REGULATION OF MITOTIC EXIT IN *S. POMBE* THROUGH ACTIVATION OF A CDC14

FAMILY PHOSPHATASE

By

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LIST OF ABBREVIATIONS

A	alanine
aa	amino acid
Ade	adenine
Ala	alanine
APC/C	Anaphase-Promoting Complex/Cyclosome
Arg	arginine
Asp	aspartic acid
Asyn	asynchronous
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
C	carboxy/Celsius
cdc	cell division cycle
cDNA	complimentary DNA
Cdk	Cyclin-dependent kinase
Ce	*Caenorhabditis elegans*
Clp1	Cdc14-like phosphatase
co-IP	co-immunoprecipitation
Cys	cysteine
2D-LC	2-dimensional liquid chromatography
Δ	deletion strain/truncation
D	aspartic acid
DAPI	4,6-diamidino-2-phenylindole
D Box	destruction box
DIC	differential contrast interference
DiFMUP	6,8-difluoro-4-methylumbelliferyl phosphate
DTT	dithiothriotol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid

FEAR	Cdc14 early anaphase release
5-FOA	*5*-fluororotic acid
G	gap phase
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
Gln	glutamine
Glu	glutamic acid
GST	glutathione-S-transferase
h	human
Hs	*Homo sapiens*
HA	influenza hemaggluttinin epitope
His	histidine
HRP	horseradish peroxidase
IP	immunoprecipitation
Kan^R	kanamycin resistant
kDa	kiloDalton
KGY	Kathy Gould yeast
l	liter
λ	lambda
Leu	leucine
Lys	lysine
μg	microgram
μCi	microCurie
ml	milliliter
mM	milimolar
μM	micromolar
M	molar, mitosis
MBP	maltose binding protein
MEN	mitotic exit network
MPF	maturation promoting factor

Myc	myc epitope
N	amino
ng	nanogram
nm	nanometer
NaCl	sodium chloride
NaH$_2$PO$_4$	sodium phosphate, dibasic
NaOH	sodium hydroxide
nmt	no message in thiamine promoter
NP-40	Nonidet® P-40
ORF	open reading frame
p	protein
^{32}P	phosphorous-32
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PD	phosphatase dead
Phe	phenylalanine
pKG	plasmid Kathy Gould
PTP	protein tyrosine phosphatase
pNPP	*p*-nitrophenylphosphate
Pro	proline
PVDF	polyvinyldifluoride
S	synthesis phase
Sc	*Saccharomyces cerevisiae*
SCF	Skp/Cullen/F-box
SDS	sodium dodecyl sulfate
Ser	serine
SIN	septation initiation network
Sp	*Schizosaccharomyces pombe*
SPB	spindle pole body
T	thiamine
TAP	tandem affinity purification

Thr	threonine
Trp	tryptophan
Tyr	tyrosine
U	unit
Unt	untagged
WPD	tryptophan-proline-aspartic acid
X	*Xenopus*/any amino acid
YE	yeast extract
Z	any hydrophobic amino acid

CHAPTER I

INTRODUCTION

The eukaryotic cell division cycle

In order for multicellular organisms to fully develop, they must precisely undergo many rounds of cell division. The process of cell division involves the duplication and accurate segregation of the genetic material to two daughter cells. Research over the past 30 years from a number of model systems has illuminated both the complex nature and the strict conservation involved in cell cycle control. Although many of the key regulators have been identified through genetic and biochemical studies, the precise coordination among the regulators still remains relatively obscure. Cell division is fundamental for life, and inaccuracies in the transmission of genetic material to the progeny are a hallmark of many forms of cancer as well as many developmental disorders. Therefore, a more complete understanding of the events that regulate the cell division cycle will provide us with information pertinent to a fundamental biological process, as well as potential therapeutic tools with which to fight these processes in the event they go awry.

The basic somatic cell division cycle of eukaryotes involves alternating rounds of DNA replication followed by segregation of the duplicated sister chromatids to opposite poles of the cell prior to cell cleavage (Figure 1). These events occur during DNA Synthesis phase (S phase) and Mitosis (M phase), respectively, just two of the four major cell cycle transitions found in eukaryotes. During S phase, the entire cellular genome is replicated error-free, and a linkage is established between the newly duplicated sister chromatids. Mitosis is coincident with dissolution of this linkage and the segregation of these sisters during anaphase. Cytokinesis is the final event of the cell cycle and results in cell cleavage to produce two identical daughter cells. Two intervening gap phases, G_1 and G_2, separate S and M phases. While most of the real labor of the cell cycle is accomplished during S and M phases, the two gap phases are responsible for most of the cellular growth as well as for monitoring the success and fidelity of previous cell cycle events. This success is monitored by checkpoints, which act as molecular stop signals to prevent transitions into subsequent cell cycle phases when the conditions are not right to do so.

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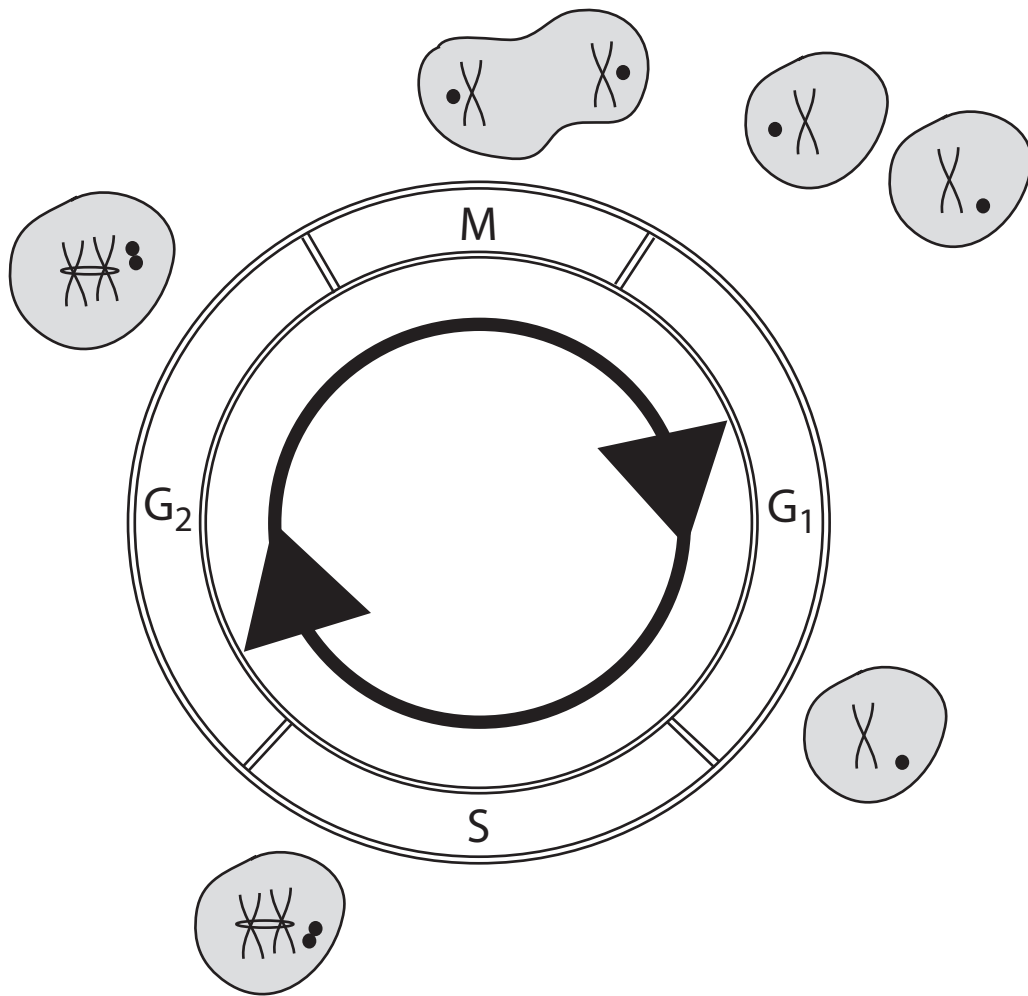
Figure 1. The eukaryotic cell division cycle. The somatic cell cycle is divided into four distinct phases: G_1, S, G_2, and M. During the G1/S transition, the centrosome (black circle) is duplicated but remains linked. During S phase, each chromatid is precisely replicated and a linkage is established that ties the sister chromatids together. Upon progression through mitosis, this linkage is broken and the sister chromatids separate to opposite poles of the cell. Cytokinesis produces two identical daughter cells as the membrane is cleaved between the segregated sister chromatids.

These restraints inhibit the activities of a conserved family of protein kinases, the Cyclin-dependent kinases (Cdks) that act at the center stage of the eukaryotic cell cycle.

Cdks are highly conserved across the eukaryotic lineage from yeast to humans. As their name implies, this family of kinases is defined by its requirement for association with a cyclin subunit, an event necessary for its catalytic activity and substrate targeting. Cyclin subunits are characterized by their cell cycle periodicity as they are synthesized and destroyed during each cell cycle (Murray and Kirschner, 1989), and therefore, play a large role in the irreversibility of many aspects of cell cycle progression. The Cdk complex phosphorylates a number of critical substrates necessary for transition through different cell cycle phases, and Cdk activity rises and falls as the phase controlled is traversed. A team of Cdks and cyclins exist in higher eukaryotes that collaborate to drive distinct and sometimes overlapping cell cycle events by targeting different substrates (reviewed by Morgan, 1997). Cdks not only are responsible for driving the cell cycle through these major transitions, they also play a critical role in ensuring that certain events occur only once per cell cycle.

Cdk1p and Its Regulation

A rise in the activity of one Cdk, the Cdk1-Cyclin B complex (hereafter referred to as Cdk1p), is required for entry into mitosis in all eukaryotes. This activity was first described as Maturation Promoting Factor (MPF), a biochemical activity capable of driving *Xenopus* eggs from an interphase to a mitotic state (Masui and Markert, 1971; Newport and Kirschner, 1984). Genetic screens in unicellular eukaryotes like *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* aimed at identifying critical regulators of the cell cycle identified homologous genes required for traversing both the G_1/S and G_2/M transitions (Nurse and Bissett, 1981). These genes encode the protein kinase constituent of MPF (Gautier et al., 1988). Strong conservation among cell cycle regulators was readily apparent from experiments that demonstrated that the human *cdk1*[+] gene could rescue the temperature sensitive lethality of the *S. pombe cdk1*[-] mutant (Lee and Nurse, 1987).

Cdk1p activity peaks at the G_2/M transition and falls precipitously after sister chromatid separation upon destruction of cyclin B. Such tight control over the activity of this complex suggests a complex mode(s) of regulation. Indeed, Cdk1p activity is not just regulated at the level of cyclin binding. Multiple highly conserved phosphorylation events impinge upon Cdk1p

during interphase to maintain its activity in check. These phosphorylations occur on conserved Thr and Tyr residues within the ATP binding pocket of the Cdk1p enzyme (Gould and Nurse, 1989; Krek and Nigg, 1991) and preclude Cdk1p's ability to bind ATP and thus function as a protein kinase. At the G_2/M transition, these inhibitory phosphorylation events are removed from Cdk1p, and mitotic entry is initiated. As Cdk1p accumulates in late G_2 in the phosphorylated state, the removal of these phosphorylation events acts as a rate-limiting step in the activation of Cdk1p.

Identification of Key Regulators of Cell Cycle Progression in *S. pombe*

The biochemical regulation of Cdk1p during the G_2/M transition was first predicted based on genetic studies in the fission yeast, *S. pombe* (Nurse and Bissett, 1981; Russell and Nurse, 1986; Russell and Nurse, 1987; Gould and Nurse, 1989). In *S. pombe*, a single Cdk1p-cyclin B complex is capable of driving the entire cell cycle, and its activity rises and falls as cells progress through mitosis (Figure 2) (Fisher and Nurse, 1996). The *wee1*[+] and *cdc25*[+] gene products were first identified as dose dependent inhibitors and inducers of mitotic entry, respectively (Russell and Nurse, 1986; Russell and Nurse, 1987). Evidence that these two regulators functioned independently and antagonistically to one another, and that they converged at the level of Cdk1p phosphorylation, was gained from a number of genetic observations. First, whereas the single mutants are inviable at the restrictive temperature, double mutants between *cdc25*[-] and *wee1*[-] are viable (Russell and Nurse, 1987). Second, overexpression of Cdc25p in the absence of *wee1*[-] function is additive and leads to mitotic catastrophe (Russell and Nurse, 1986). Additionally, mutation of Tyr-15 of Cdk1p to a non-phosphorylatable Phe mimicks *wee1Δ* in its ability to drive premature mitotic commitment (Gould and Nurse, 1989). Biochemical evidence subsequently confirmed these genetic predictions with evidence that the Wee1 (and the closely related Myt1) family of tyrosine kinases were responsible for inhibitory phosphorylation events at Thr-14 and Tyr-15 (Lundgren et al., 1991; Parker et al., 1992; Mueller et al., 1995). Conversely, the Cdc25 family of protein phosphatases was shown to catalyze the reversal of these phosphorylation events (Kumagai and Dunphy, 1991; Gautier et al., 1991; Lee et al., 1992).

Although these predictions were made nearly 20 years ago, *S. pombe* continues to serve as an excellent model system for the study of the biochemical events that regulate the cell
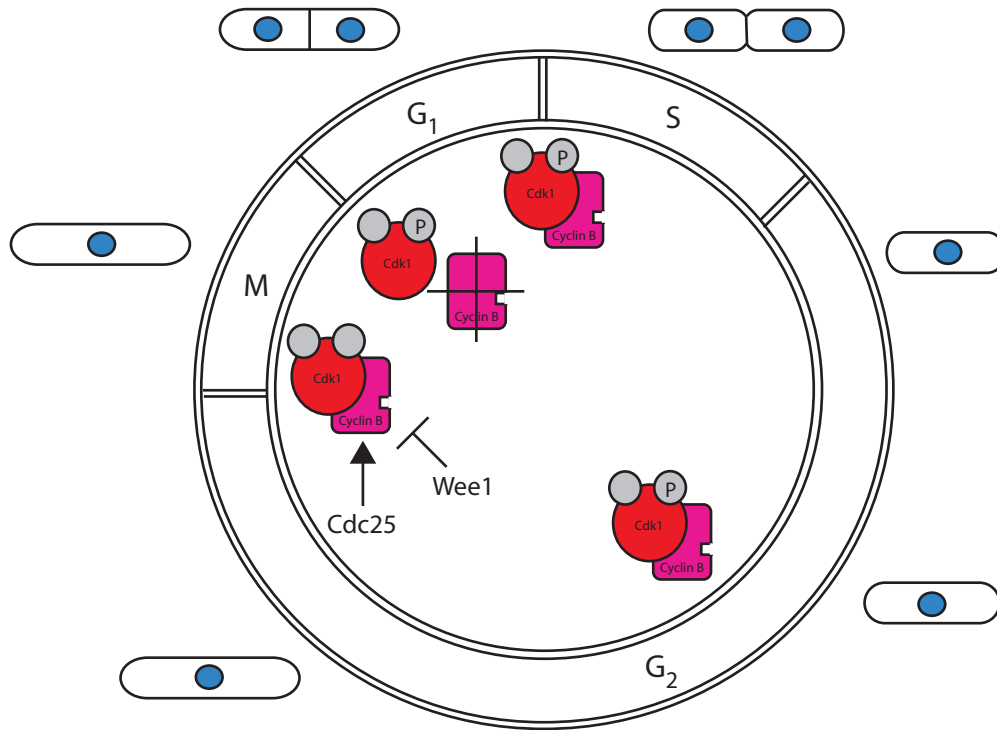
Figure 2. Progression through the cell cycle in *S. pombe*. A) Schematic representation of the *S. pombe* cell cycle. Cells grow by tip elongation at both ends and divide by medial fission at a precise cell size. Cdc25p and Wee1p coordinate cell cycle position with mitotic entry through modulation of the Tyr-15 phosphorylation state of Cdk1p. Mitotic exit is achieved by destruction of cyclin B (Cdc13p).

division cycle. *S. pombe* as a model system combines facile genetics and biochemistry with the ability to infer cell cycle stage as a function of its cell size (Nurse, 1975). Additionally, *S. pombe* is an excellent system for the study of cytokinesis, as it divides through the use of a medially placed contractile ring similar to animal cells (reviewed by Feierbach and Chang, 2001). These combinations make it particularly appealing for the study of the events that regulate mitotic progression.

Regulation of Wee1 and Cdc25

The balance in activities between the inhibitory kinases and the activating phosphatases determines the timing of mitotic initiation (reviewed by O'Farrell, 2001). It is not surprising then that both sets of enzymes are under stringent regulation throughout the course of the cell cycle. Wee1 and Cdc25 are both targets of the DNA damage and replication checkpoints. These checkpoints prevent Cdk1p activation by sustaining Wee1 activity and either inactivating or destabilizing Cdc25 (reviewed by Raleigh and O'Connell, 2000; Donzelli and Draetta, 2003). This regulation is mediated through phosphorylation of checkpoint response sites present on Wee1 and Cdc25. Even in the absence of checkpoint activation, Wee1 and Myt1 activities far outweigh those of Cdc25 during interphase to maintain Cdk1p in the inhibited state (Figure 3A). Strikingly, the same checkpoint response sites are phosphorylated on Cdc25 (Human Ser-216, *Xenopus* Ser-287) during an unperturbed interphase by separate kinases not involved in checkpoint signaling, such as C-Tak1 in mammals (Peng et al., 1998), protein kinase A in *Xenopus* (Duckworth et al., 2002), and the stress activated kinase, Srk1p in *S. pombe* (Lopez-Aviles et al., 2005). That different cell cycle inputs would use the same sensors reveals a remarkable conservation among cell cycle regulation. These phosphorylation events promote Wee1 and Cdc25 binding to 14-3-3 molecules (Lee et al., 2001; Kumagai et al., 1998; Lopez-Girona et al., 1999; Peng et al., 1998). Surprisingly, 14-3-3 interactions with Wee1 or Cdc25 result in dramatically different effects. Wee1 phosphorylation by Chk1 promotes 14-3-3 binding and enhances Wee1 ability to arrest cell division in response to damage (Lee et al., 2001). With regard to Cdc25, this protein-protein interaction plays two important inhibitory roles: first, it acts as a tether for Cdc25 to localize to the cytoplasm (presumably away from Cdk1p in the nucleus), and second, it prevents dephosphorylation of the phosphorylated response site (Margolis et al., 2003). Disruption of this binary complex has been postulated to play a critical role in

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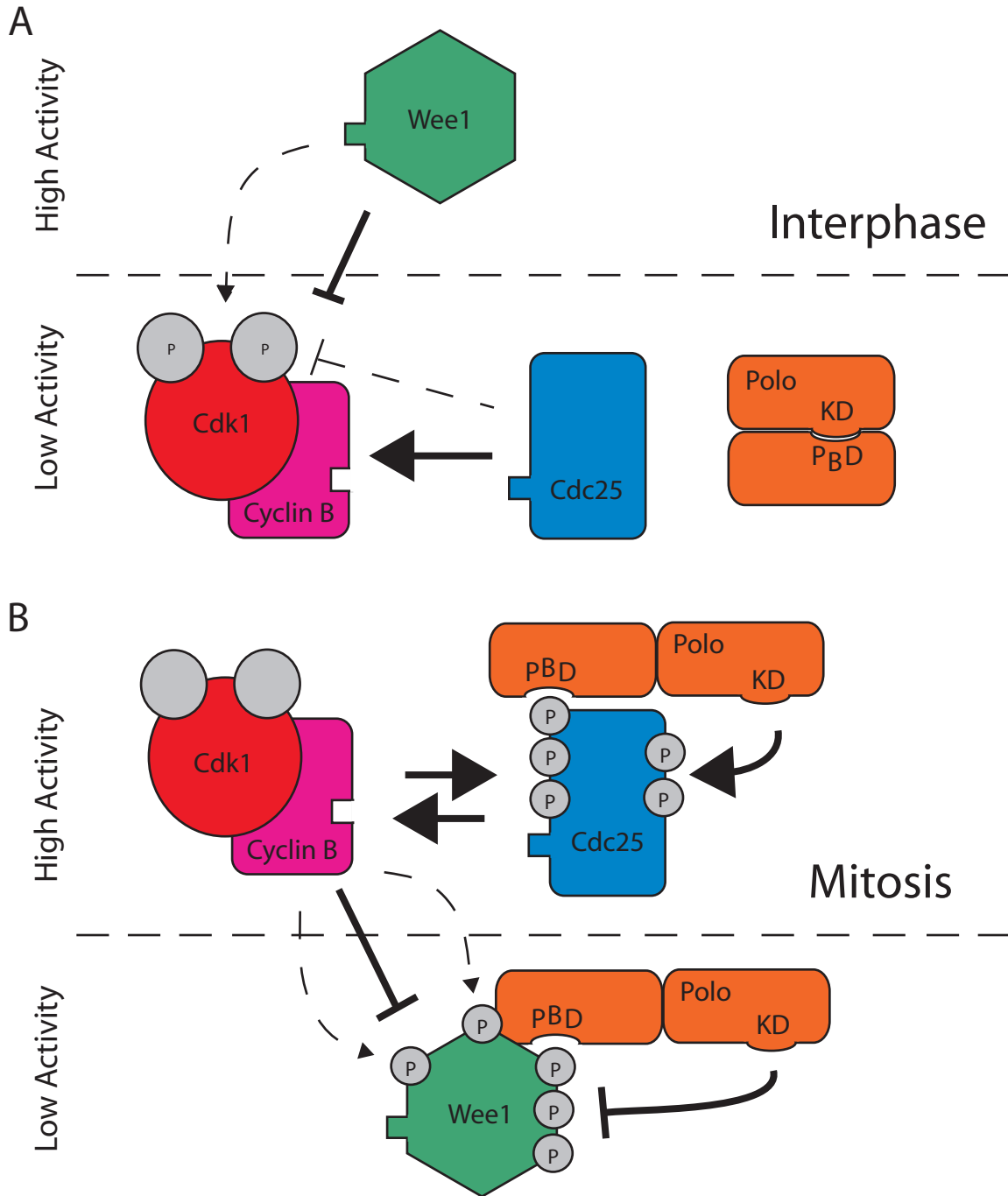
Figure 3. Mitotic initiation in eukaryotes involves a Cdk1p auto-amplification loop. A) During interphase, Cdk1-cyclin B complex is maintained inactive due to inhibitory phosphorylations by elevated Wee1 (Myt1 as well) activity. Cdc25 is unable to reverse these phosphorylations because of attenuated activity and interaction with 14-3-3 (not shown). Polo kinase (orange) remains inactive due to intra-molecular interaction through its kinase domain (KD) and its Polo Box Domain (PBD). B) The balance is switched upon entry into mitosis as Cdc25 activity increases and activates Cdk1-cyclin B complex. Cdk1-cyclin B enters into a positive amplification loop whereby it phosphorylates and activates Cdc25 and phosphorylates and inactivates Wee1. These phosphorylation events relieve Polo auto-inhibition and allow Polo to bind to and target Cdc25 for additional activation and Wee1 for additional inactivation.

determining the timing of mitosis. Accordingly, a mutant of Cdc25 that fails to interact with 14-3-3 is a more potent activator of mitotic initiation (Margolis et al., 2003).

In addition to these regulatory factors that maintain this balance in the interphase state, many additional elements impinge upon Cdc25 and Wee1 and Myt1 activities at the G_2/M transition to promote mitotic initiation. Mitotic commitment is coincident with an attenuation of the inhibitory kinase activities and burst in activity of the activating phosphatases. Wee1 proteins are phosphorylated and inactivated upon entry into mitosis through multiple mechanisms involving a number of kinases (reviewed by Kellogg, 2003). The Nim1p/Cdr1p kinase in fission yeast targets the C-terminal catalytic domain of Wee1p for phosphorylation and attenuates the tyrosine kinase activity of the enzyme (Wu and Russell, 1993; Parker et al., 1993). A second mitotic specific phosphorylation has been described for *Xenopus* Wee1 (Mueller et al., 1995a). This phosphorylation also renders the kinase less active towards its physiological substrate (dephosphorylated Cdk1p) and involves phosphorylation in the N-terminal regulatory domain of *X*Wee1 by Cdk1p. Similarly, the fission yeast Wee1p N-terminus is inhibitory to its *in vivo* function (Aligue et al., 1997; Tang et al., 1993). Additionally, human Wee1 becomes a target for protein degradation by the SCF ubiquitin ligase in response to mitotic phosphorylation events (Watanabe et al., 2004). Myt1 activity is also attenuated upon the transition into M phase (Mueller et al., 1995b; Inoue and Sagata, 2005).

