cdk- dependent phosphorylation. Mol Cell *2*, 709-718.

Visintin, R., Hwang, E. S., and Amon, A. (1999). Cfi1 prevents premature exit from mitosis by anchoring Cdc14 phosphatase in the nucleolus [see comments]. Nature *398*, 818-823.

Visintin, R., Prinz, S., and Amon, A. (1997). CDC20 and CDH1: a family of substrate-specific activators of APC-dependent proteolysis. Science *278*, 460-463.

Visintin, R., Stegmeier, F., and Amon, A. (2003). The role of the polo kinase Cdc5 in controlling Cdc14 localization. Mol Biol Cell *14*, 4486-4498.

Wang, W. Q., Bembenek, J., Gee, K. R., Yu, H., Charbonneau, H., and Zhang, Z. Y. (2004). Kinetic and mechanistic studies of a cell cycle protein phosphatase Cdc14. J Biol Chem *279*, 30459-30468.

Wheatley, S. P., Hinchcliffe, E. H., Glotzer, M., Hyman, A. A., Sluder, G., and Wang, Y. (1997). CDK1 inactivation regulates anaphase spindle dynamics and cytokinesis in vivo. J Cell Biol *138*, 385-393.

Wolfe, B. A., and Gould, K. L. (2004). Fission yeast Clp1p phosphatase affects G(2)/M transition and mitotic exit through Cdc25p inactivation. Embo J *23*, 919-929.

Wu, J. Q., Kuhn, J. R., Kovar, D. R., and Pollard, T. D. (2003a). Spatial and temporal pathway for assembly and constriction of the contractile ring in fission yeast cytokinesis. Dev Cell *5*, 723-734.

Wu, S., Huang, J., Dong, J., and Pan, D. (2003b). hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. Cell *114*, 445-456.

Xu, S., Huang, H. K., Kaiser, P., Latterich, M., and Hunter, T. (2000). Phosphorylation and spindle pole body localization of the Cdc15p mitotic regulatory protein kinase in budding yeast. Curr Biol *10*, 329-332.

Xu, T., Wang, W., Zhang, S., Stewart, R. A., and Yu, W. (1995). Identifying tumor suppressors in genetic mosaics: the Drosophila lats gene encodes a putative protein kinase. Development *121*, 1053-1063.

Yabuta, N., Fujii, T., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Nishiguchi, H., Endo, Y., Toji, S., Tanaka, H., Nishimune, Y., and Nojima, H. (2000). Structure, expression, and chromosome mapping of LATS2, a mammalian homologue of the Drosophila tumor suppressor gene lats/warts. Genomics *63*, 263-270.

Yamaguchi, S., Murakami, H., and Okayama, H. (1997). A WD repeat protein controls the cell cycle and differentiation by negatively regulating Cdc2/B-type cyclin complexes. Mol Biol Cell *8*, 2475-2486.

Yamano, H., Gannon, J., and Hunt, T. (1996). The role of proteolysis in cell cycle progression in Schizosaccharomyces pombe. Embo J *15*, 5268-5279.

Yang, X., Li, D. M., Chen, W., and Xu, T. (2001). Human homologue of Drosophila lats, LATS1, negatively regulate growth by inducing G(2)/M arrest or apoptosis. Oncogene *20*, 6516-6523.

Yang, X., Yu, K., Hao, Y., Li, D. M., Stewart, R., Insogna, K. L., and Xu, T. (2004). LATS1 tumour suppressor affects cytokinesis by inhibiting LIMK1. Nat Cell Biol *6*, 609-617.

Yoshida, S., and Toh-e, A. (2001). Regulation of the localization of Dbf2 and mob1 during cell division of saccharomyces cerevisiae. Genes Genet Syst *76*, 141-147.

Zachariae, W., Schwab, M., Nasmyth, K., and Seufert, W. (1998). Control of cyclin ubiquitination by CDK-regulated binding of Hct1 to the anaphase promoting complex. Science *282*, 1721-1724.