Cdc25 family members are similarly regulated at the post-translational level via phosphorylation upon entry into mitosis. In contrast to the inhibition witnessed with Wee1p and Myt1p family members, Cdc25 activity can be stimulated through mitotic phosphorylation. Mammals contain three different Cdc25 isoforms (A, B, and C), which differ in their regulation and cell cycle timing (reviewed by Donzelli and Draetta, 2003). Both *h*Cdc25C and *h*Cdc25A are mitotic phosphoproteins (Izumi et al., 1992; Hoffmann et al., 1993); however, this phosphorylation differs in its effects. *h*Cdc25C catalytic activity is stimulated upon mitotic phosphorylation, whereas *h*Cdc25A phosphorylation protects the protein from proteolysis and has no influence on activity (Mailand et al., 2000). *S. pombe* Cdc25p is also hyperphosphorylated and hyperactive at the G_2/M transition (Kovelman and Russell, 1996). Similar to mitotic phosphorylation of *h*Cdc25C and *Xenopus* Cdc25, elimination of this phosphorylation returns *Sp*Cdc25p activity to basal levels (Kovelman and Russell, 1996; Kumagai and Dunphy, 1992; Izumi et al., 1992). The mitotic phosphorylation and activation of Cdc25 proteins requires the

activity of Cdk1p, and *in vitro* Cdk1p phosphorylation of *h*Cdc25C and *Xenopus* Cdc25 stimulates its activity (Hoffmann et al., 1993; Izumi and Maller, 1993). *In vivo*, Cdk2p catalyzes the removal of 14-3-3 from *Xenopus* Cdc25 through phosphorylation of its amino terminus (Margolis et al., 2003). Thus, an involvement for Cdk1p directly in the activation of Cdc25 (and inactivation of Wee1 and Myt1) has led to the proposal of an auto-amplification loop involving Cdk1p activation at mitotic onset (Figure 3B). This model has been used to explain why addition of small amounts of active Cdk1p leads to the creation of large amounts of active kinase complex, as well as to account for the rapid and irreversible entry of cells into the mitotic state (reviewed by O'Farrell, 2001). Cdk1p is also responsible for the phosphorylation-dependent stabilization of *h*Cdc25A observed in mitosis (Mailand et al., 2002b). Adding to the complexity of this self-amplification loop is the finding that the conserved Polo family of protein kinases targets these G_2/M regulators for additional levels of phosphorylation and activation/inactivation after prior phosphorylation by Cdk1p (Elia et al., 2003; Watanabe et al., 2004; Inoue and Sagata, 2005).

At exit from mitosis, the dephosphorylation of *h*Cdc25A leads to its recognition and ubiquitination by the Anaphase-Promoting Complex/Cyclosome (APC/C) ubiquitin ligase (Mailand et al., 2002b). Although *h*Cdc25C similarly is dephosphorylated at mitotic exit, this dephosphorylation does not affect its turnover, but rather returns its activity to basal levels (Mailand et al., 2002b). *Sp*Cdc25p has been predicted to be destabilized during late mitosis (Ducommun et al., 1990; Moreno et al., 1990), but it is unclear whether its stability is linked to its phosphorylation status. Additionally, in *S. pombe* the Cdc25p -14-3-3 interaction is resumed during late mitosis (Lopez-Girona et al., 1999). While Ser/Thr protein phosphatases (PPs), such as PP2A, have been predicted to reverse the activating phosphorylations on *h*Cdc25C (Clarke et al., 1993), it is not known if PP2A accounts for all phosphatase activity counteracting activating phosphorylations of Cdc25 homologs during the exit from mitosis.

Regulation of Mitotic Exit

In addition to these G_2/M regulators, Cdk1p phosphorylates a number of mitotic substrates essential for a coordinated progression through mitosis. Cdk1p activity must subsequently be attenuated in late mitosis to allow the exit from mitosis, a period where mitotic structures are disassembled and mitotic events are reversed, as well as for cytokinesis to occur

(Wheatley et al., 1997). Similar to the mitotic inhibition of Wee1 and Myt1, Cdk1p phosphorylates many of its inhibitors and attenuates their activities during mitotic progression. For instance, *S. cerevisiae* Cdk1p phosphorylates the Cdk1p inhibitor, Sic1p, which is then targeted for degradation by the SCF ubiquitin ligase (Verma et al., 1997). Cdk1p additionally phosphorylates the transcription factor for Sic1p, Swi5p, to prevent its accumulation in the nucleus (Moll et al., 1991). The signal that determines the degradation of Sic1p is exquisitely fine tuned to the amount of Cdk1p activity present in cells. Maximal Sic1p turnover requires 6 of the 9 potential phosphorylation sites to be occupied (Nash et al., 2001). During mitotic exit and subsequent reduction in Cdk1p activity, Sic1p is stabilized and further reduces Cdk1p activity.

If high levels of Cdk1p activity inhibit its attenuation, then how is the mitotic state ever inactivated to allow for exit from mitosis? Somewhat anomalously, Cdk1p activity in mitosis has also been reported to initiate the signals that eventually destroy it. Destruction of mitotic cyclin B levels during anaphase drives Cdk1p inactivation at mitotic exit. This destruction is mediated by the ubiquitin system of protein degradation (reviewed by Hochstrasser, 1996). Cyclin B is modified by the addition of a poly-ubiquitin chain, which targets the protein to the proteasome to be degraded. The APC/C adds the poly-ubiquitin chain to cyclin B and other select molecules during anaphase and mitotic exit (reviewed by Morgan, 1999). The APC/C is activated at anaphase when complexed with specific activators capable of targeting the ubiquitin ligase to the correct substrates. One such activator, Cdc20/Fizzy, initiates anaphase by catalyzing the ubiquitination of the anaphase inhibitor, Securin, and cyclin B (Visintin et al., 1997; Lim et al., 1998). Phosphorylations of APC/C components and Cdc20/Fizzy by Cdk1p stabilize the interaction (Rudner and Murray, 2000; Golan et al., 2002), and thus initiate anaphase progression and cyclin proteolysis. However, this model for APC/C activation may not be as simple as presented. Cdk1p activity is also required for maintenance of the spindle checkpoint (D'Angiolella et al., 2003), and high levels of Cdk1p also inhibit the activity of separase (Stemmann et al., 2001), a protease required for sister chromatid separation. It seems likely then that anaphase initiation and the exit from mitosis respond to different amounts of Cdk1p activity. Consistent with this, mitotic Cdk1p inactivation occurs in two phases in *S. cerevisiae* (Yeong et al., 2000). The first begins at the metaphase to anaphase transition and requires the activity of the APC^{Cdc20} to attenuate Cdk1p activity. The second phase requires a separate APC/C complex involving the activator, Cdh1p/Hct1p, and culminates in complete abrogation of Cdk1p activity

at mitotic exit. During these phases, other elements may contribute to a reduction in Cdk1p activity. The Cdk1p auto-amplification loop is turned off at this time, but it is not clear with what kinetics it is extinguished or whether this may play an active role in attenuating Cdk1p activity.

Regulation of Cytokinesis

Critical for maintaining normal patterns of growth and division is the precise coupling of the nuclear division cycle with that of the cytoplasmic division cycle, the process known as cytokinesis. Cytokinesis occurs at the end of mitosis and results in the physical cleavage of one cell into two daughter cells. An outstanding question in mitotic control concerns how cytokinesis and mitosis are coupled to ensure the fidelity of the entire cell division process. Again, model systems, particularly budding and fission yeast, have paved the way for the development of an understanding as to how these events are coordinated. In these simple eukaryotes, a large amount of evidence points to the fact that while Cdk1p activity is necessary for constructing structures which will bring about cytokinesis, its elevated activity during mitotic progression inhibits the elements that trigger cytokinesis (Wheatley et al., 1997; Hwa Lim et al., 2003; Guertin et al., 2000).

Initiation of cytokinesis in eukaryotes involves the regulated contraction of an actin and myosin based ring positioned between the segregated sister chromatids (reviewed by Balasubramanian et al., 2004; Wolfe and Gould, 2005). This structure is temporally regulated to only initiate ring contraction once sister chromatid separation is complete. In budding and fission yeast, analogous pathways termed the Mitotic Exit Network (MEN) and Septation Initiation Network (SIN), respectively, function at the end of mitosis to promote ring contraction and, in these organisms, synthesis of a cell wall (reviewed by Bardin and Amon, 2001; Simanis, 2003). These pathways are composed of a GTPase signaling cascade, which transmits the signal for cytokinesis through a number of effector protein kinases (Table 1).

During late mitosis, these components become activated at the spindle pole body, the yeast counterpart to the mammalian centrosome, and transduce a signal for ring contraction, resulting in the movement of effector kinase(s) to the site of cell division (Sparks et al., 1999; Frenz et al., 2000). Component localization to the spindle pole body allows both the SIN and MEN pathways to be influenced by the nuclear division cycle via their close proximity to Cdk1p

Table 1. MEN and SIN proteins in *S. cerevisiae* and *S. pombe*

Function	*S. cerevisiae*	*S. pombe*
Coiled-coil scaffold	?	Sid4p
Coiled-coil scaffold	Nud1p	Cdc11p
ras-like GTPase	Tem1p	Spg1p
GEF	Lte1p	?
2-component GAP	Bfa1p	Byr4p
2-component GAP	Bub2p	Cdc16p
Protein kinase	Cdc15p	Cdc7p
Protein kinase	?	Sid1p
Accessory protein	?	Cdc14p
Protein kinase	Dbf2p/Dbf20p	Sid2p
Accessory protein	Mob1p	Mob1p
Protein kinase	Cdc5p	Plo1p
Protein phosphatase	Cdc14p	Clp1p/Flp1p
Ring finger protein/inhibitor	Dma1p/Dma2p	Dma1p

(Morrell et al., 2004) as well as by the position of the elongating anaphase spindle where it comes into contact with cortical elements capable of triggering activation of the pathways. Such a mechanism has been very well documented for the inhibition of MEN activity achieved through the two-component GAP system for the Tem1p GTPase, composed of Bub2p and Bfa1p. Upon spindle pole body passage through the mother-bud neck, Bub2p and Bfa1p are inactivated and Tem1p comes into contact with its putative GEF, which initiates MEN signaling (Pereira et al., 2001; Bardin et al., 2000). *S. pombe* utilize a similar strategy for SIN inhibition during interphase and late mitosis. Byr4p and Cdc16p, the two-component GAP system operating in fission yeast, maintain SIN inactive through inhibition of the GTPase, Spg1p (Fankhauser et al., 1993; Furge et al., 1998). Premature inactivation of this GAP complex uncouples cytokinesis from the nuclear division cycle.

Cdk1p activity works with the GAP complexes to maintain the MEN and SIN inactive during mitotic progression. For example, budding yeast Cdc15p effector kinase is inhibited *in vivo* by Cdk1p-dependent phosphorylations (Jaspersen and Morgan, 2000). Similarly, high levels of Cdk1p inhibit fission yeast Sid1p kinase localization to the spindle pole body (Guertin et al., 2000). Inhibiting the activity of certain SIN and MEN components creates an ideal mechanism to couple nuclear and cytoplasmic divisions, such that cytokinesis can only proceed upon elimination of Cdk1p activity (Figure 4A and 4B) (reviewed by Balasubramanian et al., 2000).
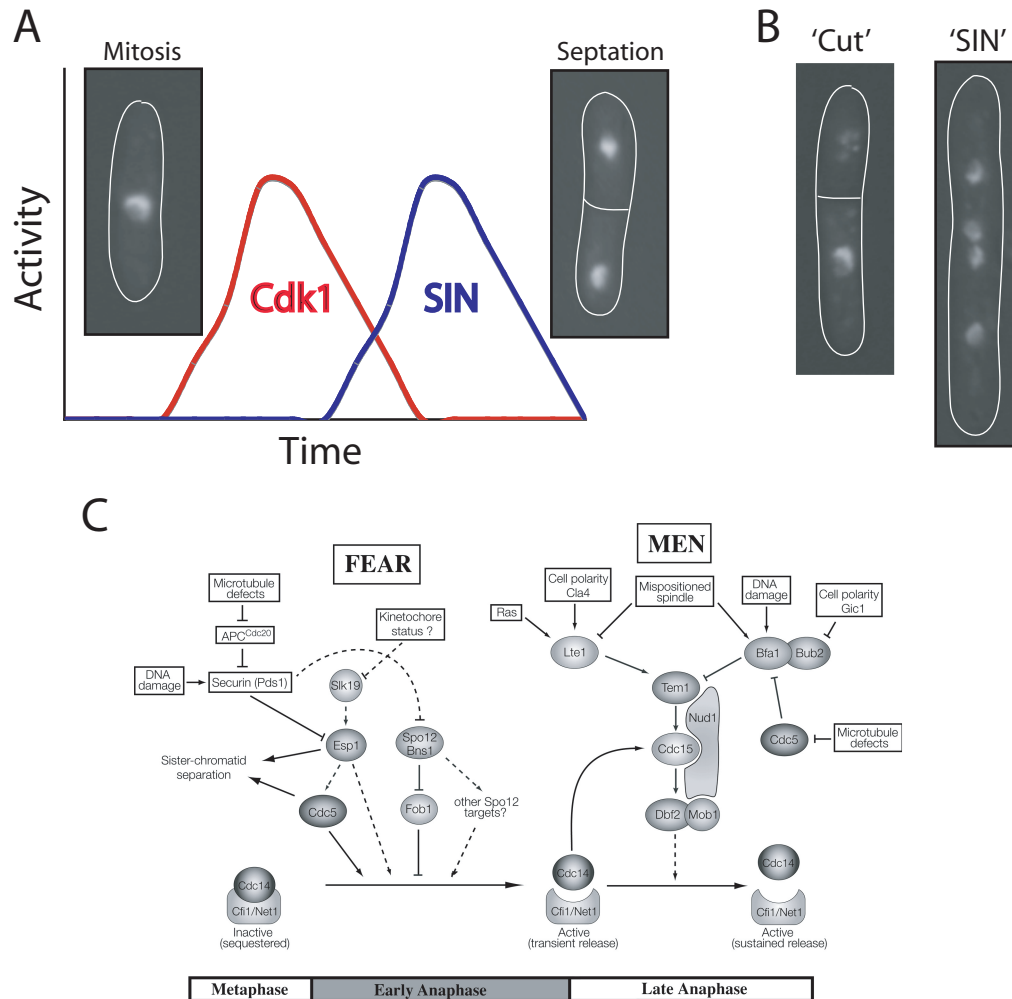
Figure 4. Coordination of nuclear and cytoplasmic divisions in yeast. A) Relationship between activities of Cdk1p versus SIN during mitotic progression. DAPI stained images showing normal coupling of nuclear and septation pathways. Cells are outlined in white to delineate cell boundary. B) DAPI stained images of instances where septation and the nuclear cycle are uncoupled. 'Cut' phenotype results from septation in the absence of chromosome segregation. 'SIN' phenotype results from a failure to prevent further rounds of nuclear division in the absence of septation, and is indicative of SIN mutants. C) Relationships among FEAR (Fourteen Early Anaphase Release) and MEN (Mitotic Exit Network) pathways in *S. cerevisiae* regulate the release of the Cdc14p phosphatase from its nucleolar inhibitor. Adapted from Stegmeier and Amon, 2004.

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While both the SIN and MEN control cytokinesis, only the MEN plays an additional essential function in the inactivation of mitotic Cdk1p activity. MEN mutants fail to exit mitosis and arrest as binucleate cells with segregated sister chromatids, high levels of the B type cyclin, Clb2p, and mitotic Cdk1p substrates in the hyperphosphorylated state (Visintin et al., 1998; Jaspersen et al., 1998). These mutants fail to activate the dual specificity phosphatase, Cdc14p, which catalyzes the dephosphorylation of mitotic Cdk1p substrates, and acts as the terminal effector of the MEN pathway (Figure 4C) (Jaspersen et al., 1998; Shou et al., 1999; Visintin et al., 1999). Although not essential for Cdk1p inactivation at mitotic exit, the SIN is required to prevent additional rounds of nuclear division in the absence of a complete cytokinesis event (Liu et al., 2000; Le Goff et al., 1999). This cytokinesis checkpoint monitors some element of the actin cytoskeleton and additionally requires the function of the inhibitory Wee1p kinase to inhibit Cdk1p and arrest cell division (Liu et al., 2000). In the absence of these functional pathways, nuclear division is not blocked. Whether this inhibition of Cdk1p activity occurs through a Cdc14 family member is unclear.

Cdc14 protein phosphatases

The Cdc14 family of protein phosphatases is conserved from yeast to humans and plays numerous roles during cell cycle progression (Figure 5A) (reviewed by Trautmann and McCollum, 2002). One constant among family members is the ability to specifically dephosphorylate substrates of the cyclin-dependent kinases (Gray et al., 2003; Kaiser et al., 2002). These phosphatases are members of the dual specificity subfamily of protein tyrosine phosphatases. Structurally, they adopt a 2-domain architecture, arranged in tandem with each domain possessing a fold indicative of dual specificity phosphatases (Gray et al., 2003). While the more proximal domain (A domain) lacks the catalytic core required for phosphatase activity, it forms a pocket with the B domain (catalytic domain) necessary for substrate interactions. The B domain contains both the WPD (Trp-Pro-Asp) loop and the PTP (protein tyrosine phosphatase) motif, within which sit critical residues required for the hydrolysis of the phosphoester bond (Figure 5A and 5B) (Gray et al., 2003; Wang et al., 2004).

The best characterized member of this conserved family is the founding member, *S. cerevisiae* Cdc14p. Mutants in *cdc14⁻* display a MEN phenotype and prevent mitotic exit and cytokinesis by failing to properly inactivate Cdk1p in late mitosis (Visintin et al., 1998; Shou et

A

HsCdc14A   1
HsCdc14B   1
CeCdc14   1
SpCdc14   1
ScCdc14   1

WPD Motif   PTP Motif

B   A Domain   B Domain

Nucleolar Targeting   PTP Signature Motif
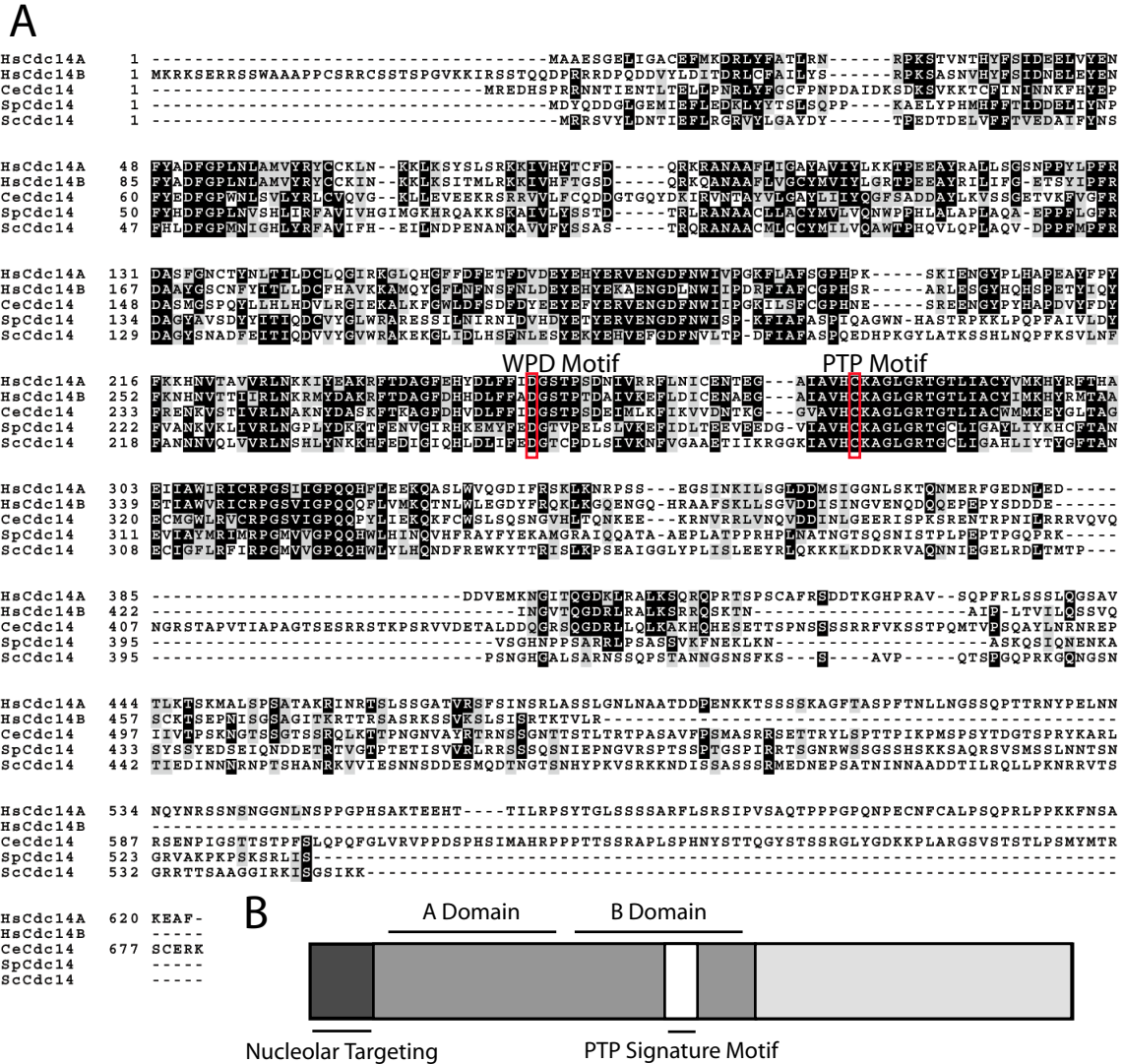
Figure 5. Cdc14 family of protein phosphatases. A) Multiple sequence alignment of Cdc14 family members (*H. sapiens* Cdc14A, *H. sapiens* Cdc14B, *C. elegans* Cdc14, *S. pombe* Clp1p, *S. cerevisiae* Cdc14p) using ClustalW. Boxshade was used to delineate identical amino acids (black background) and similar amino acids (gray background). A red box within the WPD and PTP motifs indicates Asp and Cys residues critical for phosphatase activity. B) Schematic representation of Cdc14 primary amino acid sequence. Nucleolar targeting sequence is shown by a dark gray box, PTP signature motif is shown in white box, and non-conserved C-terminus is shown by light gray box.

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al., 1999). Cdc14p acts as the terminal effector of this pathway since Cdc14p overproduction can rescue growth defects of all MEN mutants for their mitotic exit defects (Jaspersen et al., 1998). Many studies have established that Cdc14p brings about mitotic exit and Cdk1p inactivation by dephosphorylating at least three key substrates: the APC activator Cdh1p/Hct1p, the Cdk inhibitor Sic1p, and its transcription factor Swi5p (Visintin et al., 1998). In addition, Cdc14p targets a number of mitotic substrates at the end of mitosis to promote efficient cell cycle exit. It is not surprising then that Cdc14p is under intense regulation. Cdc14p is sequestered in an inactive state, bound to a competitive inhibitor Net1p/Cfi1p throughout the majority of the cell cycle, only being released at the metaphase to anaphase transition, where it relocates to the mitotic spindle, the spindle pole body, the kinetochore, as well as throughout the nucleus and cytoplasm (Shou et al., 1999; Stegmeier et al., 2002; Visintin et al., 1999; Pereira et al., 2002; Yoshida et al., 2002). Cdc14p release from this inhibition is achieved in two successive waves. The first, termed the FEAR network, occurs at the onset of anaphase and enables Cdc14p to perform a variety of functions necessary for a coordinated progression through anaphase (reviewed by D'Amours and Amon, 2004). The MEN pathway controls the second phase of Cdc14p release and promotes its full activation. A high degree of interplay occurs between Cdc14p and MEN components (Figure 4C). FEAR controlled release of Cdc14p in early anaphase initiates a positive feedback loop for MEN activation involving dephosphorylation of inhibitory phosphorylations on the MEN protein kinase Cdc15p (Stegmeier et al., 2002). Additionally, Cdc14p is also thought to control the termination of this signaling cascade as dephosphorylation and reactivation of the two-component GAP complex by Cdc14p at mitotic exit further silences MEN signaling (Pereira et al., 2002). Only subsequent to MEN activation can Cdc14p inactivate Cdk1p. How this inactivation is restricted temporally is not known.

Due to the unique manner in which budding yeast divide, it is currently unclear whether all organisms will rely so heavily on Cdc14 phosphatases for exit from mitosis. Knockdown experiments on *C. elegans* Cdc14 using RNA*i* results in embryonic lethality. However, these organisms die from a failure to complete cytokinesis, and not from a failure to exit mitosis (Gruneberg et al., 2002). *Ce*Cdc14 function seems to be required for stabilization of the midbody through localizing key spindle midzone factors. While a single Cdc14 homolog exists in *S. cerevisiae*, *S. pombe*, *D. melanogaster*, and *C. elegans*, two isoforms exist in mammals, Cdc14A and Cdc14B. Human Cdc14A and B have different patterns of localization in that throughout

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interphase *h*Cdc14A resides on the centrosome while *h*Cdc14B is sequestered in the nucleolus (Mailand et al., 2002a) (Kaiser et al., 2002). Both of these isoforms are redistributed upon entry into mitosis as the centrosomes participate in formation of the mitotic spindle and the nuclear envelope breaks down. Because of these distinct localizations between the two isoforms, *h*Cdc14A and *h*Cdc14B are thought to regulate distinct cell cycle events. By localizing to the centrosome, *h*Cdc14A is positioned to influence not only microtubule function, but also events that regulate G_2/M transition as active Cdk1p first appears on the centrosomes (Jackman et al., 2003). Consistent with this, deregulation of *h*Cdc14A results in defects in the centrosome cycle as well as in cytokinesis defects (Kaiser et al., 2002; Mailand et al., 2002a). Because of the disparities between different Cdc14 family members and the processes they regulate, there is a need to study additional family members in other eukaryotes to determine whether there is a cohesive nature to their activities. While the negative regulation of Cdk does represent a conserved feature of Cdc14 family members (Gruneberg et al., 2002; Kaiser et al., 2002; Mailand et al., 2002a), their mechanisms of action may differ and be tailored to the unique challenges faced by each organism. The goal of this dissertation is to understand the function and regulation of the *S. pombe* Cdc14 family member, Clp1p, and how these processes contribute to the exit from mitosis.

Summary

A rise in the activity of the conserved Cdk1-cyclin B complex initiates mitotic commitment and drives the phenotypic changes that occur during mitotic progression. Its activity must be attenuated after sister chromatid segregation has occurred to allow for mitotic exit and cytokinesis. As inappropriate Cdk1p activation is often associated with deregulated cell cycle events and genomic instabilities, a more complete understanding of its inhibitors is essential, as these gene products may represent potential tumor suppressors. One such negative regulator of Cdk1p activity is the conserved Cdc14 family of protein phosphatases. These phosphatases reverse Cdk1p dependent phosphorylation events during the exit from mitosis. Here, we examine the role that the Cdc14 family member in *S. pombe*, Clp1p, plays in Cdk1p activity attenuation during the exit from mitosis (Chapter III). We find that Clp1p disrupts the Cdk1p auto-amplification loop in late mitosis to attenuate Cdk1p activity below a threshold that allows for activation of the SIN. We go on to show a mechanism as to how this disruption is restricted

temporally to occur only after the onset of anaphase (Chapter IV). This regulation involves Cdk1p mediated inhibition of Clp1p catalytic activity, and underscores the importance of positive and negative feedback loops that drive progression into and out of mitosis.

CHAPTER II

MATERIALS AND METHODS

Strains, media, and methods

The *S. pombe* strains used in this study (Table 2) were grown in YE or minimal medium with the appropriate supplements as previously described (Moreno et al., 1991). Strains were constructed by tetrad dissections or random spore analysis. DNA transformations were done by lithium acetate transformation (Keeney and Boeke, 1994). Induction of the *nmt* promoter (Maundrell, 1993) was achieved by growing cells in thiamine (repressing conditions) and then washing cells three times in medium lacking thiamine (inducing conditions).

The *clp1*[+] and *cdc25*[+] ORFs were tagged at their 3' ends with either the MYC-Kan[R], HA-Kan[R], GFP-Kan[R], or TAP-Kan[R] cassettes as previously described (Bahler et al., 1998). Kan[R] transformants were screened by whole cell PCR and then visualized using fluorescent microscopy or detected through immunoblot analysis to ensure proper integration and expression of the fusion protein.

Gene replacements involving the *clp1*[+] ORF were performed as described (Boeke et al., 1984). Briefly, *clp1::ura4*^{+} cells were transformed with a genomic construct of *clp1* in pIRT2 vector carrying desired mutation(s). Recombinants were selected for in the presence of the drug, 5-fluororotic acid (5-FOA) to counterselect against the presence of *ura4*[+]. Candidate colonies were screened for proper integration at the *clp1*[+] locus by PCR.

For synchronous cell cycle experiments, strains were centrifugally elutriated using a Beckman JE-5.0 rotor as described (Gould and Feoktistova, 1996). Early G$_2$ cells were collected onto filters and resuspended in appropriate medium concentration. For *cdc25-22* block and release experiments, cells were shifted to the restrictive temperature at 36°C for 4 hours. Cells were then released to the permissive temperature at 25°C to undergo a synchronous mitosis. For *nda3-KM311* block and release experiments, cells were shifted to the restrictive temperature at 18°C for 7 hours, and then released to the permissive temperature at 32°C to undergo a synchronous anaphase.

Table 1. **Strains used in this study**

Strain	Genotype	Source/Reference
KGY 98	h^{90} wee1-50 mik1::ura4$^+$ clp::ura4$^+$ uar4-D18 ade6-M21X leu1-32	This study
KGY 100	h^- mts3-1 cdc25-myc::ura4$^+$ lid1-6 ura4-D18 ade6-M21X leu1-32	This study
KGY 102	h^- mts3-1 cdc25-myc::ura4$^+$ skp1-A4 ura4-D18 ade6-M21X leu1-32	This study
KGY 110	h^- mts3-1 cdc25-myc::ura4$^+$ clp1::ura4$^+$ ura4-D18 ade6-M21X leu1-32	This study
KGY 139	h^+ clp1-HA:kan^R ura4-D18 ade6-210 leu1-32	This study
KGY 148	h^- cdc25-GFP:kan^R sid4-GFP:kan^R clp1::ura4$^+$ ura4-D18 ade6-M210 leu1-32	This study
KGY 246	h^- ade6-M210 ura4-D18 leu1-32	Lab stock
KGY 297	$h^?$ nda3-KM311 cdc25-myc::ura4$^+$ pub1::ura4$^+$ ura4-D18 ade6-M21X leu1-32	This study
KGY 434	h^+ cdc3-124 ura4-D18 leu1-32	Lab Stock
KGY 570	$h^?$ mts3-1 rad1::ura4$^+$ cdc25-myc::ura4$^+$ ura4-D18 ade6-M21X leu1-32	This study
KGY 574	h^- mts3-1 leu1-32	Lab stock
KGY 612	h^- wee1-50 mik1::ura4$^+$ uar4D18 ade6M21X leu1-32	Lab stock
KGY 655	$h^?$ cdc3-124 clp1::ura4$^+$ ura4-D18 ade6-216 leu1-32	Trautmann, et al, 2001
KGY 851	h^- cdc25-22 ura4-D18 ade6-M21X leu1-32	Lab stock
KGY 877	h^+ cdc25-GFP:kan^R sid4-GFP:kan^R ura4-D18 ade6-M210 leu1-32	This study
KGY 878	h^- mts3-1 cut2-myc:kan^R clp1::ura4$^+$ ura4-D18 ade6-M21X leu1-32	This study
KGY 1296	MATa trp1-190 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ lys2::GAL1-HIS3 GAL2-ADE2 met2::GAL-LacZ	James, et al. 1996
KGY 1687	h^+ mts3-1 rad1::ura4$^+$ ura4-D18	Lab Stock
KGY 1744	h^- cdc10-v50 ura4-D18 leu1-32	Lab stock
KGY 1923	h^- mts3-1 cut2-myc:kan^R leu1-32	Berry et al, 1999
KGY 1948	h^- mts3-1 cut2-myc:kan^R lid1-6 ade6-M21X ura4-D18 leu1-32	Berry et al, 1999
KGY 2752	h^+ cdc10-v50 clp1::ura4$^+$ ura4-D18 ade6-M21X leu1-32	This study
KGY 2877	h^- leu1::pJK148-nmt1-clp1$^+$ ura4-D18 ade6-210	This study
KGY 2882	h^- clp1-myc:kan^R ura4-D18 ade6-M21X leu1-32	Trautmann, et al, 2001
KGY 2940	h^- leu1::pJK148-nmt1-clp1::C286S ura4-D18 ade6-M210	This study
KGY 3159	h^- leu1::pJK148-nmt1-clp1-TAP ura4-D18 ade6-210	This study
KGY 3225	$h^?$ leu1::pJK148-nmt1-clp1-TAP wee1::ura4$^+$ ura4-D18 ade6-M21X his3-237	This study
KGY 3374	h^- clp1-GFP:kan^R ura4-D18 ade6-216 leu1-32	Trautmann, et al, 2001
KGY 3377	h^- cdc25-myc::ura4$^+$ ura4-D18 leu1-32	Lopez-Girona et al, 1999
KGY 3380	h^- cdc25-22 clp1::ura4$^+$ ura4-D18 ade6-M21X leu1-32	This study
KGY 3381	h^- clp1::ura4$^+$ ade6-M210 ura4-D18 leu1-32	Trautmann, et al, 2001
KGY 3388	h^+ nda3-KM311 clp1-myc:kan^R ade6-M216 leu1-32	This study
KGY 3499	h^+ cdc25-myc::ura4$^+$ clp1::ura4$^+$ ade6-M21X leu1-32	This study
KGY 3612	h^- nda3-KM311 ura4-D18 leu1-32	Lab stock
KGY 3766	h^+ cdc25-22 clp1-myc:kan^R ura4-D18 ade6-M21X leu1-32	This study
KGY 3783	h^+ nda3-KM311 clp1::ura4$^+$ ura4-D18 ade6-M21X leu1-32	This study
KGY 3916	h^+ nda3-KM311 cdc25-myc::ura4$^+$ ura4-D18 ade6-M21X leu1-32	This study
KGY 3920	$h^?$ nda3-KM311 clp1-TAP:kan^R ura4-D18 ade6-M21X leu1-32	This study
KGY 4213	h^+ nda3-KM311 cdc25-myc::ura4$^+$ clp1::ura4$^+$ ura4-D18 ade6-M21X leu1-32	This study
KGY 4337	h^- cdc25-GFP:kan^R ura4-D18 ade6-M210 leu1-32	This study
KGY 4341	h^- cdc25-GFP:kan^R clp1::ura4$^+$ ura4-D18 ade6-M210 leu1-32	This study
KGY 4366	h^+ mts3-1 cdc25-myc::ura4$^+$ ura4-D18 ade6-M21X leu1-32	This study
KGY 4593	$h^?$ cdc25-22 clp1::6A-myc:kan^R ura4-D18 ade6-M21X leu1-32	This study
KGY 4594	h^- clp1::6A-myc:kan^R ura4-D18 ade6-216 leu1-32	This study
KGY 4604	$h^?$ nda3-KM311 clp1::6A-myc:kan^R ura4-D18 ade6-M21X leu1-32	This study
KGY 4605	$h^?$ nda3-KM311 clp1::D257A-myc:kan^R ura4-D18 ade6-M21X leu1-32	This study
KGY 4630	h^+ nda3-KM311 clp1-GFP:kan^R ura4-D18 ade6-M21X leu1-32	This study
KGY 4687	h^- clp1::D257A-myc:kan^R ura4-D18 ade6-216 leu1-32	This study
KGY 4807	h^- clp1::8A-myc:kan^R ura4-D18 ade6-216 leu1-32	This study
KGY 4826	$h^?$ cdc3-124 clp1::6A-myc:kan^R ura4-D18 ade6-216 leu1-32	This study
KGY 4827	$h^?$ cdc3-124 clp1::8A-myc:kan^R ura4-D18 ade6-216 leu1-32	This study
KGY 4828	$h^?$ cdc3-124 clp1::D257A-myc:kan^R ura4-D18 ade6-216 leu1-32	This study
KGY 4884	h^- clp1::6A/D257A-myc:kan^R ura4-D18 ade6-216 leu1-32	This study
KGY 4891	h^+ nuf2-2	Nabetani, *et al.* 2001
KGY 5018	h^- clp1::C286S-myc:kan^R ura4-D18 ade6-216 leu1-32	This study

Strain	Genotype	Source/Reference
KGY 5080	h^-/h^+ clp1-myc:kan/clp1-HA:kan^R ura4-D18/ura4-D18 ade6-216/ade6-210 leu1-32/leu1-32	This study
KGY 5124	$h^?$ cdc3-124 clp1-myc:kan^R ura4-D18 ade6-216 leu1-32	This study
KGY 5184	h^- clp1::6D-myc:kan^R ura4-D18 ade6-216 leu1-32	This study
KGY 5364	$h^?$ nuf2-2 clp1-myc:kan^R leu1-32	This study
KGY 5365	$h^?$ nuf2-2 plo1-24C clp1-myc:kan^R ura4-D18 ade6-M21X leu-32 his3-D1	This study
KGY 5366	$h^?$ cdc3-124 clp1::6D-myc:kan^R ura4-D18 ade6-216 leu1-32	This study
KGY 5367	$h^?$ cdc3-124 clp1::C286S-myc:kan^R ura4-D18 ade6-216 leu1-32	This study

$h^?$ denotes the mating type was not determined in these studies

Molecular biology techniques

All plasmid constructions were performed by standard molecular biology techniques. Those used in this study are listed in Table 3. All DNA oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Iowa). All sequences were PCR amplified with Taq-Plus Precision (Stratagene) according to manufacturer's protocol. Site-directed mutagenesis was carried out with Chameleon (Stratagene) or Quickchange (Stratagene) according to manufacturer protocols. Automated sequencing confirmed the presence of mutations and the fidelity of the sequence. The $clp1^+$ ORF was amplified from a cDNA clone with oligonucleotides containing Nde1 and BamH1 at the 5' and 3' ends, respectively. The $cdc25^+$ open reading frame was amplified from a genomic clone with oligonucleotides containing Nde1 and Sma1 at the 5' and 3' ends, respectively. All other constructs were subcloned from this construct by restriction digests or through PCR amplification.

Cytology and Microscopy

Cdc25p-GFP and Clp1p-GFP cells were grown in YE medium at 25°C and visualized live using an Ultraview LCI confocal microscope equipped with a 488-nm Ar ion laser (Perkin-Elmer). Z-series optical sections were taken at 0.5 µm spacing, and images captured using Ultraview LCI software (version 5.2, Perkin-Elmer) and processed using Velocity software (version 1.4.2; Improvision). To visualize DNA and microtubules, cells fixed with ethanol were stained with DAPI (4,6-diamidino-2-phenylindole) and anti-TAT1 (tubulin) antibodies, respectively, as described (Chang et al., 2001). FACS analysis was performed as described (Breeding et al., 1998).

Table 3. **Plasmids Used in this Study**

Plasmid Name	Vector	Insert
pKG 45	pMAL-2c	$clp1^+$
pKG 47	pMAL-2c	clp1-C286S
pKG 49	pREP1	$cdc25^+$
pKG 367	pREP81	none
pKG 368	pREP1	none
pKG 596	pRMH41	none
pKG 606	pRHA41	none
pKG 1743	pGAD424	none
pKG 1826	pMAL-2c	none
pKG 2042	pJK148	$nmt1-clp1^+$
pKG 2064	pJK148	nmt1-clp1-C286S
pKG 2105	pGBT9	$clp1^+$
pKG 2106	pGBT9	clp1-C286S
pKG 2120	pJK148	nmt1-clp1-TAP
pKG 2128	pGAD424	$cdc25^+$
pKG 2191	pGAD424	cdc25 1-373
pKG 2229	pGAD424	cdc25 303-596
pKG 2430	pGAD424	cdc25 1-79
pKG 2431	pGAD424	cdc25 1-197
pKG 2432	pGEX4T	cdc25 1-191
pKG 2439	pRHA41	$clp1^+$
pKG 2440	pRHA41	clp1-C286S
pKG 3420	pMAL-2c	clp1-D257A
pKG 3421	pMAL-2c	clp1-6A
pKG 3422	pMAL-2c	clp1-8A
pKG 3425	pMAL-2c	clp1-6D
pKG 3426	pSK	$clp1^+$
pKG 3427	pSK	clp1-C286S
pKG 3428	pSK	clp1-D257A
pKG 3429	pSK	clp1-6A
pKG 3430	pSK	clp1-8A
pKG 3432	pSK	clp1-6A/D257A
pKG 3433	pSK	clp1-6D
pKG 3434	pIRT2	$clp1^+$
pKG 3435	pIRT2	clp1-C286S
pKG 3436	pIRT2	clp1-D257A
pKG 3437	pIRT2	clp1-6A
pKG 3438	pIRT2	clp1-8A
pKG 3440	pIRT2	clp1-6A/D257A
pKG 3441	pIRT2	clp1-6D
pKG 3569	pMAL-2c	cdc25 1-79
pKG 3570	pMAL-2c	cdc25 1-197
pKG 3571	pMAL-2c	cdc25 1-373
pKG 3572	pMAL-2c	$cdc25^+$
pKG 3573	pMAL-2c	cdc25 79-197
pKG 3574	pMAL-2c	cdc25 191-373
pKG 3575	pMAL-2c	cdc25 191-596
pKG 3576	pMAL-2c	cdc25 373-596
pKG 3578	pMAL-2c	cdc25-15A
pKG 3579	pRMH41	$cdc25^+$
pKG 3580	pRMH41	cdc25-C480S
pKG 3581	pRMH41	cdc25-15A
pKG 3582	pRMH41	cdc25 79-596
pKG 3583	pRMH41	cdc25 123-596
pKG 3584	pRMH41	cdc25 191-596
pKG 3585	pREP1	cdc25-15A
pKG 3586	pREP81	cdc25-15A
pKG 3587	pREP81	$cdc25^+$

Protein Lysates, Immunoprecipitations, Lysate Bindings, TAP Purifications, and Immunoblots

Whole cell lysates were prepared by glass bead disruption in NP-40 buffer supplemented with protease inhibitors either in native or denaturing conditions as described (Gould et al., 1991). Native lysates were subjected to immunoprecipitation with anti-Cdk1p (4711), anti-HA (12CA5), or anti-MYC antibodies (9E10). Immunoblot analysis was performed as described (Tomlin et al., 2002) using either HRP conjugated secondary antibodies coupled with enhanced chemiluminescence or using fluorescent labeled secondary antibodies coupled with laser scanning on an Odyssey machine (LI-COR, NE) as described (Ohi et al., 2005). Tandem Affinity Purifications were performed as described (Tasto et al., 2001). For phosphatase assays, native lysates were prepared as before, and immunoprecipitated with 9E10 antibodies. Beads were washed extensively in NP-40 buffer, and then two to three times in phosphatase assay buffer (50 mM Imidazole [pH 6.9], 1 mM EDTA, 1 mM DTT). Beads were then incubated at 30°C for 30 to 45 minutes in phosphatase assay buffer containing recombinant phosphatases. Reactions were separated by SDS PAGE and visualized through immunoblot analysis as described (Tomlin et al., 2002). *In vivo* ubiquitination assays were performed as described (Benito et al., 1998; Berry et al., 1999). Lysate binding experiments were performed as described (Carnahan and Gould, 2003). Yeast-2 hybrid analysis was performed as described (Carnahan and Gould, 2003).

In vitro Kinase and Phosphatase Assays

All recombinant bacterially produced proteins were purified on either glutathione beads (GST) or amylose beads (MBP) as described (Tomlin et al., 2002). Approximately 100 ng of recombinant Cdk1p kinase complex, purified from baculovirus infected insect cells as described (Yoon et al., 2002), were used to phosphorylate approximately 1 μg of bacterially produced MBP-Cdc25p or MBP-Clp1p in HB15 buffer supplemented with 10 μM cold ATP and 5 μCi γ P^{32} ATP. Reactions were incubated at 30°C for 30 minutes and terminated by addition of sample buffer. Samples were boiled and separated by SDS PAGE. Coomassie staining or autoradiography was used for detection of proteins. For phosphatase assays, bacterially produced, soluble GST-Cdc25p 1-197 or GST-Clp1p 348-537 was phosphorylated by kinase active Cdk1p complex as before and recovered on glutathione beads. Beads were washed extensively in TB1 buffer (50 mM Tris-HCl [pH 8.0], 120 mM NaCl, 1 mM EDTA, 1 mM DTT) and eluted in TB1 buffer

containing 20 mM glutathione. Eluted phosphorylated proteins were incubated in the presence of varying concentrations of recombinant phosphatase at 30°C for 45 minutes in phosphatase assay buffer. Reactions were terminated by addition of sample buffer and subsequent boiling. Reactions were separated by SDS PAGE and proteins visualized by Coomassie staining or autoradiography. Histone H1 kinase assays of Cdk1p immunoprecipitates were performed as described (Gould et al., 1991).

Endogenous and Recombinant Cdc25p Activity Assays

Endogenously tagged Cdc25p-MYC activity assays were performed as described (Kovelman and Russell, 1996) except lysates were immunoprecipitated with 9E10 antibodies. Briefly, immunoprecipitates containing Cdc25p-MYC were washed as described in Buffer C (Kovelman and Russell, 1996) supplemented with 0.1% Triton X-100, and incubated with inactive Cdk1p obtained from *cdc25-22* (G_2) lysates. Cdk1p was then re-isolated via immunoprecipitation and Histone H1 kinase assays were performed as described (Gould et al., 1991). Histone H1 assays were quantified by Cherkov scintillation counting.

Between 0.25 – 1 µg of recombinant MBP-Cdc25p were incubated with lysates containing inactive Cdk1p from *cdc25-22* (G_2). Cdk1p was then isolated via immunoprecipitation and Histone H1 kinase assays performed as described (Gould et al., 1991).

Phosphatase Assays Involving Artificial Substrates

pNPP (Sigma, MO) assays were performed as described (Montalibet et al., 2005). Briefly, approximately 2 µg of recombinant MBP-Clp1p were incubated in the presence of phosphatase assay buffer containing 20 mM *pNPP* at 30°C for 30 minutes. Reactions were terminated by addition of 0.25N NaOH and absorbance measured at 410 nm. DiFMUP (Invitrogen, CA) continuous assays were performed on Clp1p immune complexes obtained from 20 OD_{595} of cells as described (Gould et al., 1991) with the exception that cells were lysed in a modified NP-40 buffer lacking sodium vanadate but containing instead 60 mM β-glycerophosphate. Clp1p immune complexes were resuspended in phosphatase assay buffer, supplemented with bovine serum albumin (New England Biolabs, MA) to 250 µg/mL, in a 96-well plate. DiFMUP was added via a FlexStation (Molecular Devices, CA) to a final concentration of 250 µM and fluorescence monitored over time with excitation at 385 nm and emission measured at 455 nm.

Fluorescent readings were plotted using Excel, and the rates of the reaction were determined using linear regression analysis. Protein quantifications for normalization were measured from 1/5 of the assay input using Odyssey software (LI-COR, NE).

In Vivo Labeling and Tryptic Phosphopeptide Mapping

In vivo labeling was performed as described (Gould et al., 1991). Briefly, cells were grown overnight in phosphate-free medium containing 50 μM NaH_2PO_4 and appropriate supplements. Harvested cells were resuspended in phosphate-free medium containing 50 μM NaH_2PO_4 and 2-8 mCi of [^{32}P] orthophosphate and grown at 36°C for 4 hours. Extracts were prepared as described (Gould et al., 1991) and immunoprecipitated for desired protein. Immune complexes were resolved on SDS PAGE and transferred to PVDF membrane and exposed to film for autoradiography. Tryptic phosphopeptide mapping of labeled proteins was performed as described (Den Haese et al., 1995).

CHAPTER III

Clp1p DISRUPTS THE Cdk1p AUTO-AMPLIFICATION LOOP DURING MITOTIC EXIT

Introduction

Cdc14p plays an essential role in mitotic exit in *S. cerevisiae* by targeting cyclin B for degradation as well as activating an inhibitor of Cdk1p. Because budding yeast delay mitotic exit until both sister chromatid separation and traversal into the daughter bud have occurred, it has not been clear whether Cdc14p protein homologs from different eukaryotes play similar essential roles in mitotic exit. We have identified the *S. pombe* Cdc14p family member, Clp1p. While *clp1^{+}* is not essential for mitotic exit as in budding yeast, it is critical for maintaining the normal balance of Cdk1p activity at both the G$_2$/M transition and during mitotic exit. Here, we detail Clp1p's function in inhibiting Cdk1p activity through disruption of the Cdk1p auto-amplification loop during these transitions.

Results

Clp1 regulates the G$_2$/M transition by promoting inhibitory Cdk1p phosphorylation

The following section describes work performed in collaboration with the laboratory of Dr. Dannel McCollum at the University of Massachusetts Medical School. Examination of the *S. pombe* genome database from the Sanger Centre revealed a predicted protein homolog (ORF SPAC1782.09c) of the *CDC14* gene from *S. cerevisiae*. *S. pombe* Cdc14-like phosphatase (Clp1p, also known as Flp1p) shares 40% identity with budding yeast Cdc14p. Unlike *CDC14*, *clp1^{+}* is not essential for vegetative growth as disruption of the ORF with the *ura4^{+}* marker in a diploid strain resulted in four viable spores (data not shown). Although viable, *clp1Δ* cells have noticeable growth defects, the most significant of which are a reduction in cell size at division and defects in cytokinesis. Whereas wild type cells divide precisely at 14 μm, *clp1Δ* cells divide at an average of 9.3 μm at 36°C (Figure 6A). This phenotype is indicative of a deregulated G$_2$/M transition, occurring when the balance of activities between Cdc25p and Wee1p is tipped in favor of the mitotic state prematurely (Russell and Nurse, 1987). Consistent with this, strong overproduction of Clp1p from the inducible *nmt1* promoter is toxic and led to a prolonged delay
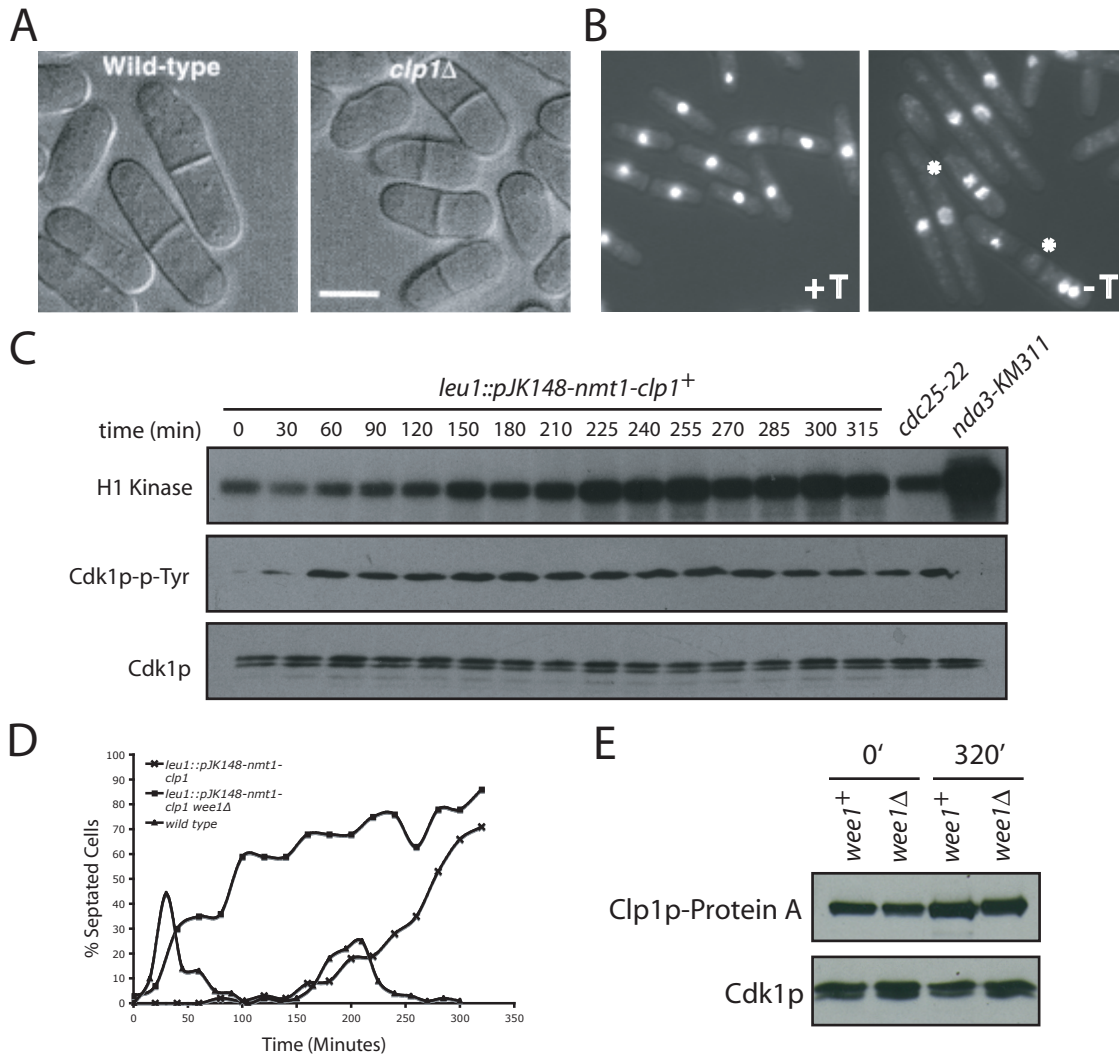
Figure 6. Clp1p regulates the G_2/M Transition. A) Differential Contrast Images (DIC) of *wild type* (KGY 246) or *clp1Δ* (KGY 3381) cells grown at 36ºC. Scale bar represents 5 µm (Images taken from Trautmann, *et al*, 2001). B) *leu1::pJK148-nmt1-clp1* (KGY 2877) cells grown in either the presence of thiamine (repressing conditions, +T) or in the absence of thiamine (inducing conditions, -T) were fixed with ethanol and stained with DAPI to visulaize nuclei. Asterisks indicate cells that have failed to septate between the segregating chromosomes. C) *leu1::pJK148-nmt1-clp1* (KGY 2877) cells were grown in the absence of thiamine for 14.5 hours at 32ºC, at which point small G_2 cells were isolated by centrifugal elutriation. Samples were processed at the indicated time points for Histone H1 kinase activity, and levels of Cdk1p-p-tyr and Cdk1p by immunoblotting. D) *leu1::pJK148-nmt1-clp1-Protein A* (KGY 3159) and *leu1::pJK148-nmt1-clp1-Protein A wee1Δ* (KGY 3225) cells were grown in the absence of thiamine for 12 hours at 32ºC, at which point small G_2 cells were isolated by centrifugal elutriation. Septation index was determined by light microscopy. *Wild type* (KGY 246) cells were synchronized by centrifugal elutriation and used as control. E) Samples from elutriations in D) were prepared and the levels of Clp1p-Protein A and Cdk1p were determined by immunblot analysis.

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in G$_2$ phase of the cell cycle (Figure 6B). Synchronous cells overproducing $clp1^+$ produced a delay in G$_2$ for around 3 hours, a time period in which wild type cells underwent 2 successive divisions (Figure 6D). *S. cerevisiae* Cdc14p overproduction is also lethal, and promotes premature destruction of cyclin B prior to mitotic initiation (Visintin et al., 1998). During this delay upon overproduction of Clp1p, cyclin B was not destabilized prematurely (data not shown), but rather Cdk1p was maintained in the inhibited and Tyr-15 phosphorylated state (Figure 6C). As a result, Histone H1 kinase activity remained low throughout the time course. Once Clp1p overproducing cells entered mitosis, they suffered additional defects such as mitotic spindle defects (data not shown), nuclear positioning defects (data not shown), and often times septation occurring in the absence of proper sister chromatid segregation (Figure 6B, see asterisks). Because Wee1p is the primary kinase regulating Cdk1p Tyr-15 phosphorylation in *S. pombe* (Parker et al., 1992; Russell and Nurse, 1987), we tested whether the G$_2$ delay required a functional Wee1p. When a similar amount of Clp1p was overproduced in a strain deleted for $wee1^+$ (Figure 6E), no delay in mitotic progression was observed (Figure 6D). Overproduction of Clp1p in a $wee1\Delta$ background leads to an accumulation of multiple rounds of septation after entry into mitosis and as a result cells lose viability (Figure 6D and data not shown). Therefore, Wee1p lies functionally downstream of Clp1p in the regulation of the G$_2$/M transition, and these data suggest that Clp1p overproduction results in the stabilization of activated Wee1p and potentially the inactivation of Cdc25p. An inability to activate Wee1p and inactivate Cdc25p in cells deleted for $clp1^+$ could also account for its reduced cell size at division (see below). These events would likely be mediated through Clp1p dephosphorylation of Cdk1p phosphorylation sites on Wee1p and Cdc25p, thereby preventing mitotic entry through premature disruption of the Cdk1p auto-amplification loop. Consistent with a phosphatase dependent function for Clp1p in this process, Clp1p phosphatase activity was required for the imposed G$_2$ delay (Figure 7A). In fact, strong overproduction of a catalytically inactive mutant, Clp1p-C286S, resulted in only a slight delay in the kinetics of progression through G$_2$. Instead, these cells delayed progression through mitosis and delayed Cdk1p inactivation for approximately 80 minutes (Figure 7C). A high percentage of cells overproducing Clp1p-C286S failed to properly construct a bipolar spindle (Figure 7D), the failure of which often times led to incomplete genome segregation to both daughter cells (data not shown). The toxic effect of Clp1p-C286S overproduction is most

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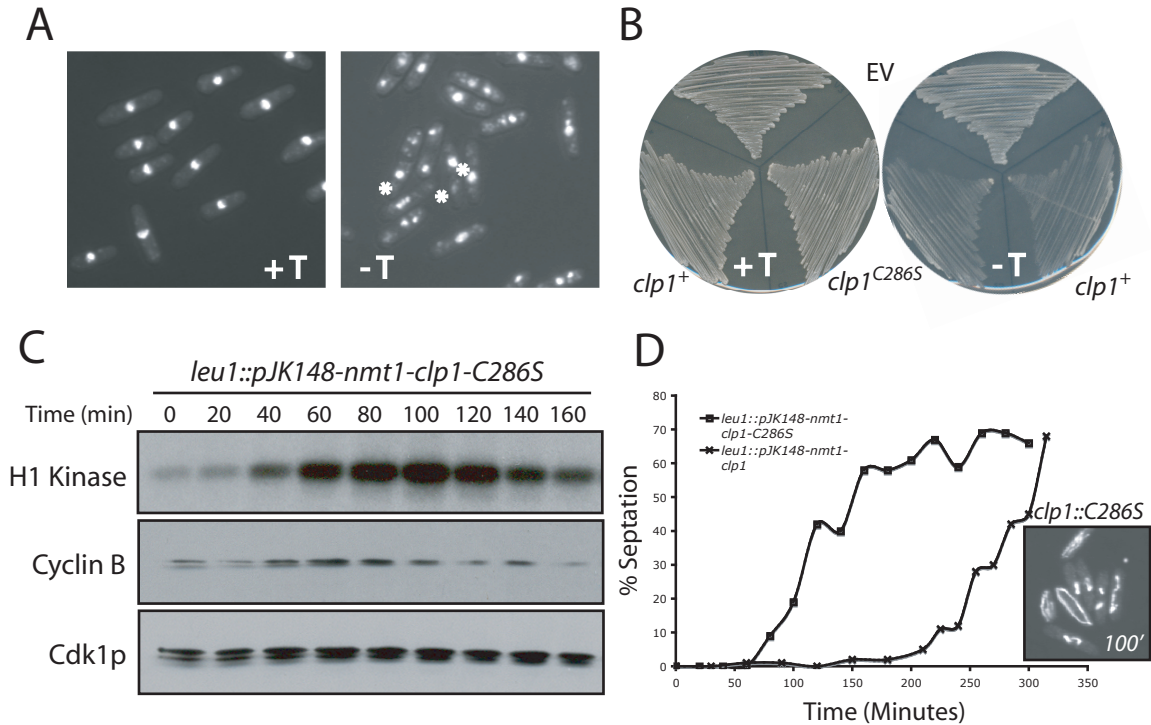
Figure 7. Clp1p-C286S delays progression through mitosis. A) *leu1::pJK148-nmt1-clp1-C286S*
(KGY 2940) were grown overnight at 32ºC in the presence (repressing conditions, +T) or absence of
thiamine (inducing conditions, -T) prior to being fixed with ethanol and stained with DAPI to visualize
nuclei. Asterisks indicate presence of cells that have undergone septation in the absence of chromosome
segregation. B) *wild type* (KGY 246) cells transformed with *pREP1:Empty vector (EV)*,
leu1::pJK148-nmt1-clp1 (KGY 2877), and *leu1::pJK148-nmt1-clp1-C286S* (KGY 2940) were struck
onto plates containing (+T) or lacking (-T) thiamine and grown for 48 hours at 32ºC.
C) *leu1::pJK148-nmt1-clp1-C286S* (KGY 2940) cells grown in the absence of thiamine for 14.5 hours
were synchronized to select for small G_2 cells by centrifugal elutriation. Samples were taken at the
indicated time points and processed for Histone H1 activity and cyclin B (Cdc13p) and Cdk1p levels
through immunoblot analysis. D) *leu1::pJK148-nmt1-clp1* (KGY 2877) and *leu1::pJK148-nmt1-clp1-
C286S* (KGY 2940) were grown in the absence of thiamine as in C) and processed for septation index by
light microscopy. Inset, *leu1::pJK148-nmt1-clp1-C286S* (KGY 2940) cells from C) at 100' were fixed
with ethanol and visualized for microtubules via indirect immunofluorescence.

likely due to a failure to prevent septation in the absence of undergoing proper chromosome segregation (Figure 7A, see asterisks, and 7B).

Regulation of Clp1p Localization

In this collaboration, our labs also demonstrated that, similar to budding yeast Cdc14p (Shou et al., 1999; Visintin et al., 1999), *S. pombe* Clp1p shows a dynamic pattern of localization during the course of the cell cycle. In particular, throughout interphase a functional Clp1p-GFP fusion localizes to the spindle pole body, the yeast counterpart to the mammalian centrosome, and to the non-DAPI staining region of the nucleus, which corresponds to the nucleolus (Figure 8A). Upon entry into mitosis, Clp1p-GFP exits the nucleolus and localizes to the contractile ring and the forming mitotic spindle, as well as throughout the nucleus and cytoplasm (Figure 8B). This localization pattern appears to be a major source of Clp1p regulation as bulk protein levels do not fluctuate through the cell cycle (Figure 8D). Clp1p-GFP release prior to initiation of anaphase is distinct from the release of budding yeast Cdc14p, which does not occur until anaphase onset (Stegmeier et al., 2002; Pereira et al., 2002; Yoshida et al., 2002). To confirm this observation, we examined Clp1p-GFP localization in cells arrested with the *nda3-KM311* mutant, a mutation in β-tubulin that activates the spindle checkpoint in response to perturbations in the microtubule cytoskeleton (Hiraoka et al., 1984). Clp1p-GFP nucleolar signal is absent during this block, and Clp1p-GFP becomes distributed throughout the nucleus and cytoplasm, and is concentrated to the contractile ring and the kinetochore (Figure 8C). These data suggest that Clp1p is fully released from the nucleolus prior to the initiation of anaphase, and that Clp1p may play important roles during the early stages of mitotic progression (Trautmann et al., 2004).

Additional data went on to support a model whereby Clp1p exits the nucleolus during early mitosis in a SIN independent manner, but its return to the nucleolus is prevented through sustained SIN activation (Trautmann et al., 2001; Cueille et al., 2001). That SIN activity sustained the release of Clp1p from the nucleolus was revealed through analysis of the cytokinesis checkpoint mutant, *cps1-191*. *cps1-191* mutant cells, in response to a failure to initiate cytokinesis, arrest as binucleates possessing two G_2 nuclei that are blocked prior to initiation of the next round of mitosis (Le Goff et al., 1999; Liu et al., 1999). Although they fail to undergo septation and cytokinesis, *cps1-191* mutants arrest with active SIN signaling (Liu et al., 2000). Maintenance of the mitotic arrest requires the Wee1p inhibitory kinase as well as an
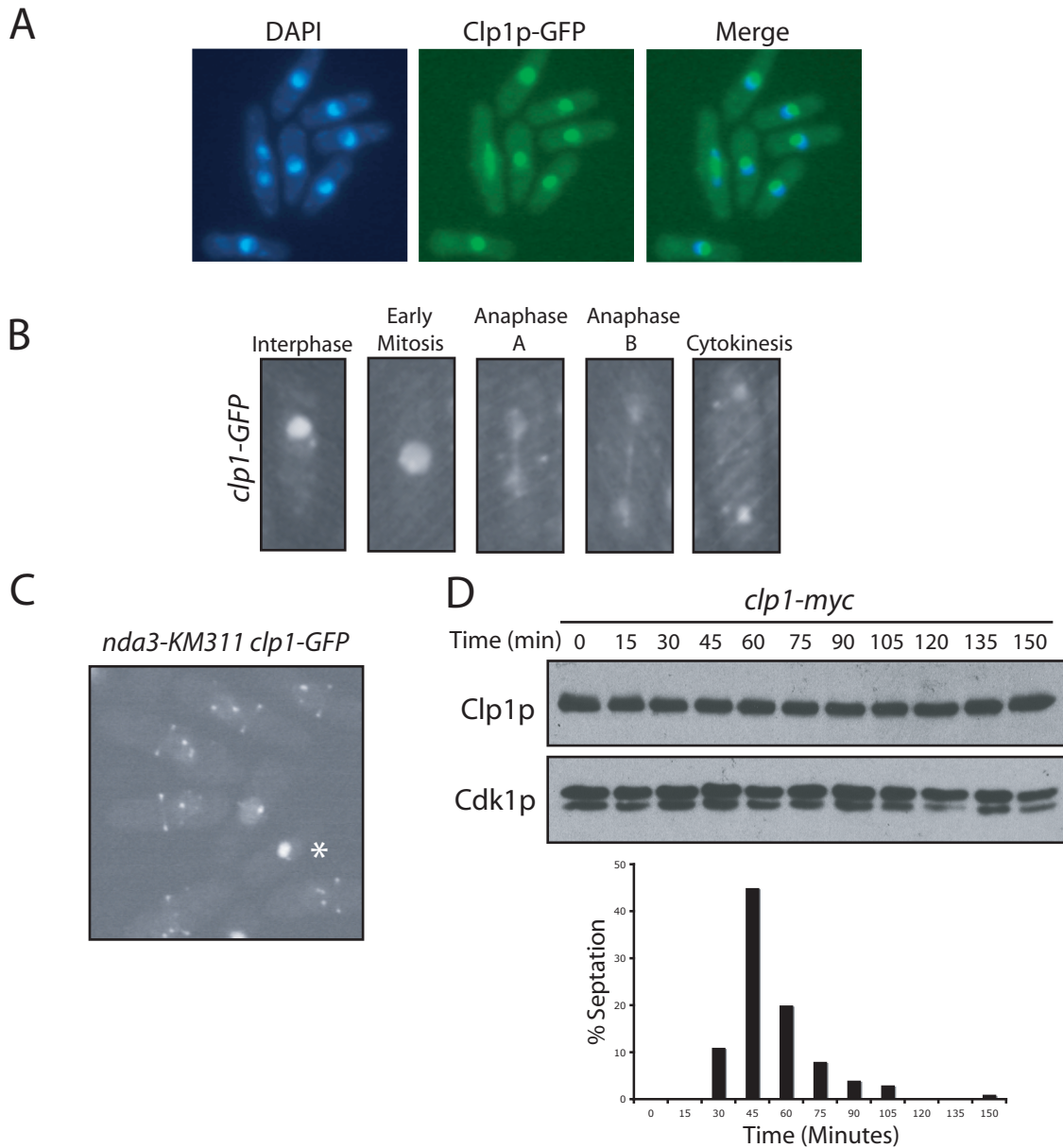
Figure 8. Regulation of Clp1p through the cell cycle. A) *clp1-GFP* (KGY 3374) cells were grown at 25ºC, fixed with Methanol, and stained with DAPI to visualize nuclei. B) Compression of image stacks of asynchronous *clp1-GFP* (KGY 3374) cells grown at 25ºC in YE media. C) *nda3-KM311 clp1-GFP* (KGY 4630) cells grown at 18ºC were viewed live for GFP fluorescence. Asterisk indicates position of cell not yet arrested at the block point with Clp1p-GFP retained in the nucleolus. D) *clp1-myc* (KGY 2882) cells were grown at 32ºC overnight prior to synchronization by centrifugal elutriation. Samples were processed for septation index via light microscopy (bottom panel) and for Clp1p and Cdk1p levels via immunoblotting.

active SIN (Le Goff et al., 1999; Liu et al., 2000). During this block, Clp1p-GFP fails to localize to the nucleolus even though the cells are biochemically arrested in G_2, a point in the cell cycle in which Clp1p is normally found in the nucleolus (data not shown). Providing a link between the SIN and Wee1p, Clp1p is additionally required to maintain this checkpoint (Figure 25B) (Trautmann et al., 2001; Cueille et al., 2001). This is presumably due to Clp1p indirect effects on contractile ring stability through its ability to maintain an active SIN (Mishra et al., 2004).

A small percentage of *clp1Δ* cells fail at cytokinesis and show strong negative genetic interactions with mutants in the SIN pathway (data not shown). *clp1Δ* cytokinesis failure is exacerbated when combined with activating mutations of Cdk1p or inactivating mutations of Wee1p (data not shown). These data underscore the antagonistic action that elevated Cdk1p activity plays to SIN signaling during mitotic progression. Consistent with a role for Clp1p in cytokinesis, SIN signaling is attenuated in *clp1Δ* mutants (data not shown).

<center>Clp1p targets Cdc25p for dephosphorylation at mitotic exit</center>

Having established that Clp1p regulates the G_2/M transition by promoting the inhibited, Tyr-15 phosphorylated form of Cdk1p, and that the Wee1p inhibitory kinase is necessary for this effect (Cueille et al., 2001; Trautmann et al., 2001), we wished to address whether or not Clp1p also influenced the function of the counterbalancing phosphatase, Cdc25p. To this end, we created two populations of synchronous cells through centrifugal elutriation both expressing endogenously tagged Cdc25p-MYC, either in a *clp1+* background or in a deletion of it. Cdc25p levels were elevated in the absence of *clp1+* at all cell cycle stages examined (Figure 9A).

We next examined whether Cdc25p, a highly phosphorylated protein during mitosis (Kovelman and Russell, 1996; Moreno et al., 1990), was a target for dephosphorylation by Clp1p at mitotic exit. We utilized the reversible, cold-sensitive *nda3*-KM311, to arrest cells in an early mitotic state either in the absence or presence of *clp1+*. These cells were then released to the permissive temperature and monitored for Cdc25p levels and phosphorylation state by immunoblot analysis. As the *clp1+* exited mitosis and septated (Figure 9C), Cdc25p became hypophosphorylated and slightly destabilized at the completion of mitotic exit. In the absence of *clp1+*, Cdc25p remained at high levels and in the hyperphosphorylated state (Figure 9B).

Clp1p may target Cdc25p at mitotic exit for dephosphorylation, and hence, inactivation, to help downregulate Cdk1p activity. To test if the hyperphosphorylated Cdc25p observed in the

<center>32</center>
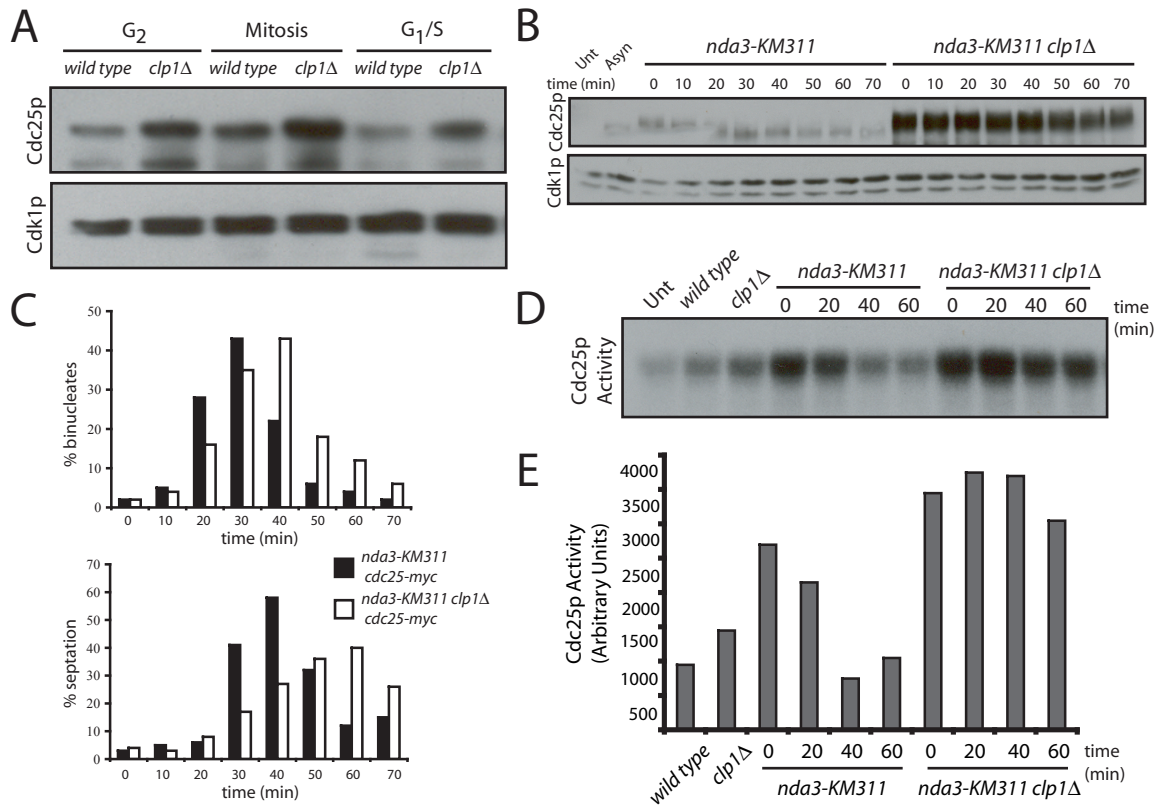
Figure 9. *clp1*[+] regulation of Cdc25p. A) *cdc25-myc* (KGY 3377) or *cdc25-myc clp1Δ* (KGY 3499) were grown to mid-log phase and synchronized by centrifugal elutriation. Samples were taken after synchronization and processed for protein (A) and cell cycle stage by DAPI staining and cell measurement analysis as follows: G2 cells were uni-nucleate and 8-12 μm in length, Mitotic cells were uninucleate or binucleate cells with condensed chromatin, G1/S cells contained two interphase nuclei and a septum (data not shown). Cdc25p-myc and Cdk1p were detected by immunoblotting with 9E10 and anti-PSTAIR antibodies, respectively. B) *nda3-KM311 cdc25-myc* (KGY 3916) and *nda3-KM311 cdc25-myc clp1Δ* (KGY 4213) were grown to mid-log phase and synchronized by cold shift to 18°C for 7 hours. Cells were then released to the permissive temperature (32°C), and samples were collected at the indicated time points. Extracts were prepared from these and processed for immunoblot analysis. Completion of mitotic exit was monitored by determining binucleate formation (C, top panel) and septation index (C, lower panel). For the Cdc25p immunoblot, samples were immunoprecipitated with 9E10 antibody and immunoblotted with anti-Cdc25p antibodies. D) Cells were synchronized as in B) and samples were collected at the indicated time points. Extracts were prepared and immunoprecipitated with 9E10 antibody. Samples were processed for Cdc25p activity and quantified and normalized to basal levels of activity (Unt) in E) indirectly via activation of Cdk1p's Histone H1 activity as described in material and methods. In this experiment, Cdc25p activity was a saturable reaction (data not shown).

absence of *clp1*[+] at mitotic exit is still active, we looked at Cdc25p activity from an *nda3-KM311* block/release experiment as before. In *clp1Δ* cells, Cdc25p activity was elevated and persisted even as cells exited mitosis, whereas wild type cells inactivated Cdc25p within 40 minutes after release (Figure 9D and 9E).

Cdc25p localizes within the nucleus where it presumably encounters its substrate, Cdk1p, from the late stages of interphase until the mid stages of anaphase (Lopez-Girona et al., 1999). To determine if the lack of Clp1p led to increased amounts of nuclear Cdc25p, the *cdc25*[+] endogenous locus was tagged at its C-terminus with a *gfp* cassette (Bahler et al., 1998) in the presence or absence of *clp1*[+]. *clp1Δ* cells showed a higher percentage of binucleates with nuclear Cdc25p-GFP staining than did wild type cells (Figure 10A, arrows). To quantify this, we created a *cdc25*[+]-*gfp* strain that also contained endogenously *gfp*-tagged *sid4*[+]. Sid4p is a constitutive spindle pole body component (a subcellular localization we have not observed for endogenous Cdc25p-GFP), and served as a marker for mitotic progression (Tomlin et al., 2002). In *clp1*^{+} cells, Cdc25p-GFP nuclear staining was observed in only 17% of late anaphase cells, whereas in the absence of *clp1*[+] this percentage increased to 67% (Figure 10B and 10C). At this time, we can not distinguish whether the increased Cdc25p levels observed in the absence of *clp1*[+] enhanced our ability to detect late anaphase nuclear fluorescence, as opposed to Clp1p specifically regulating rates of Cdc25p nuclear import/export.

Clp1p promotes the destabilization and ubiquitination of Cdc25p

Cdc25p homologs in higher eukaryotes are degraded via ubiquitin-mediated proteolysis, often in response to DNA damage checkpoints (reviewed by Donzelli and Draetta, 2003). To address whether *S. pombe* Cdc25p is destabilized during G_1 phase and whether or not this was *clp1*[+] dependent, we expressed the catalytically inactive Cdc25p-C480S from the inducible *nmt* promoter in cells arrested in G_1 with a *cdc10-V50* allele. Thiamine (repressing conditions) was then added to the cultures along with cyclohexamide to prevent protein translation, and Cdc25p levels were monitored via immunoblot analysis. Cdc25p was nearly undetectable (see Asterisk in Long Exposure) in *cdc10-V50* arrested cells (Figure 11A). However, in the absence of *clp1*[+], Cdc25p was stabilized. FACS analysis confirmed that these cells remained in G_1 at the end of the time course (Figure 11B).

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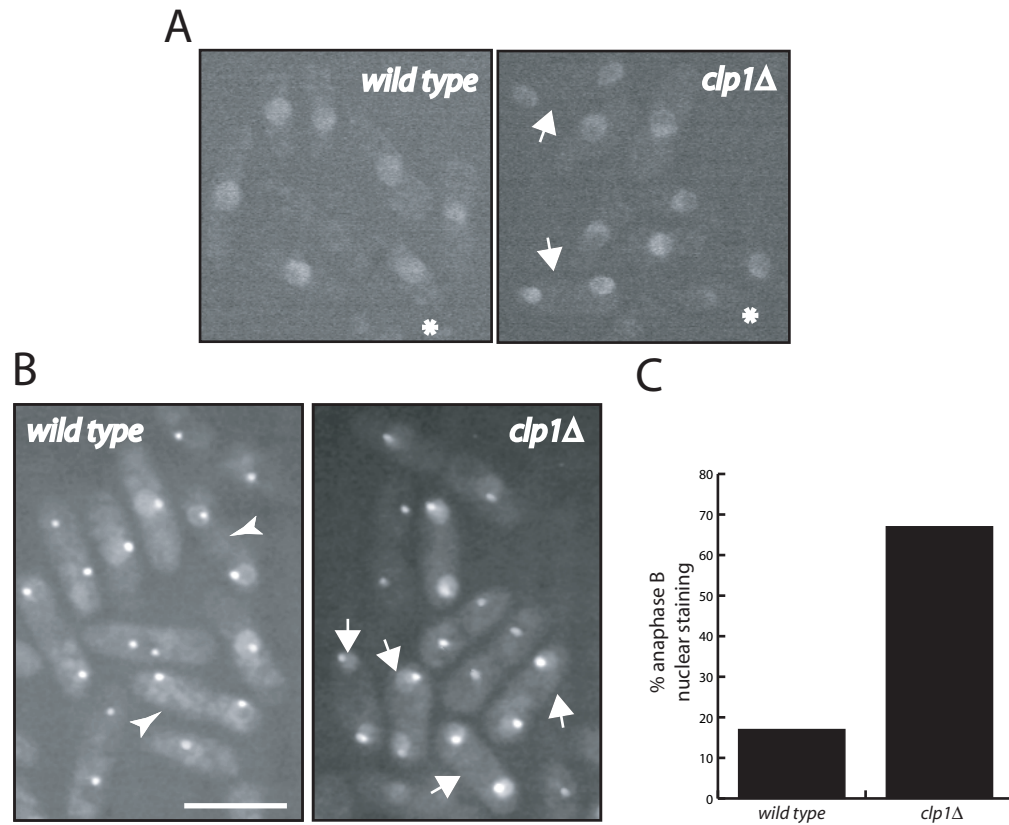
Figure 10. Cdc25p remains nuclear in *clp1Δ* anaphase B cells. A) Images of *cdc25-GFP* (KGY 4337) and *cdc25-GFP clp1Δ* (KGY 4341) asynchronous cells grown at 25°C in YE medium. Asterisks (*) indicate binucleate cells without Cdc25p-GFP nuclear fluorescence. B) Compression of image stacks of *cdc25-GFP sid4-GFP* (KGY 877) and *cdc25-GFP sid4-GFP clp1Δ* (KGY 148) asynchronous cells grown at 25°C in YE medium. Pointed arrows indicate binucleate cells with Cdc25p-GFP staining at the nuclear periphery. Solid arrowheads indicate binucleate cells with nuclear Cdc25p-GFP. Scale bar indicates 10 μM. C) Percentage of anaphase B cells with nuclear Cdc25p-GFP from (B). Anaphase B cells were those with 2 well separated spindle pole bodies (Sid4p-GFP) and no septum.
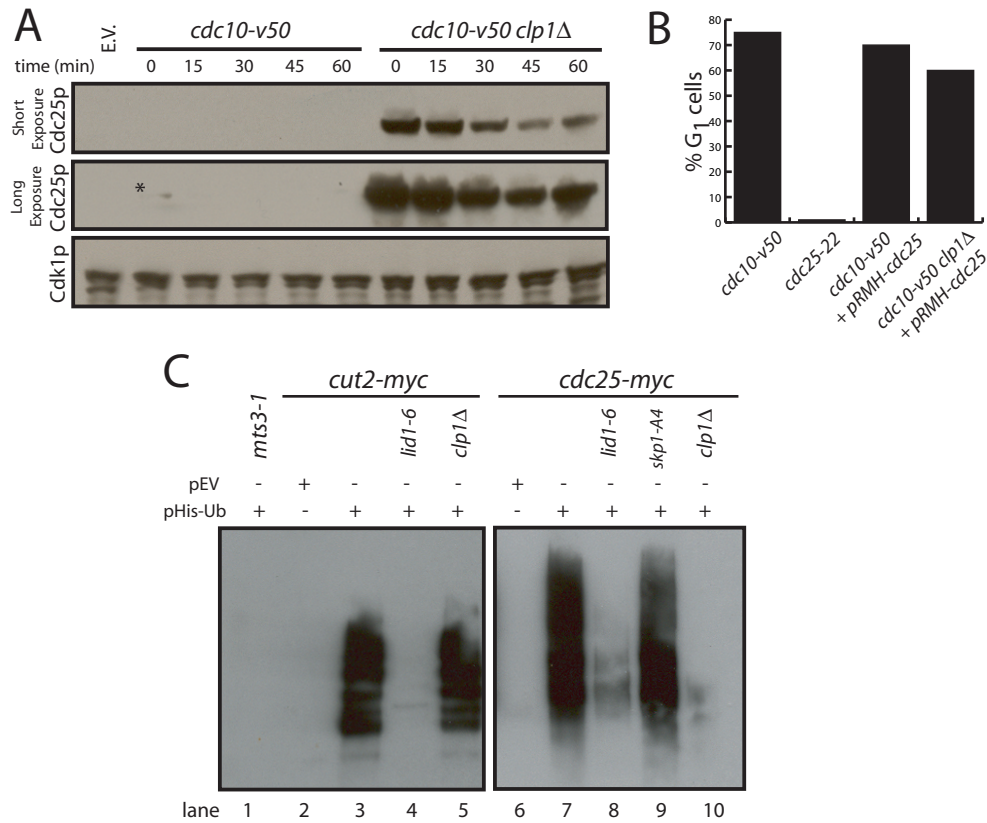
Figure 11. Cdc25p destabilization and ubiquitination require Clp1p and APC/C. A) *cdc10-V50* (KGY 1744) and *cdc10-V50 clp1Δ* (KGY 2752) cultures expressing *pREP41myc-cdc25^{C480S}* were grown in the absence of thiamine for 20 hours at the permissive temperature (25°C), shifted to the non-permissive temperature (36°C) for 3.5 hours, at which point excess thiamine (4 μM) and cyclohexamide (100 μg/ml) were added to the cultures. Samples were taken at the indicated time points. Extracts were prepared and immunoblotted with 9E10 and anti-PSTAIR to detect Cdc25p and Cdk1p, respectively. B) Percentage of G1 cells from A) as determined by Sytox Green (Molecular Probes) staining and flow cytometry. C) In vivo ubiquitination assays. *mts3-1* (KGY 574) (lane 1), *mts3-1 cut2-myc* (KGY 1923) (lane 3), *mts3-1 cut2-myc lid1-6* (KGY 1948) (lane 4), *mts3-1 cut2-myc clp1Δ* (KGY 878) (lane 5), *mts3-1 cdc25-myc* (KGY 4366) (lane 7), *mts3-1 lid1-6 cdc25-myc* (KGY 100) (lane 8), *mts3-1 skp1-A4 cdc25-myc* (KGY 102) (lane 9), and *mts3-1 clp1Δ cdc25-myc* (KGY 110) (lane 10) strains transformed with *pREP1-His6-Ubiquitin*, and *mts3-1 cut2-myc* (KGY 1923) (lane 2) and *mts3-1 cdc25-myc* (KGY 4366) (lane 6) transformed with empty vector were grown at 25°C for 22 hours in the absence of thiamine to induce His6-Ubiquitin expression and then shifted to 36°C for an additional 4 hours. Samples were collected and extracts were prepared. Ubiquitin-conjugates were purified on Ni-NTA beads, separated on SDS PAGE, and immunoblotted with 9E10 antibodies to detect Cut2p and Cdc25p.

Human Cdc25A and Cdc25C have been reported to be APC/C substrates, and in the case of Cdc25A, the SCF-ubiquitin dependent pathways as well (reviewed by Donzelli and Draetta, 2003). To examine if destabilization of Cdc25p was due to ubiquitination, and which ubiquitin ligase(s) may be responsible, we employed an *in vivo* ubiquitination assay. As previously reported (Nefsky and Beach, 1996), Cdc25p is ubiquitinated *in vivo* (Figure 11C, lane 7). Use of temperature sensitive mutations in *lid1-6*, an APC/C mutant (Berry et al., 1999), and *skp1-A4*, a mutation that renders the SCF defective (Yamano et al., 2000), revealed that APC/C activity (Figure 11C, lane 8) was required for the *in vivo* ubiquitination of Cdc25p, while the SCF was dispensable (Figure 11C, lane 9). Interestingly, Clp1p was also required for Cdc25p ubiquitination (Figure 11C, lane 10). This is not due to a general APC/C defect in *clp1Δ* cells as ubiquitination of the APC/C substrate Cut2p was unaffected (Figure 11C, lane 5).

We additionally examined potential *cis* and *trans* acting factors involved in Cdc25p destabilization during mitotic exit. Cdc25p contains 5 putative destruction boxes (D-Box) matching the minimum consensus of Arg-X-X-Leu that could provide recognition by the APC/C (Figure 12A) (Glotzer et al., 1991). Again using the *cdc10-v50* mutant to examine stability during G$_1$, we expressed truncations of Cdc25p under the control of the exogenous *nmt* promoter. Whereas full length Cdc25p was highly destabilized during this arrest, deletion of the first 78 amino acids from Cdc25p greatly stabilized the protein (Figure 12B). Because truncation of this N-terminal region eliminates only one putative D-Box, further truncations were made. Elimination of the first 122 and 190 amino acids resulted in a similar stabilization of Cdc25p during G$_1$ (Figure 12B). These data suggest that N-terminal sequences present in Cdc25p target it for degradation during G$_1$. Still, other elements intrinsic to Cdc25p must be acting to modulate its stability, since the turnover of these truncations did not fully mimic the absence of Cdc25p turnover observed in *clp1Δ* mutants.

Cdk1p phosphorylation of Cdc25A during mitosis protects the phosphatase from recognition by the APC/C (Mailand et al., 2000). Because Cdc25p is not efficiently dephosphorylated during mitotic exit in *clp1Δ* cells, it is possible that a similar mechanism may be operating within *S. pombe* Cdc25p. To address whether dephosphorylation of Cdc25p during mitotic exit by Clp1p targets it for destruction, we expressed a mutant of Cdc25p lacking the 15 putative Cdk1p phosphorylation sites during a G$_1$ arrest either in the presence or absence of *clp1$^+$*. Elimination of these putative phosphorylation sites prevents Cdc25p
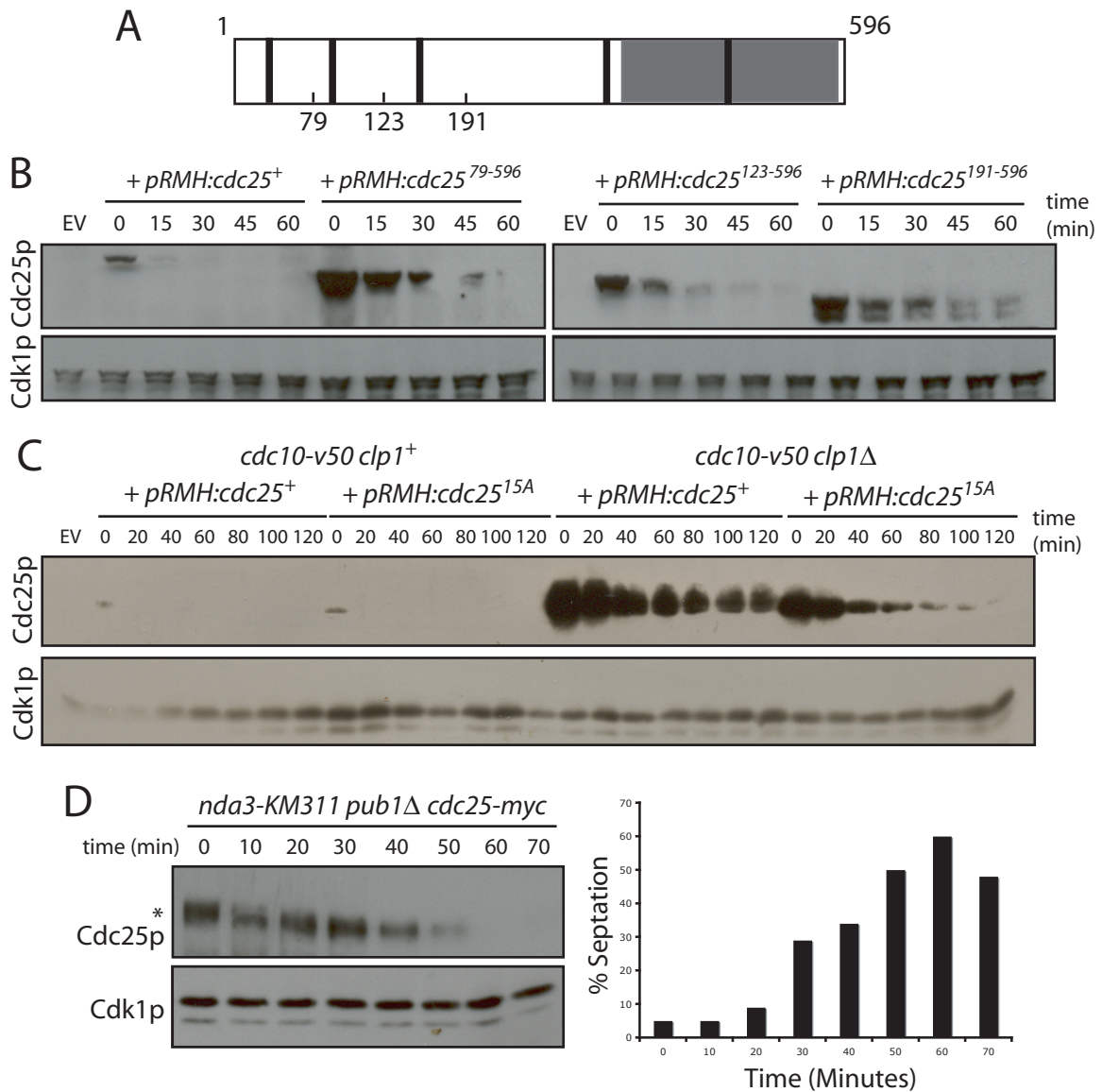
Figure 12. *cis* and *trans* acting elements controlling Cdc25p stability. A) Schematic of Cdc25p depicting catalytic domain in gray, and presence of 5 putative destruction boxes shown by black vertical lines. Numbers underneath represent position of amino acids used for truncations B) *cdc10-v50* (KGY 1744) transformed with the indicated vectors were grown in the absence of thiamine for 20 hours at 25ºC before being shifted to 36ºC for 3 hours, at which point cyclohexamide (100 µg/mL) and excess thiamine (4 µM) were added to repress expression. Samples were processed at the indicated time points for Cdc25p and Cdk1p levels by immunoblot analysis. C) *cdc10-v50* (KGY 1744) and *cdc10-v50 clp1Δ* (KGY 2752) transformed with the indicated vectors were grown in the absence of thiamine for 20 hours at 25ºC before being shifted to 36ºC for 3 hours, at which point cyclohexamide (100 µg/mL) and excess thiamine (4 µM) were added to repress expression. Samples were processed at the indicated time points for Cdc25p and Cdk1p levels by immunoblot analysis. D) *nda3-KM311 pub1Δ cdc25-myc* (KGY 297) was shifted to the restrictive temperature for 7 hours at 18ºC, before being released to the permissive temperature of 32ºC. Samples were collected at the indicated time points and processed for Cdc25p levels and phosphorylation status and Cdk1p levels through immunoblot analysis, as well as for % septation through light microscopy (right panel).

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hyperphosphorylation in *clp1Δ* cells; however, Cdc25p-15A remains stable in the absence of *clp1+* function (Figure 12C). These data suggest that mitotic phosphorylation of Cdc25p does not play a protective role, and that Clp1p plays a more direct role in Cdc25p destabilization than merely reversing phosphorylation events.

The HECT-domain containing protein Pub1p has also been implicated in Cdc25p destabilization (Nefsky and Beach, 1996). To address whether Pub1p may play a role in promoting the destabilization of Cdc25p during mitotic exit, we utilized the reversible *nda3-KM311* mutant to block cells in early mitosis in the absence of *pub1+* function. These cells were then released to the permissive temperature to exit mitosis and monitored for Cdc25p phosphorylation state and levels through immunoblot analysis. At the block point, Cdc25p levels are elevated in the absence of *pub1+*. However, as *pub1Δ* cells exit mitosis and undergo septation, Cdc25p became hypophosphorylated and destabilized (Figure 12D). These data suggest that while *pub1+* does regulate Cdc25p stability, it does not do so during the exit from mitosis.

<u>Clp1p and Cdc25p interact *in vivo*</u>

To determine whether we could detect an interaction between Clp1p and Cdc25p *in vivo*, we employed a reciprocal co-immunoprecipitation assay. In this experiment, catalytically inactive Clp1p (C286S) was expressed because it has been predicted to bind with higher affinity to its substrate (Tonks and Neel, 1996). Cdc25p-MYC specifically associated with a minor amount of Clp1p-HA when expressed from a multi-copy plasmid (Figure 13A). We obtained similar results when wild type Clp1p was expressed (data not shown). The low level of interaction between Cdc25p and Clp1p likely represents both the transient nature of the interaction as well as the possibility that many proteins may compete for Clp1p binding *in vivo*.

To identify which domains of Cdc25p (Figure 13B) might be required for interaction with Clp1p, we utilized the yeast two-hybrid assay. Interaction of Clp1p and Cdc25p depended on the N-terminal 200 amino acids of Cdc25p (Figure 13C). To confirm this, Cdc25p fragments produced as MBP fusions were tested for their ability to bind Clp1p-MYC in cell lysates. All MBP-Cdc25p fragments containing the N-terminus but not MBP alone were able to interact with Clp1p-MYC confirming the two-hybrid data (Figure 13D).
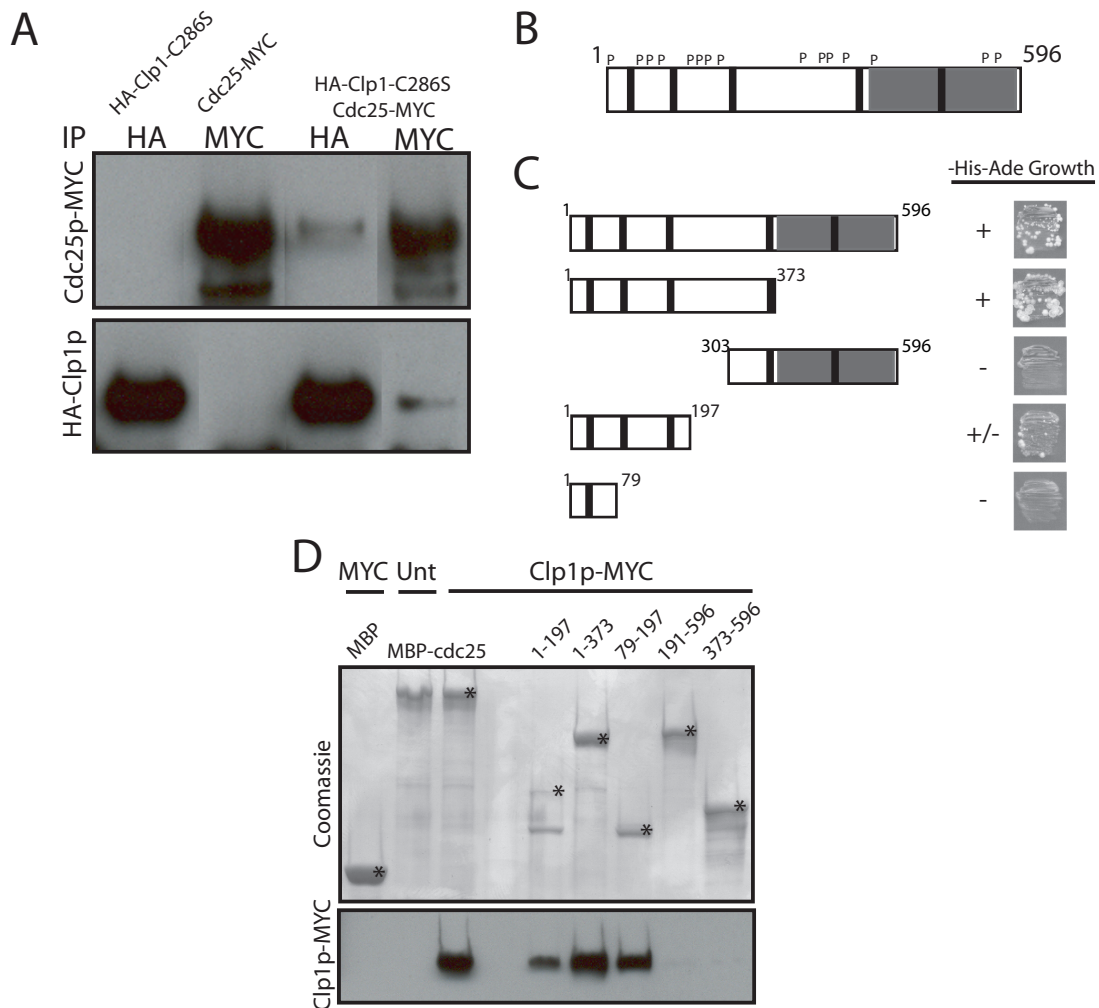
Figure 13. Clp1p interacts with the N-terminus of Cdc25p. A) *wild type* (KGY 246) or *cdc25-myc* (KGY 3377) cells were transformed with empty vector or *pREP41- HA-clp1^{C286S}*, and expression was induced by growth in media lacking thiamine for 18 hours at 32°C. Samples were collected and extracts were prepared. Native lysates were split, immunoprecipitated with 9E10 and 12CA5 antibodies, and separated by SDS PAGE. Membranes were probed with 9E10 and 12CA5 antibodies to detect MYC and HA tagged proteins, respectively. B) Schematic representation of Cdc25p depicting carboxy terminal catalytic domain (gray box), putative Cdk1p phosphorylation sites (P), and 5 putative destruction boxes shown by black vertical lines. C) Strain *PJ206* (KGY 1296) was co-transformed with bait plasmid *pGBT9:clp1* and the indicated *cdc25* fragments in the prey plasmid, *pGAD424*, and then screened for ability (+/-) to support growth on –His –Ade (double selection) plates. D) Extracts were prepared from either *wild type* (KGY 246) (Unt) or *clp1-myc* (KGY 2882) strains grown in YE media at 32°C, and incubated in the presence of the indicated MBP-Cdc25p fragments bound to amylose beads. Beads were washed extensively and samples separated by SDS PAGE. Proteins were either detected by Coomassie blue staining or transferred to PVDF and subsequently probed with 9E10 antibodies to detect Clp1p. Asterisks (*) indicate the position of the predicted size of the fusion protein on Coomassie stained gel.

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Cdk1p phosphorylates Cdc25p to Drive Mitotic Initiation

Knowing that Cdc14p homologs specifically remove phosphorylation events positioned by proline-directed kinases (Gray et al., 2003; Kaiser et al., 2002; Visintin et al., 1998), coupled with evidence from *Xenopus* and human cells demonstrating that Cdk1p phosphorylates Cdc25p homologs both *in vitro* and *in vivo* (Izumi and Maller, 1993; Hoffmann et al., 1993), we tested whether recombinant *S. pombe* Cdk1p could phosphorylate Cdc25p *in vitro*. Cdc25p contains 15 putative Cdk1p consensus sites for phosphorylation (Figure 14B). Recombinant Cdk1p complex, but not the kinase inactive complex, phosphorylated recombinant MBP-Cdc25p, but failed to phosphorylate MBP alone (Figure 14A). Truncation analysis coupled with site-directed mutagenesis revealed that Cdk1p possesses the potential to phosphorylate nearly all of the 15 consensus sites *in vitro* (Figure 14C and 14D). In fact, all 15 Ser-Pro/Thr-Pro sites must be mutated to non-phosphorylatable Ala residues to abolish Cdk1p phosphorylation of Cdc25p *in vitro* (Figure 14D). *In vitro*, Cdk1p phosphorylates 4 major tryptic peptides, 3 of which reside in the N-terminal 200 amino acids (Figure 14E). Only a subset of these sites appear to be major *in vivo* sites of phosphorylation during a mitotic arrest, since mixing of phosphorylated tryptic peptides from *in vivo* and *in vitro* phosphorylation revealed many non-overlapping sites (Figure 14F).

Mitotic phosphorylation and activation of Cdc25p by Cdk1p has been predicted to affect the timing of mitotic commitment (Izumi and Maller, 1993; Hoffmann et al., 1993). In order to gain insight into how these phosphorylations may facilitate entry into mitosis, we expressed a mutant of Cdc25p lacking all 15 potential sites of Cdk1p phosphorylation, Cdc25p-15A, in cells. Both high and low levels of expression of Cdc25p and Cdc25p-15A from the inducible *nmt* promoter were sufficient to rescue growth of the *cdc25-22* mutant allele at 36°C (Figure 15A). However, under repressing conditions (+ thiamine) from the low level *nmt81* promoter, expression of Cdc25p-15A was unable to rescue temperature sensitivity, whereas expression of the wild type protein still rescued the growth defect (Figure 15A). High level expression of Cdc25p in *S. pombe* cells shortens the length of G_2, and produces cells dividing with a characteristic "wee" phenotype (Figure 15B) (Russell and Nurse, 1986). However, full strength expression of Cdc25p-15A failed to advance mitosis prematurely (Figure 15B). Instead, expression of Cdc25p-15A delays progression through mitosis with a large number of cells possessing highly condensed chromatin (Figure 15B, see asterisks). These data demonstrate that
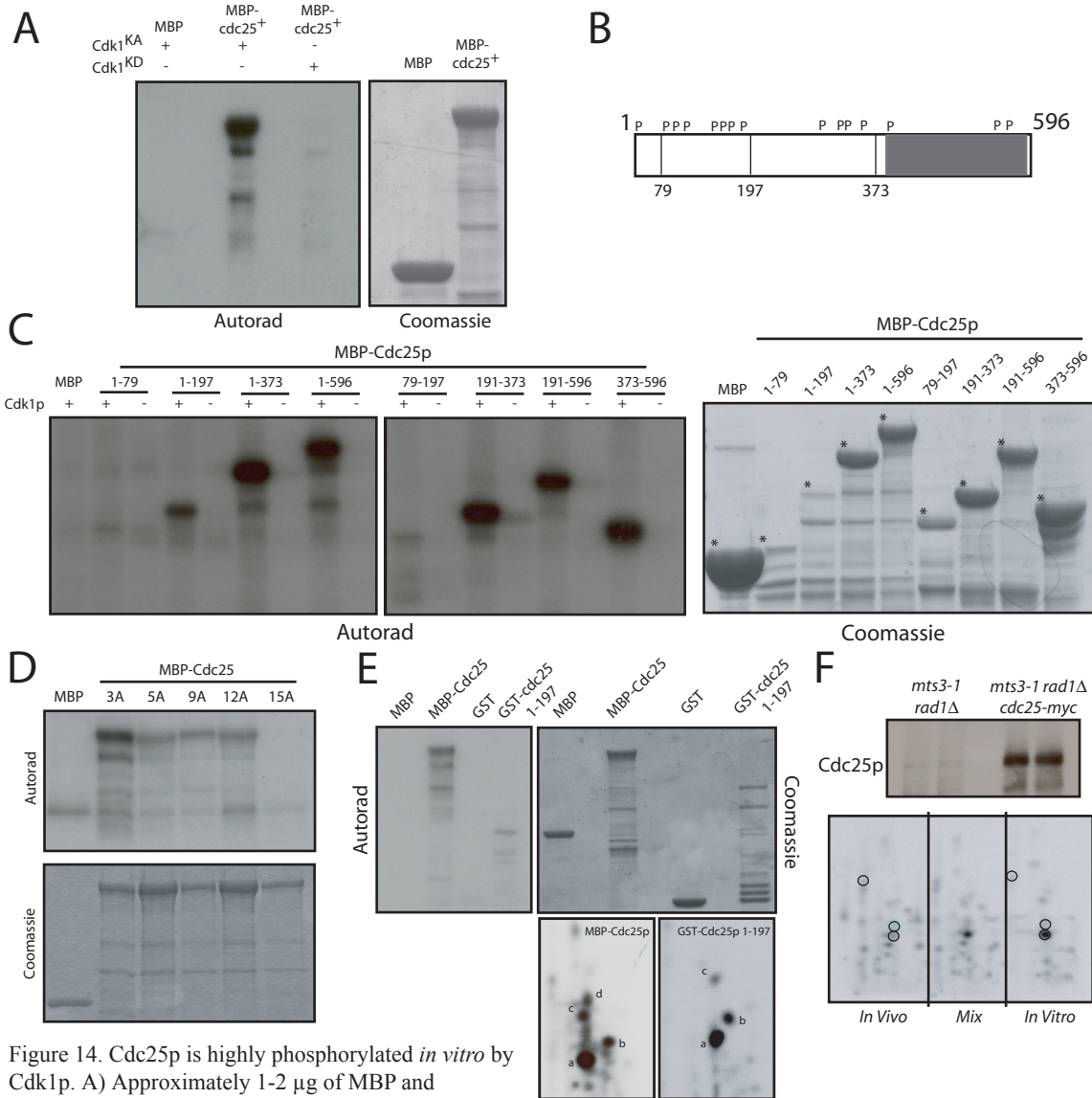
Figure 14. Cdc25p is highly phosphorylated *in vitro* by Cdk1p. A) Approximately 1-2 µg of MBP and MBP-Cdc25p were phosphorylated *in vitro* with baculoviral produced and purified recombinant active (KA) or kinase dead (KD) Cdk1p complex. Reactions were separated by SDS PAGE and analyzed by Coomassie blue staining and autoradiography. B) Schematic representation of Cdc25p depicting putative Cdk1p phosphorylation consensus sites. The catalytic domain is shaded in gray, and the vertical lines refer to the amino acids used to begin or end various Cdc25p fragments. C) The indicated Cdc25p fragments fused to MBP were incubated in the presence of Kinase active (+) Cdk1p or Kinase dead (-) Cdk1p and radiolabeled ATP. Reactions were separated by SDS PAGE and either stained with Coomassie to visualize protein bands (right panel) or exposed to film for autoradiography (left 2 panels). D) MBP and MBP-Cdc25p phosphosite mutants were subjected to *in vitro* phosphorylation by kinase active Cdk1p. Reactions were resolved on SDS PAGE and stained with Coomassie to visualize bands (bottome panel) and exposed to film for autoradiography (top panel). D) MBP, MBP-Cdc25p, GST, and GST-Cdc25 1-197 were subjected to *in vitro* phosphorylation by kinase active Cdk1p. Reactions were resolved on SDS PAGE and stained with Coomassie to visualize bands (right upper panel) or transferred to PVDF membrane and exposed to film for autoradiography (left upper panel). Bands corresponding to phosphorylated MBP-cdc25p and GST-cdc25p 1-197 were excised from membrane and subjected to tryptic phosphopeptide mapping as described in materials and methods. F) *mts3-1 rad1*Δ (KGY 1687) and *mts3-1 rad1*Δ *cdc25-myc* (KGY 570) cells were grown for 4 hours at 36ºC in the presence of ^{32}P orthophosphate. Cdc25p-myc immunoprecipitates were resolved on SDS PAGE and exposed to film for autoradiography (top panel). Tryptic peptides from the *in vivo* labeling were mixed with those produced upon incubation of Cdk1p with MBP-Cdc25p *in vitro* and separated in 2 dimensions on TLC plates and exposed to film for autoradiography (lower panel). Circles represent tryptic fragments that are present in both the *in vivo* and *in vitro* maps.
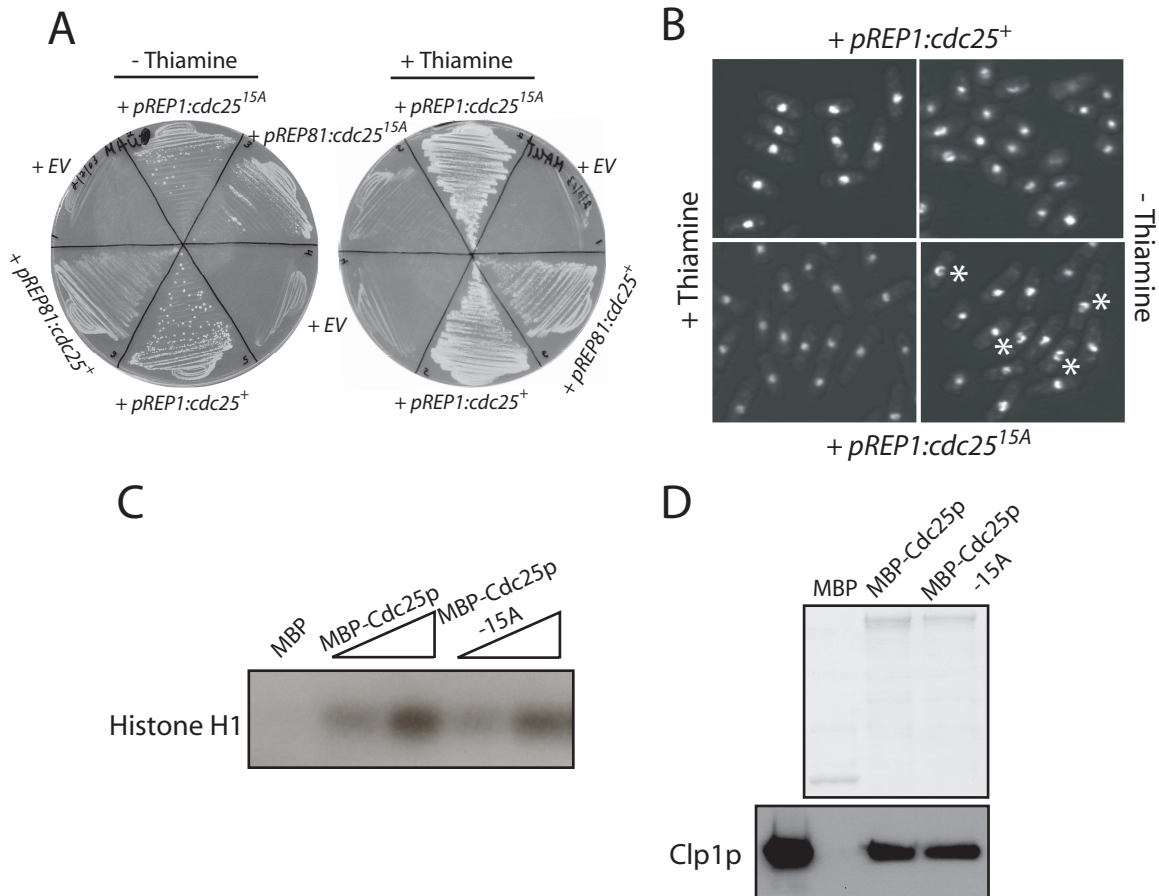
Figure 15. Cdc25p-15A shows reduced activity *in vivo* but not *in vitro*. A) *cdc25-22* (KGY 851) cells transformed with the indicated vectors were struck out onto Minimal Medium either in the presence (+) or absence (-) of Thiamine. Plates were incubated at 36ºC for 48 hours to observe rescue. B) *wild type* (KGY 246) cells were transformed with the indicated vectors and grown in the presence (+ T) or absence (- T) of Thiamine for 20 hours at 32ºC. Cells were fixed with Ethanol and stained with DAPI to visualize nuclei. Asterisks indicate position of cells containing condensed chromatin. C) Inactive Cdk1p from *cdc25-22* (KGY 851) was incubated in the presence of MBP, or increasing concentrations of MBP-Cdc25p or MBP-Cdc25p-15A, and then measured for its ability to phosphorylate Histone H1 *in vitro*. D) Lysates prepared from *clp1-myc* (KGY 2882) were incubated in the presence of MBP, MBP-Cdc25p, or MBP-Cdc25p-15A. Samples were resolved on SDS PAGE and Clp1p levels were detected via immunoblot analysis and MBP and MBP-Cdc25p were detected through Coomassie staining.

Cdc25-15A has reduced activity *in vivo*. To determine whether this reduced *in vivo* activity stems from a reduction in catalytic activity *in vitro*, we assayed Cdc25p-15A *in vitro* activity as a function of its ability to activate Tyr-15 phosphorylated Cdk1p, obtained from *cdc25-22* mutant cells (Kovelman and Russell, 1996). Recombinant MBP-Cdc25p and MBP-Cdc25p-15A activate Cdk1p's Histone H1 kinase activity with similar concentration dependencies, whereas MBP alone failed to activate Cdk1p (Figure 15C). Additionally, MBP-Cdc25p-15A retains the ability to interact with Clp1p-MYC from *S. pombe* cell lysates (Figure 15D). Together, these data suggest that despite Cdc25p-15A possessing full catalytic activity *in vitro* and interacting with known interacting proteins, the phosphosite mutant displays a reduced ability to drive mitotic progression *in vivo*.

Clp1p reverses Cdk1p-dependent phosphorylation events on Cdc25p

We next examined whether or not recombinant MBP-Clp1p could dephosphorylate recombinant GST-Cdc25p 1-197 when phosphorylated *in vitro* by active Cdk1p complex. MBP-Clp1p, but not a phosphatase dead version in which the catalytic Cysteine-286 is replaced by Serine (MBP-C286S) (Denu et al., 1996), removed radioactive phosphate from Cdc25p in a dose-dependent fashion (Figure 16A). Furthermore, both MBP-Clp1p and λ phosphatase, but not MBP-C286S, could dephosphorylate Cdc25p immunoprecipitated from mitotically arrested *S. pombe* cells (Figure 16B). Approximately five times the amount of λ PPase was added (~500 ng) to achieve similar levels of dephosphorylation.

clp1Δ cells delay mitotic Cdk1p inactivation and advance G$_2$ due to stabilized Cdc25p

Having established that in the absence of *clp1$^+$* Cdc25p is upregulated during many stages of the cell cycle, we wanted to know if this resulted in higher Cdk1p activity at inappropriate times. To this end, we examined synchronous cell populations either in the presence or absence of *clp1$^+$* using a *cdc25-22* arrest/release protocol. 90 minutes after release of the wild type population, the completion of cell division had nearly been reached (Figure 17A). However, in the absence of *clp1$^+$* at the 90 minute time point, the septation peak had not been reached and did not occur for an additional 20 minutes (Figure 17A and data not shown). This could result from a septation defect or a delay at any point prior to this. To pinpoint the delay, we examined the microtubule cytoskeleton of the cells and quantified the percentages of metaphase

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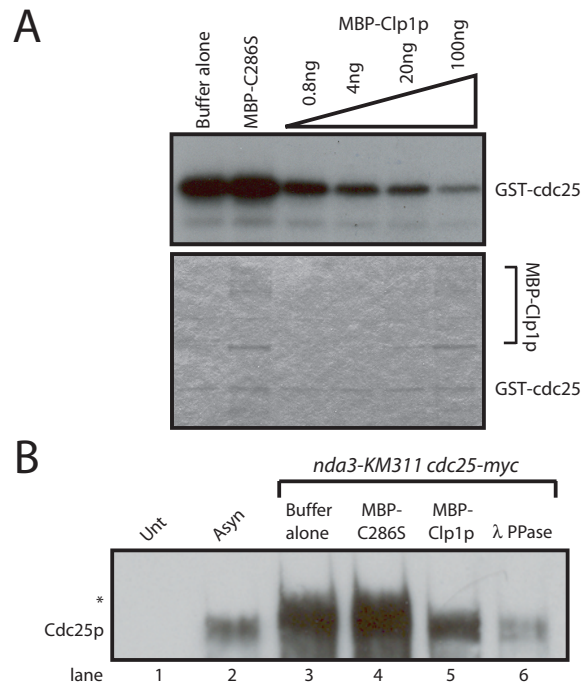
Figure 16. Clp1p reverses Cdk1p dependent phosphorylation of Cdc25p. A) Approximately 100 ng of
GST-ΔCdc25p containing amino acids 1-197 was phosphorylated by recombinant Cdk1p complex *in
vitro*, and subsequently incubated with the indicated amounts of MBP-C286S or MBP-Clp1p.
Reactions were separated by SDS PAGE, and analyzed by Coomassie blue staining and
autoradiography. B) Cell pellets from *nda3KM311cdc25-myc* (KGY 3916) arrested at the restrictive
temperature (18°C), and *wild type* (KGY 246-Unt) and *cdc25-myc* (KGY 3377-Asyn) grown to
mid-log phase were collected. Extracts were prepared and immunoprecipitated with 9E10 antibodies
and subsequently incubated with phosphatase buffer alone (lanes 1, 2, and 3), 100 ng MBP-C286S
(lane 4), 100 ng MBP-Clp1p (lane 5), or 0.5 µg Lambda phosphatase (lane 6). Reactions were
separated by SDS PAGE and immunoblotted with anti-Cdc25p antibodies. Asterisk (*) indicates
position of the hyperphosphorylated Cdc25p.
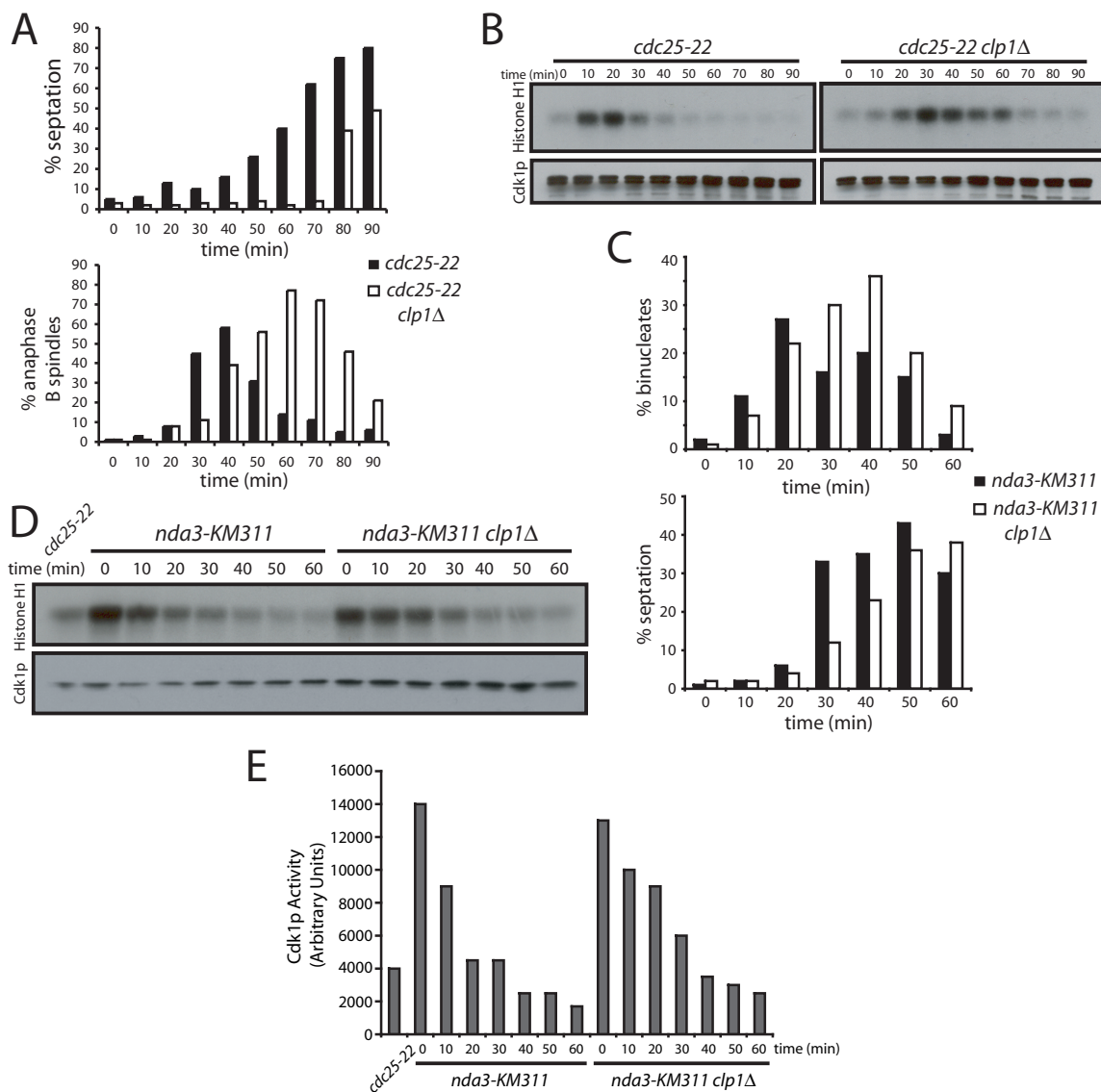
Figure 17. *clp1Δ* cells delay Cdk1p inactivation at the end of mitosis. *cdc25-22* (KGY 851) and *cdc25-22 clp1Δ* (KGY 3380) cells were arrested in G$_2$ by incubation at 36°C for 4 hours. Cultures were then released to 25°C, and samples were taken at the indicated time points and fixed with ethanol or frozen. A) Septation index was calculated using a light microscope (A, top panel). Ethanol fixed samples were subjected to indirect immunofluorescence with anti-TAT1 antibodies (A, bottom panel). The presence of elongated spindles and separated DNA masses was assessed in at least 200 cells. B) Cell pellets were lysed under native conditions and immunoprecipitated for Cdk1p with 4711 antibody. Samples were processed for Histone H1 kinase activity and immunoblotted for Cdk1p levels with anti-PSTAIR. C) *nda3-KM311* (KGY 3612) and *nda3-KM311 clp1Δ* (KGY 3783) were arrested in prometaphase by incubation at 18°C for 6.5 hours. Cells were released at 32°C, and ethanol fixed samples and cell pellets were collected at the indicated time points. Septation index was determined as in A) (C, lower panel) and fixed cells were stained with DAPI to visualize nuclei. The presence of separated DNA masses and no septum was assessed in at least 200 cells (C, top panel). D) Cell pellets were analyzed as in B) and Cdk1p activity was quantified E).

and anaphase B spindles (Figure 17A and data not shown). In the presence of *clp1⁺*, anaphase began at 30 minutes and spindles disassembled by 60 minutes as septation began (Figure 17A). This was in marked contrast to the timing of anaphase in the absence of *clp1⁺* where spindle elongation began with a 10-minute delay, and elongated spindles persisted until the end of the time course (Figure 17A). Because spindle disassembly is inhibited by elevated Cdk1p activity (Wheatley et al., 1997), we examined Cdk1p activity through immunoprecipitation kinase assays. *clp1Δ* cells delayed Cdk1p inactivation for 20-30 minutes compared to control cells (Figure 17B).

A second method of synchronization was used to assess anaphase progression in *clp1Δ* cells to eliminate the possibility that the observed delay was specific to the *cdc25-22* allele. *nda3-KM311* cells were arrested in early mitosis in the presence or absence of *clp1⁺*, and then released to the permissive temperature to allow anaphase progression. *clp1Δ* cells delayed septation by 10 minutes (Figure 17C), and mitotic Cdk1p activity was not returned to interphase levels until 40 minutes after release (Figure 17D and 17E). This is in contrast to wild type cells, which inactivated Cdk1p 20 minutes after release (Figure 17D and 17E). The more pronounced exit defect observed with the *cdc25-22* arrest/release protocol likely represents a combination of the better synchrony achieved with this protocol as well as the slower kinetics of cell cycle progression at 25°C.

To examine if the mitotic exit delay was at least in part due to hyperactive Cdc25p in *clp1Δ* cells, we again utilized the *cdc25-22* arrest/release protocol either in the presence or absence of *clp1⁺*. These cells were released to the permissive temperature to allow mitotic entry. As soon as cells entered mitosis, as judged by formation of mitotic spindles and an elevation of Cdk1p kinase activity (Figure 18B and data not shown), the cultures were divided in half. One half of the culture was shifted to the restrictive temperature to once again inactivate Cdc25-22p, while the other half was left to progress through mitosis at the permissive temperature. As before, when kept at the permissive temperature, *clp1Δ* cells exited mitosis with a 20-30 minute delay as judged by spindle disassembly and mitotic Cdk1p inactivation (Figures 18A and 18B). However, when Cdc25-22p was inactivated subsequent to Cdk1p activation, *clp1Δ* cells disassembled mitotic spindles and inactivated Cdk1p with only a 10-minute delay (Figures 18A and 18B). This slight delay could result from slow inactivation of Cdc25-22p, or suggests that Clp1p functions through additional mechanisms to promote Cdk1p inactivation. These data
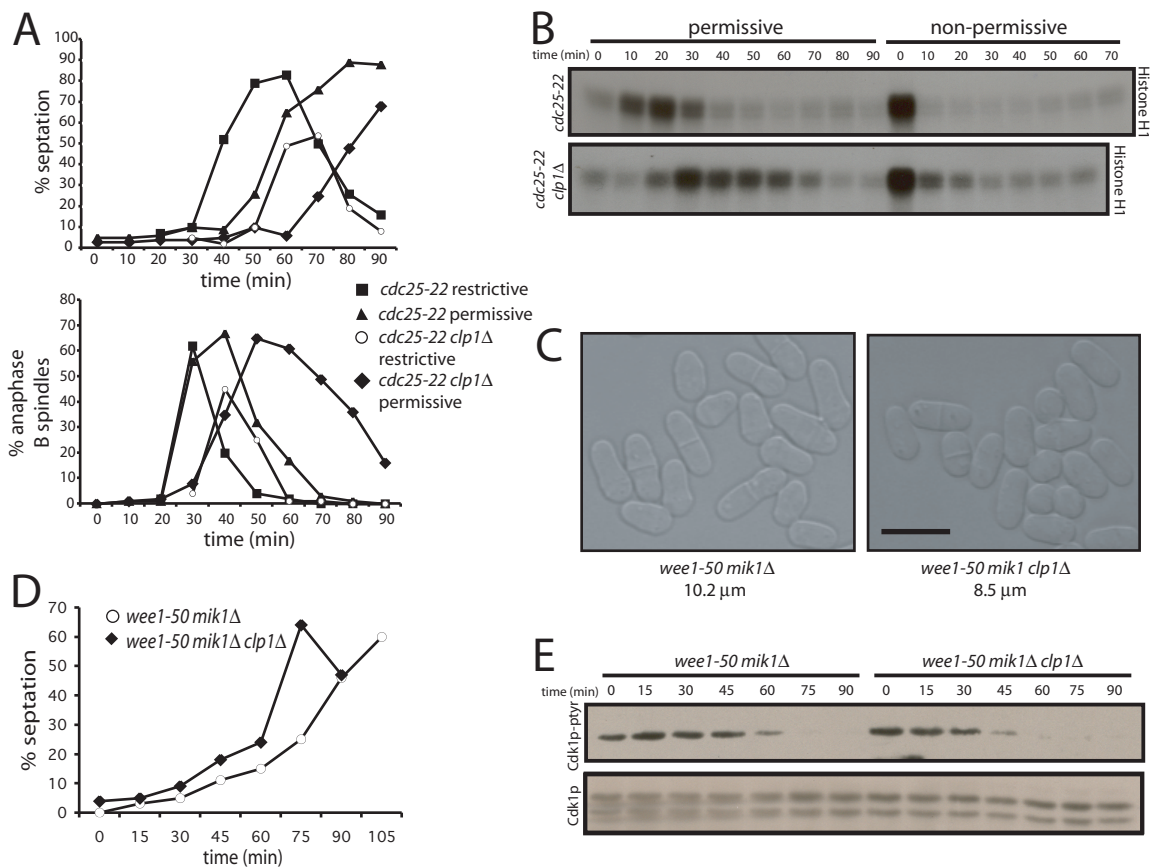
Figure 18. Exit delay and G_2 advancement depend in large on Cdc25p. A) *cdc25-22* (KGY 851) and *cdc25-22 clp1Δ* (KGY 3380) were arrested in G2 by incubation at 36°C for 4 hours and released to 25°C to undergo a synchronous mitosis. Timing of mitotic entry was judged by Histone H1 kinase assays as well as microtubule staining for metaphase spindles in several previous experiments and confirmed in this one. After cells entered mitosis (20 minutes for wild type and 30 minutes for *clp1Δ*), the cultures were spilt in half, and incubated either at 25°C (permissive) or 36°C (non-permissive). Ethanol fixed samples and cell pellets were taken at the indicated time points. A) Septation was determined by light microscopy (A, top panel). Ethanol fixed samples were subjected to indirect immunofluorescence with anti-TAT1 antibodies (A, bottom panel). The presence of elongated spindles and separated DNA masses was assessed in at least 200 cells. B) Cell pellets were lysed under native conditions and immunoprecipitated for Cdk1p with anti-4711. Lysates were processed for Histone H1 kinase activity and immunoblotted for Cdk1p with anti-PSTAIR antibodies. C) Differential Interference Contrast Images of *wee1-50 mik1Δ* (KGY 612) and *wee1-50 mik1Δ clp1Δ* (KGY 98) grown at 25°C in YE medium. Cell lengths at septation were scored from at least 20 cells using Openlab software (Improvision). Scale bar indicates 10 μm. D-E) *wee1-50 mik1Δ* (KGY 612) and *wee1-50 mik1Δ clp1Δ* (KGY 98) cells grown at 25°C were synchronized by centrifugal elutriation. Cells were released to the restrictive temperature (36°C), and samples taken at the indicated time points were processed for % septation (D) and for protein. E) Lysates were prepared under native conditions and separated on SDS PAGE. Membranes were immunoblotted for Tyr-15 phosphorylated Cdk1p and total Cdk1p with phospho-Tyr-15 Cdk1p and PSTAIR antibodies, respectively.

cumulatively argue that the mitotic exit defect of *clp1Δ* cells is due in part to the persistence of Cdc25p activity.

We and others have shown previously that *clp1Δ* cells enter mitosis at a premature cell size (Figure 6A) (Cueille et al., 2001; Trautmann et al., 2001). As previously mentioned, this could result from inhibition of Wee1p, activation of Cdc25p, or a combination of the two. Because we demonstrated defective downregulation of Cdc25p levels in *clp1Δ* cells, we determined if elevated Cdc25p activity was contributing to the G_2 advancement of *clp1Δ* cells. *wee1-50 mik1Δ* cells lose Tyr-15 kinase activity upon shift to the restrictive temperature, and therefore the rate at which these cells enter mitosis is entirely dependent upon the amount of Cdc25p activity (Rhind et al., 1997). We examined the rates of mitotic entry in *wee1-50 mik1Δ* cells in the presence or absence of *clp1⁺*. A synchronous population of G_2 cells were obtained by centrifugal elutriation and immediately shifted to the restrictive temperature to inactivate Wee1p kinase activity. *clp1Δ* cells dephosphorylated Tyr-15 of Cdk1p 15 minutes earlier than wild type cells (Figure 18E). They also reached the peak of septation 15-30 minutes earlier than control cells (Figure 18D). Even at the permissive temperature, the triple mutant (*wee1-50 mik1Δ clp1Δ*) divides at a smaller cell size (8.5 μm as opposed to 10.2 μm) than does the double mutant (Figure 18C). Together, these data suggest that loss of *clp1⁺* function acts additively with loss of *wee1⁺* function and that increased Cdc25p activity during G_2 advances mitotic commitment in the absence of *clp1⁺*.

Summary

Cdc14p protein phosphatases reverse mitotic Cdk1p phosphorylation events (Gray et al., 2003; Kaiser et al., 2002). We have described the initial characterization of the *S. pombe* Cdc14p family member, Clp1p. Unlike its *S. cerevisiae* counterpart, Clp1p is not essential for mitotic exit. However, we have shown that Clp1p plays critical roles in balancing the activity of Cdk1p during both the G_2/M transition and during the exit from mitosis. Deletion of *clp1⁺* produces small, but viable cells, whereas overproduction of Clp1p delays progression through G_2. These phenotypes result from an alteration in the balance of activities of the Wee1p inhibitory kinase and the Cdc25p phosphatase, which together regulate the Tyr-15 phosphorylation state of Cdk1p. We have shown that Clp1p directly inhibits Cdc25p activity through two potentially distinct mechanisms. First, it dephosphorylates Cdc25p during the exit from mitosis to attenuate its

increased activity, and second, Clp1p promotes the ubiquitination and destabilization of Cdc25p. Both of these elements may represent conserved features of mitotic control by Cdc14 phosphatases, as Cdc25p homologs in higher eukaryotes are regulated at the post-translational level via phosphorylation and protein degradation. We hypothesize that Clp1p functions together with the SIN to downregulate Cdk1p activity below a certain threshold competent for cytokinesis and the cycle to reset (Figure 19). A key question arising from these studies is how the disruption of the Cdk1p auto-amplification loop is temporally regulated to ensure that mitotic exit is not prematurely catalyzed.
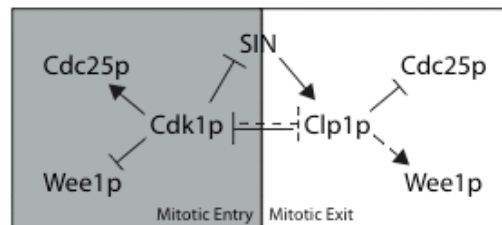
Figure 19. Positive and negative regulation of Cdk1p during mitosis. During mitosis, Cdk1p stimulates its own activity by activating its positive regulator (Cdc25p) and inactivating its negative regulators (Wee1p, SIN, Clp1p). As Cdk1p activity is sufficiently downregulated at mitotic exit, SIN and Clp1p function together to combat Cdk1p activation by reversing the auto-amplification loop and promoting cytokinesis.

CHAPTER IV

REGULATION OF Clp1p DURING MITOTIC PROGRESSION

Introduction

Clp1p is released from the nucleolus early in mitosis when it redistributes to the forming mitotic spindle, the kinetochores, and the contractile ring, as well as to the nucleus and cytoplasm (Figure 8B) (Cueille et al., 2001; Trautmann et al., 2001; Trautmann et al., 2004). This is in contrast to Cdc14p localization in budding yeast, where nucleolar release of the phosphatase does not occur until anaphase onset (Stegmeier et al., 2002; Pereira et al., 2002; Yoshida et al., 2002). Released Cdc14p correlates with its activation as a phosphatase and its ability to target substrates (Visintin et al., 1999; Shou et al., 1999). Because of the disparity in localization patterns between the two Cdc14 homologs, we wanted to determine if *S. pombe* Clp1p was under any additional modes of regulation during progression through the early stages of mitosis, which may influence its ability to interact with and dephosphorylate its substrate(s). Here, we describe mitotic specific phosphorylation of Clp1p by the mitotic regulator, Cdk1p, and show that the result of this phosphorylation is inhibition of Clp1p catalytic activity both *in vitro* and *in vivo*. As Cdk1p activity falls upon anaphase initiation, Clp1p auto-catalytically reverses these phosphorylation events, thus allowing its subsequent dephosphorylation of key substrate(s).

Results

Cdk1p phosphorylates Clp1p during early mitosis

As previously reported, Clp1p becomes hyperphosphorylated as cells progress through mitosis (Figure 20B and 21A) (Cueille et al., 2001). In particular, Clp1p accumulates in the hyperphosphorylated state during prometaphase, an arrest achieved with the *nda3-KM311* mutant (Hiraoka et al., 1984). Clp1p phosphorylation is not just a consequence of its nucleolar release since it accumulates in the hypophosphorylated state in cells arrested using the cytokinesis checkpoint mutant, *cdc3-124* (Figure 20B), a mutant in which Clp1p is maintained out of the nucleolus (Trautmann et al., 2001). In an effort to determine the role of mitotic phosphorylation of Clp1p, we biochemically purified Clp1p using a TAP (Tandem Affinity
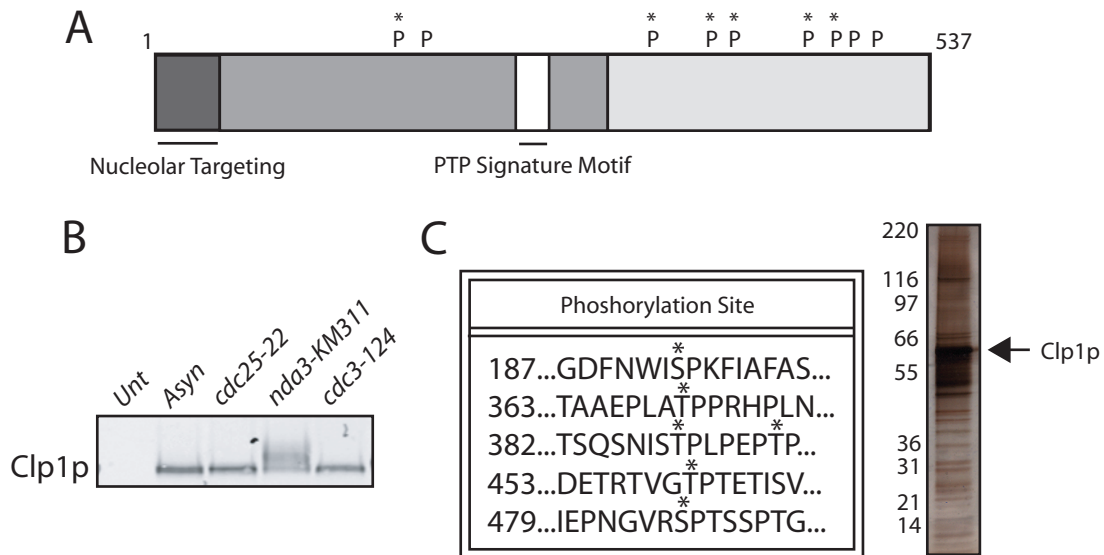
Figure 20. Clp1p is hyperphosphorylated on Ser and Thr residues in early mitosis. A) Schematic of Clp1p primary amino acid sequence. Dark box at N-terminus depicts the nucleolar targeting sequence, medium gray box depicts catalytic core domain, within which sits the characteristic PTP Signature Motif shown by a white box, and the light gray box depicts the non-conserved C-terminus. P represents putative Cdk1p consensus sites of phosphorylation. P with Asterisks (*) over them represent Cdk1p consensus sites identified through mass spectrometric analysis. B) *wild type* (KGY 246-Unt) and *clp1-myc* (KGY 2882-Asyn) cells grown at 32ºC, *cdc25-22 clp1-myc* (KGY 3766) and *cdc3-124 clp1-myc* (KGY 5124) cells grown at 36ºC, and *nda3-KM311 clp1-myc* (KGY 3388) cells grown at 18ºC were processed for Clp1p levels and phosphorylation status by immunoblot analysis. C) *nda3-KM311 clp1-TAP* (KGY 3920) cells grown at 18ºC were processed through Tandem Affinity Purification and the resulting proteins were resolved on SDS PAGE and silver stained (right panel) or phosphorylated Clp1p peptides identified by mass spectrmetric analysis. Left panel indicates partial tryptic peptides identified as being phosphorylated with amino acid residue shown at left.

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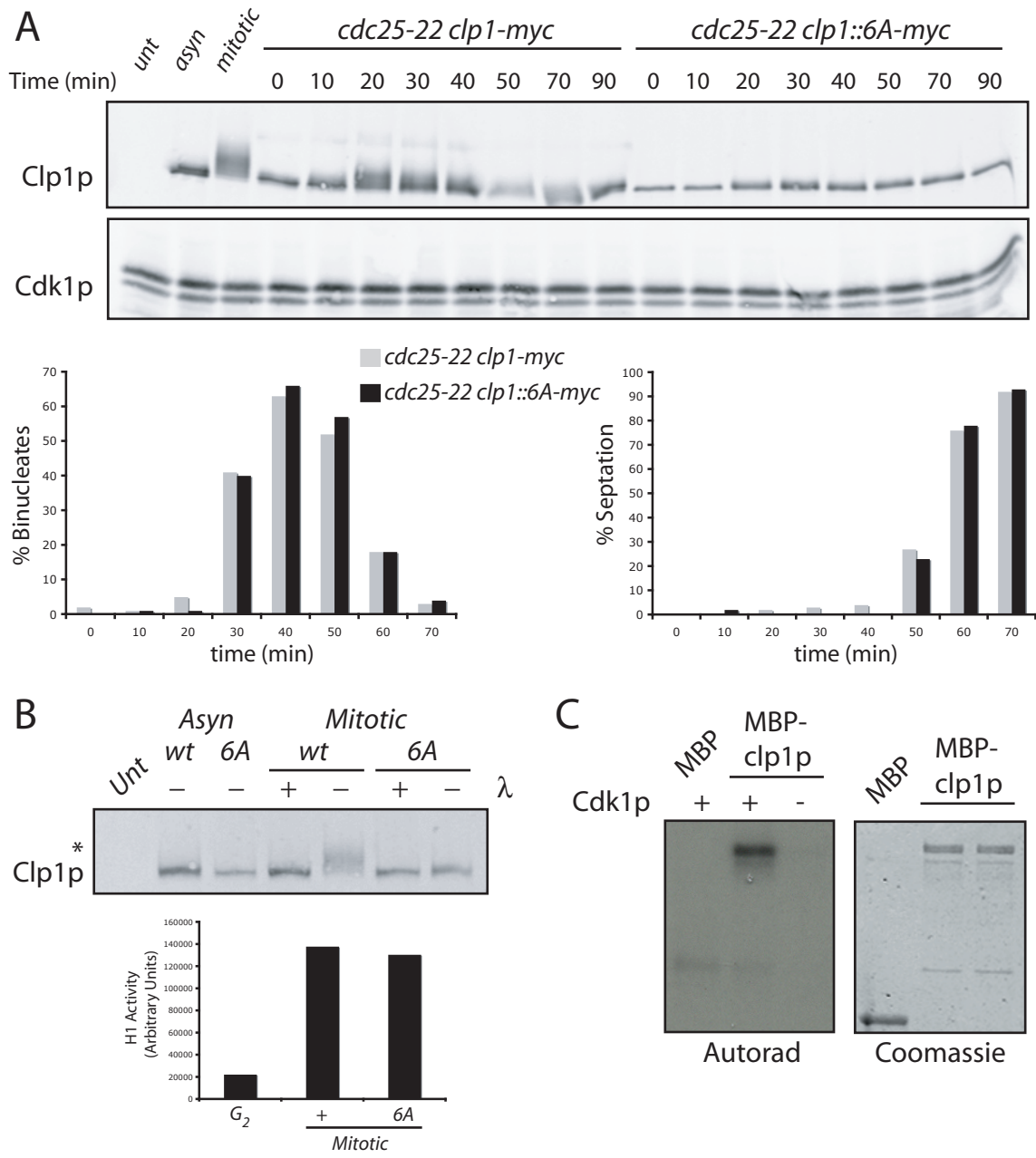
Figure 21. Clp1p is phosphorylated *in vivo* and *in vitro* by Cdk1p. A) *cdc25-22 clp1-myc* (KGY 3766) and *cdc25-22 clp1::6A-myc* (KGY 4593) were synchronized at the G_2/M boundary for 4 hours at 36ºC and released to the permissive temperature at 25ºC to enter a synchronous mitosis. Samples were taken at the indicated time points and processed for cell cycle stage by microscopy or for Clp1p and Cdk1p levels via immunblot analysis. B) *clp1-myc* (KGY 2882), *clp1::6A-myc* (KGY 4594), and *nda3-KM311 clp1-myc* (KGY 3388) and *nda3-KM311 clp1::6A-myc* (KGY 4604) cells grown at the restrictive temperature of 18ºC for 7 hours were processed for Clp1p phosphorylation status in the presence or absence (+/-) of Lambda Phosphatase (λ). Lower panel. *cdc25-22* (KGY 851), *nda3-KM311 clp1-myc* (KGY 3388), and *nda3-KM311 clp1::6A-myc* (KGY 4594) were processed for Histone H1 activity. C) Recombinant MBP and MBP-Clp1p were subjected to *in vitro* kinase assays in the presence of Kinase Active (+) or Kinase Dead (-) Cdk1p. Reactions were resolved on SDS PAGE, Coomassie stained, and exposed to film for autoradiography.

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Purification) tag from cells arrested in early mitosis with the *nda3-KM311* mutant. A portion of the purification was resolved on SDS PAGE and silver stained (Figure 20C, right panel), while the rest was treated with proteases and the peptides identified by 2D-LC tandem mass spectrometry. SEQUEST analysis identified 6 Ser and Thr phosphorylation sites (Figure 20C, left panel). Mutation of these putative phosphorylation sites to non-phosphorylatable Ala residues resulted in a Clp1p mutant severely impaired for mitotic phosphorylation (Figure 21A and 21B), despite cell cycle progression and Cdk1p Histone H1 kinase activity being largely unaffected (Figure 21A and 21B). Because these Ser and Thr sites corresponded to Cdk1p consensus sites of phosphorylation (Ser/Thr-Pro-X-Lys/Arg), we subjected recombinant Clp1p produced from bacteria to *in vitro* phosphorylation with recombinant Cdk1p. While kinase active Cdk1p phosphorylated recombinant Clp1p very well *in vitro*, it was unable to phosphorylate MBP alone. Additionally, kinase dead Cdk1p was unable to phosphorylate recombinant Clp1p (Figure 21C).

Clp1p controls its own dephosphorylation *in vivo*

Similar to the actions of Cdks, Cdc14p phosphatases are directed towards Ser and Thr residues (when phosphorylated, in the case of Cdc14p) with an additional Pro at the +1 position (Gray et al., 2003; Kaiser et al., 2002). Because Cdk1p can phosphorylate Clp1p *in vitro* and Cdk1p consensus sites are phosphorylated *in vivo*, the intriguing possibility was raised that Clp1p controlled its own dephosphorylation. To examine the kinetics of Clp1p dephosphorylation, we again utilized the *nda3-KM311* mutant to block cells in a hyperphosphorylated state in early mitosis. These cells were then released to the permissive temperature and allowed to exit mitosis synchronously. As cells exited mitosis, Clp1p became hypophosphorylated with kinetics similar to the inactivation of Cdk1p (Figure 22A and data not shown). In order to test whether or not Clp1p phosphatase activity was required for this hypophosphorylated state during mitotic exit, we generated phosphatase dead (PD) mutants of Clp1p (*C286S* and *D257A*) at the endogenous *clp1*[+] locus. These mutants behaved phenotypically identical and were used interchangeably throughout these studies (Figure 24A and data not shown). When phosphatase dead Clp1p (Clp1p-PD) was released from the *nda3-KM311* block, Clp1p-PD remained in the hyperphosphorylated state even as cells exited mitosis and underwent septation (Figure 22B). In fact, expression of phosphatase dead Clp1p produced a
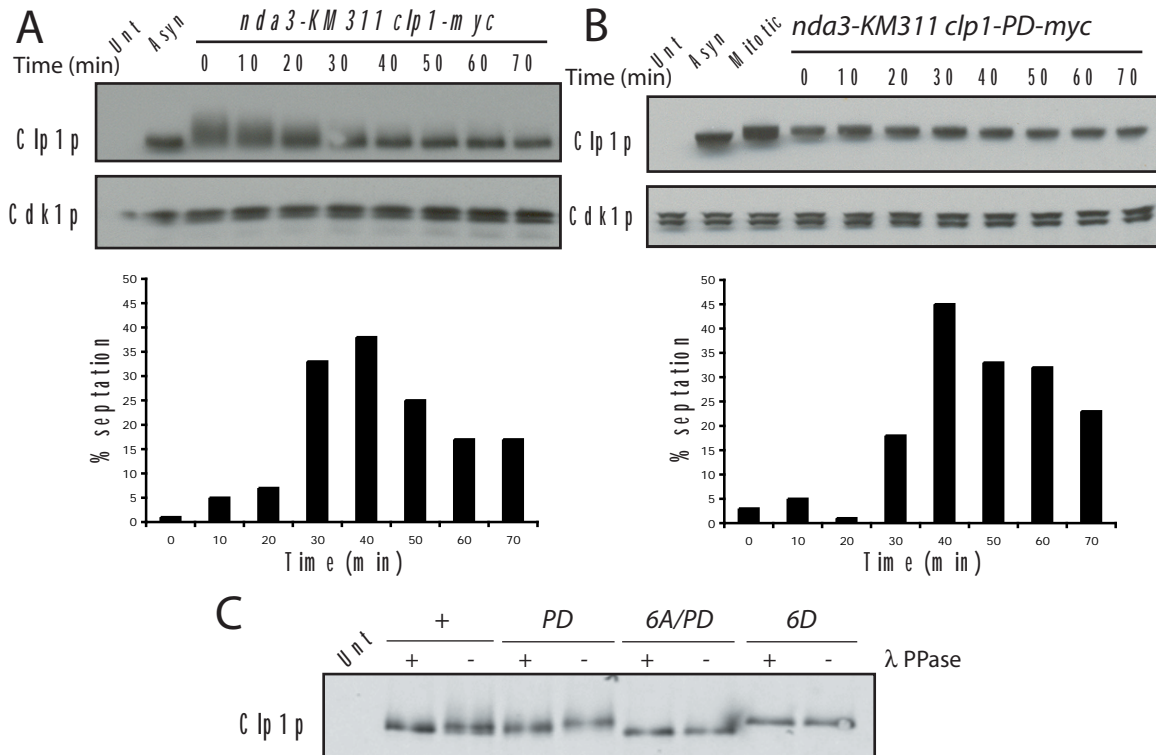
Figure 22. Clp1p auto-dephosphorylates *in vivo* during the exit from mitosis. A) *nda3-KM311 clp1-myc* (KGY 3388) or B) *nda3-KM311 clp1::D257A-myc* (KGY 4605-*PD*) were shifted to the restrictive temperature of 18ºC for 7 hours, then released to the permissive temperature of 32ºC to allow a synchronous exit from mitosis. Samples were prepared at the indicated time points and processed for septation index via light microscopy (lower panel) or for Clp1p and Cdk1p levels through immunoblot analysis (upper panel). C) Asynchronous *wild type* (KGY 246-*Unt*), *clp1-myc* (KGY 2882-+), *clp1::D257A-myc* (KGY 4687-*PD*), *clp1::6A/D257A-myc* (KGY 4884-*6A/PD*), or *clp1::6D-myc* (KGY 5184-*6D*) were processed for Clp1p phosphorylation status after treatment with (+/-) Lambda Phosphatase (λ) via immunoblot analysis.

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form of the protein that runs in the hyperphosphorylated state constitutively, a state which can be collapsed with λ phosphatase (Figure 22C). This constitutive hyperphosphorylation of the phosphatase dead mutant depends on the presence of the 6 Ser and Thr identified sites of phosphorylation (Figure 22C). These data suggest that Clp1p auto-dephosphorylates during mitotic exit, and that failure to remove Cdk1p phosphorylations mimics Clp1p hyperphosphorylation observed in mitotic arrested cells.

<u>Clp1p auto-dephosphorylates *in vitro* in *trans*</u>

We next asked whether Clp1p auto-dephosphorylates *in vitro* during incubation with Cdk1p kinase complex. Incubation of Clp1p phosphatase dead with kinase active Cdk1p yields a substantial increase in radiolabel incorporation retained than when wild type Clp1p acts as the phospho-acceptor (Figure 23A). This could result from enhanced phosphorylation of phosphatase dead Clp1p, or could result from Clp1p auto-phosphatase activity during the course of the reaction. To test the latter possibility, we again performed the kinase reactions as before but this time in the presence of sodium vanadate, a potent inhibitor of the tyrosine phosphatase superfamily. In the presence of vanadate, wild type Clp1p retained the same amount of radiolabel incorporation as did the phosphatase dead mutant after incubation in either the presence or absence of the inhibitor (Figure 23A). Thus, Clp1p auto-dephosphorylates both *in vitro* and *in vivo*.

While these data argue that Clp1p has the ability to auto-dephosphorylate, they do not demonstrate whether this occurs in *cis*, in *trans*, or both. To determine if Clp1p has the ability to dephosphorylate in *trans*, we phosphorylated a C-terminal fragment of Clp1p lacking the phosphatase domain *in vitro* with Cdk1p. This phosphorylated fragment was then used as an *in vitro* substrate for increasing concentrations of recombinant Clp1p and a phosphatase dead mutant. Whereas increasing concentrations of phosphatase active Clp1p reduced the amount of radiolabel incorporation, the highest concentration of phosphatase dead Clp1p failed to dephosphorylate this fragment (Figure 23B). To test if *trans* auto-dephosphorylation could also occur *in vivo*, we expressed *HA-clp1*+ or a *HA-clp1*^PD phosphatase dead mutant under the control of the inducible *nmt* promoter in *clp1Δ*, *clp1*+, or phosphatase dead mutant backgrounds, where endogenous *clp1*+ was tagged with the *myc* epitope. HA-Clp1p-PD expression led to an accumulation of the hyperphosphorylated species in all backgrounds tested. However, a
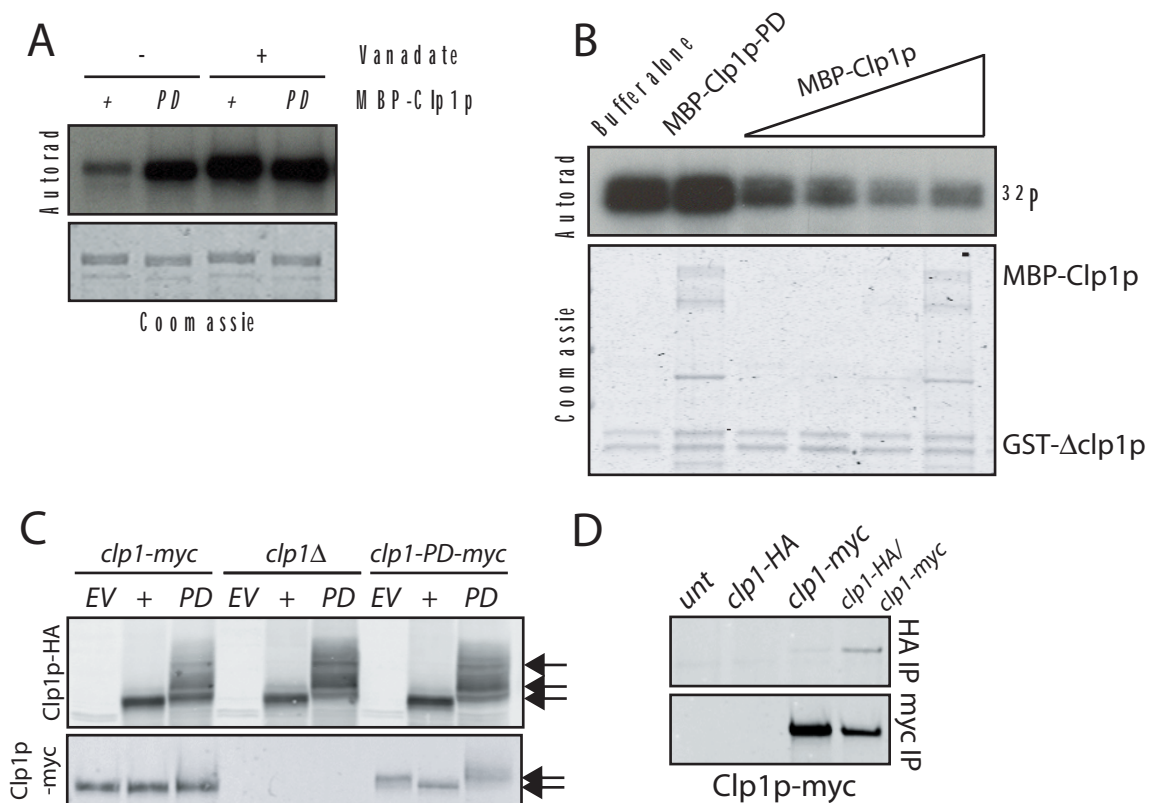
Figure 23. Clp1p auto-dephosphorylates *in trans in vitro*. A) MBP-Clp1p or MBP-Clp1p-PD (Phosphatase Dead) were subjected to *in vitro* kinase assays with kinase active Cdk1p in the presence or absence of 100 µM sodium vanadate. Reactions were resolved on SDS PAGE, Coomassie stained, and exposed to film for autoradiography. B) GST-Δclp1p containing aa 348-537 was subjected to *in vitro* phosphorylation by kinase active Cdk1p, and then incubated in the presence of buffer alone, MBP-Clp1p-PD (Phosphatase Dead), or increasing concentrations of phosphatase active MBP-Clp1p. Reactions were resolved on SDS PAGE, coomassie stained, and exposed to film for autoradiography. C) *clp1-myc* (KGY 2882), *clp1Δ* (KGY 3381), and *clp1::D257A-myc* (KGY 4687-PD) strains transformed with *pRHA:EV* (EV), *pRHA:clp1* (+), and *pRHA:clp1-PD* (PD) vectors were induced to express HA-Clp1p. Samples were processed for Clp1p-myc and Clp1p-HA phosphorylation status via immunblot analysis. Arrows indicate position of different phosphorylated species. D) *wild type* (KGY 246), *clp1-HA* (KGY 139), *clp1-myc* (KGY 2882), and *clp1-HA/clp1-myc* (KGY 5080) cells were grown at 32ºC overnight and lysates prepared for co-immunoprecipitation analysis. Immuoprecipitates were probed with 9E10 and 12CA5 to detect presence of Clp1p in the immune complex.

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significant increase in hyperphosphorylation was seen when HA-Clp1p-PD was expressed in the absence of phosphatase activity in either the *clp1Δ* or D257A-myc strains (Figure 23C, compare top two arrows). Significantly, expression of HA-Clp1p led to the accumulation of the phosphatase dead mutant in the hypophosphorylated state, suggesting that *trans* auto-dephosphorylation can also occur *in vivo*. We could not establish whether this dephosphorylation could also occur in *cis*. However, Clp1p does have the ability to self-associate *in vivo*, as analysis of a diploid strain in which one copy was tagged with the *HA* epitope and the other with the *MYC* epitope revealed that immune complexes of Clp1p-HA also specifically contained Clp1p-MYC (Figure 23D). Quantification of the relative amount of interaction suggests that about 10% of the Clp1p protein is self-associated under these experimental conditions.

Mitotic Phosphorylation of Clp1p Reduces its Catalytic Activity

Although Clp1p possesses the ability to auto-dephosphorylate, during mitosis Clp1p accumulates in the hyperphosphorylated state (Figure 24A). In fact, recombinant MBP-Clp1p incubation with mitotic Clp1p immune complexes failed to collapse the higher migrating bands despite the fact that λ phosphatase could do so (Figure 24A). Phosphatase assays for Cdc14p proteins have only been described for the two human homologs, *h*Cdc14A and *h*Cdc14B. The authors concluded from these studies that the activities varied little across the cell cycle, with *h*Cdc14A being slightly more active during S phase and in late mitosis during mitotic exit (Kaiser et al., 2002). In order to determine if Clp1p activity may be cell cycle regulated, we developed a phosphatase activity assay in which immunoprecipitated material is the source of the phosphatase. Clp1p presence in the immune complexes as well as the active site Cys and Asp residues were required to hydrolyze the artificial substrate, DiFMUP (Figure 24B). Continuous assays performed over time revealed that Clp1p activity varied little during different interphase blocks (Figure 24B). The finding that Clp1p activity was essentially the same from cells arrested in G_2 by either *cdc25-22* or *cdc3-124* mutations was surprising since Clp1p behaves dramatically differently in each of the mutants. During a G_2 arrest imposed by *cdc25-22*, Clp1p remains nucleolar and at the spindle pole body (Trautmann et al., 2001; Cueille et al., 2001). This is in contrast to the G_2 arrest achieved by activation of the cytokinesis checkpoint mutant, *cdc3-124*. During this block, Clp1p is dispersed throughout the nucleus and cytoplasm where its activity is required to maintain the block (Figure 25B) (Trautmann et al., 2001; Cueille et al., 2001). If
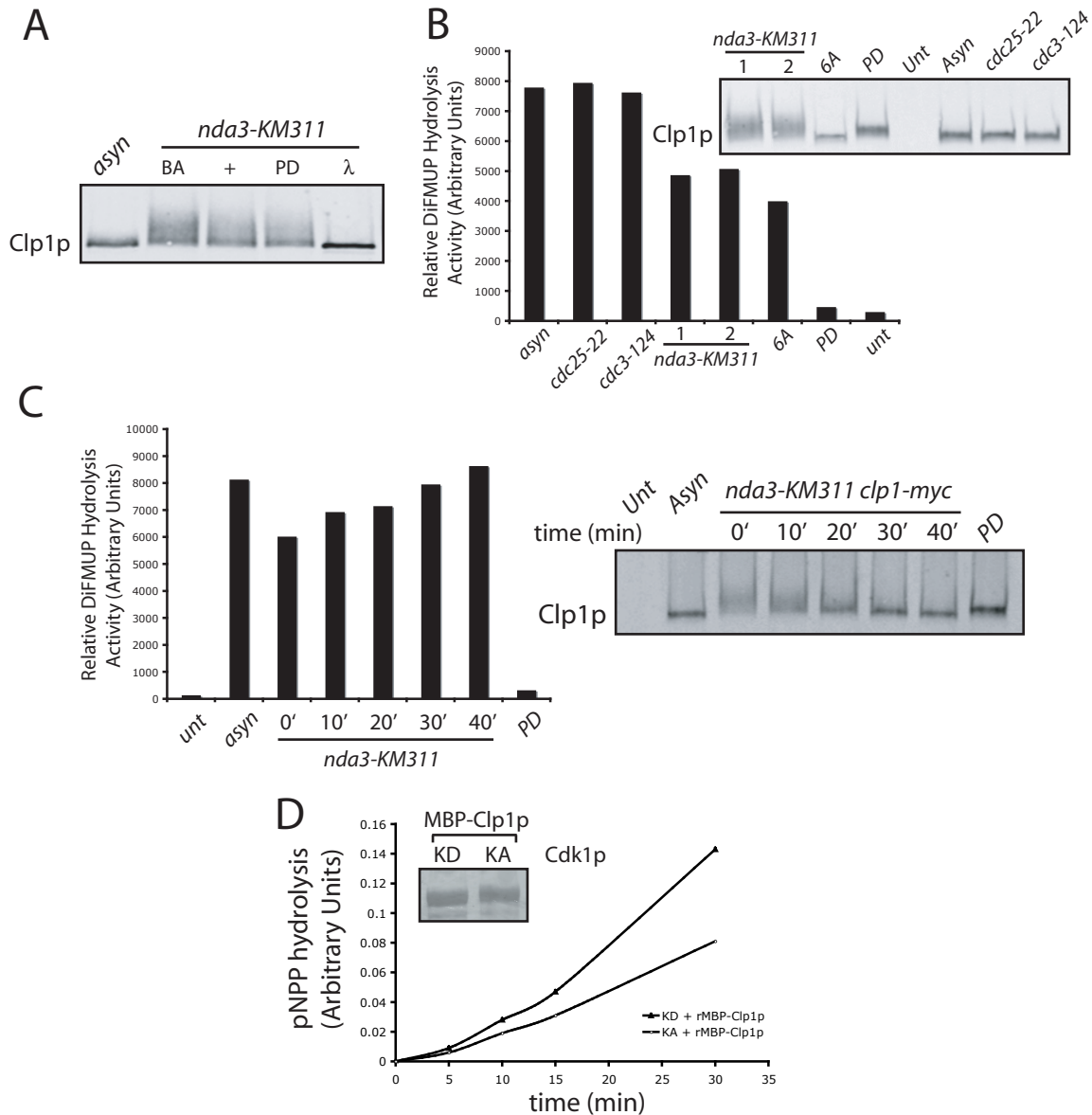
Figure 24. Cdk1p dependent phoshorylation of Clp1p reduces its cataltyic activity. A) *clp1-myc* (KGY 2882-Asyn) cells grown at 32ºC and *nda3-KM311 clp1-myc* (KGY 3388) cells grown at 18ºC were processed for Clp1p phosphorylation status via immunblot analysis after treatment with buffer alone (BA), MBP-Clp1p (+), MBP-D257A (PD), or λ phosphatase. B) *wild type* (KGY 246) (Unt), *clp1-myc* (KGY 2882-Asyn), *clp1::6A-myc* (KGY 4594) (6A), and *clp1::C286S-myc* (KGY 5018) (PD) cells grown at 32ºC, *cdc25-22 clp1-myc* (KGY 3766) and *cdc3-124 clp1-myc* (KGY 5124) cells grown at 36ºC, and *nda3-KM311 clp1-myc* (KGY 3388) cells grown at 18ºC were processed for Clp1p phosphatase activity via ability to hydrolyze DiFMUP and Clp1p phosphoylation state and levels via immunoblot analysis. C) *nda3-KM311 clp1-myc* (KGY 3388) cells were arrested at prometaphase by incubation at 18ºC for 7 hours. Cells were then released to 32ºC and samples taken at the indicated time points. Samples were processed for DiFMUP hydrolysis and Clp1p levels and phosphorylation state as in B). D) MBP-Clp1p was incubated in the presence of kinase active (KA) or kinase dead (KD) Cdk1p and ATP-γ-S at 30ºC for 7 hours, prior to being incubated in the presence of *pNPP. pNPP* reactions were continued at 30ºC and samples removed at the indicated time points and absorbance was determined by spectrophotometric analysis.

there is a nucleolar competitive inhibitor of Clp1p function analogous to Net1p/Cfi1p, the interaction must be lost under our lysis conditions. However, during early mitosis in cells arrested by the *nda3-KM311* mutant, the rate at which Clp1p hydrolyzes DiFMUP is reduced by nearly half of that observed during interphase blocks (Figure 24B). Because Clp1p is in the hyperphosphorylated state during this arrest, these data suggest that mitotic phosphorylation of Clp1p inhibits its catalytic activity and prevents substrate dephosphorylation. To examine if Clp1p dephosphorylation during mitotic exit leads to a restoration of its catalytic activity, we performed an *nda3-KM311* block and release experiment. As cells exited mitosis and underwent septation, Clp1p became hypophosphorylated and its catalytic activity increased as the phosphorylation was reversed (Figure 24C). These data suggest that Clp1p activity is inhibited during early mitosis, and regains its full activity coincidentally with its dephosphorylation during mitotic exit. We additionally tested the effects phosphorylation by Cdk1p played *in vitro* on MBP-Clp1p activity. To circumvent the issue that MBP-Clp1p auto-catalytically removes Cdk1p phosphorylation events, we included in the kinase reactions the non-hydrolyzable ATP analog, ATP-γ-S. Cdk1p uses this form of ATP less efficiently (data not shown), and therefore required longer incubation times to achieve a sub-stoichiometric hyperphosphorylation of Clp1p *in vitro*. After incubation with Cdk1p kinase active, MBP-Clp1p ability to hydrolyze the artificial substrate *pNPP* was reduced by nearly half that achieved upon incubation with kinase dead Cdk1p (Figure 24D). Fifty percent inhibition of Clp1p catalytic activity is consistent with the immunoprecipitation data, and both likely represent underestimates of the effect of phosphorylation on Clp1p activity due to a lack of stoichiometric phosphorylation under each assay condition. Again, these data corroborate a model whereby mitotic phosphorylation of Clp1p by Cdk1p reduces its catalytic activity, and then elimination of these phosphorylation events during mitotic exit restores its full activity. Inhibition of Clp1p activity in early mitosis is likely required to ensure that Clp1p substrate(s) dephosphorylation occurs only after Cdk1p activity drops.

Clp1p Phosphosite Mutants Possess Reduced Phosphatase Activity

We examined in more detail the non-phosphorylatable Clp1p mutants, as they would be predicted to be recalcitrant to Cdk1p mediated inhibition. Three methods of *in vivo* analysis of Clp1p phosphosite mutant function lead us to conclude that these mutants are not insensitive to

inhibition by Cdk1p, but rather are defective for Clp1p phosphatase activity even in the absence of phosphorylation. First, *nda3-KM311 clp1-6A* cells maintain the mitotic arrest and do not prematurely inactivate Cdk1p (Figure 22B). Second, Clp1p phosphosite mutants divide prematurely in a manner consistent with their *in vitro* phosphatase activities (Figure 25A). This is similar to cells deleted for *clp1*[+]. Third, Clp1p phosphosite mutants are unable, to varying degrees, to arrest cell division in response to activation of the cytokinesis checkpoint by the *cdc3-124* mutation (Figure 25B). Sustained checkpoint activation requires Clp1p function, as a phosphatase dead mutant fails to arrest as binucleate cells (Figure 25B).

Mutation of the 6 identified Ser and Thr sites to Asp to mimic the phosphorylated state resulted in a form of the protein that runs constitutively in the hyperphosphorylated state, whose mobility is unaffected by incubation with λ phosphatase (Figure 22C). In fact, the phosphomimetic mutant Clp1p-6D shows an even greater reduction in cell size at division as well as a reduced ability to arrest nuclear division in response to cytokinesis checkpoint activation (Figure 25A and 25B). Because of the similar results obtained for both the non-phosphorylatable and phosphomimetic Clp1p mutants, we examined their *in vitro* phosphatase activities. Whereas MBP alone and MBP-Clp1p-PD failed to hydrolyze the artificial substrate *pNPP*, MBP-Clp1p hydrolyzed the chemical in a manner that was linear with respect to time (Figure 25C). MBP-Clp1p-6A showed reduced ability to hydrolyze *pNPP* with kinetics reduced to nearly fifty percent of the wild type phosphatase (Figure 25C). Further mutation to produce a Clp1p mutant lacking 8 putative Cdk1p phosphorylation sites mimicked a phosphatase dead Clp1p mutant in its ability to hydrolyze *pNPP in vitro* as well as in its ability to sustain the cytokinesis checkpoint (Figure 25B and 25C). Again, Clp1p-6D phosphomimetic resulted in a recombinant phosphatase severely impaired for *pNPP* hydrolysis. In fact, MBP-Clp1p-6D possesses nearly half the activity of the 6A mutant both *in vitro* and *in vivo* (Figure 25B and 25D). We conclude that these phosphosite mutants of Clp1p are loss of function mutations. Because of the similar phenotypes of the 6A and 6D Clp1p mutations, the desired effect of generating mutants recalcitrant to Cdk1p mediated inhibition was not achieved.

Plo1p Involvement in Clp1p Mitotic Phosphorylation

Because recombinant Clp1p was unable to collapse the higher mobility forms of Clp1p isolated from *nda3-KM311* arrested cells (Figure 24A), we wanted to determine if secondary

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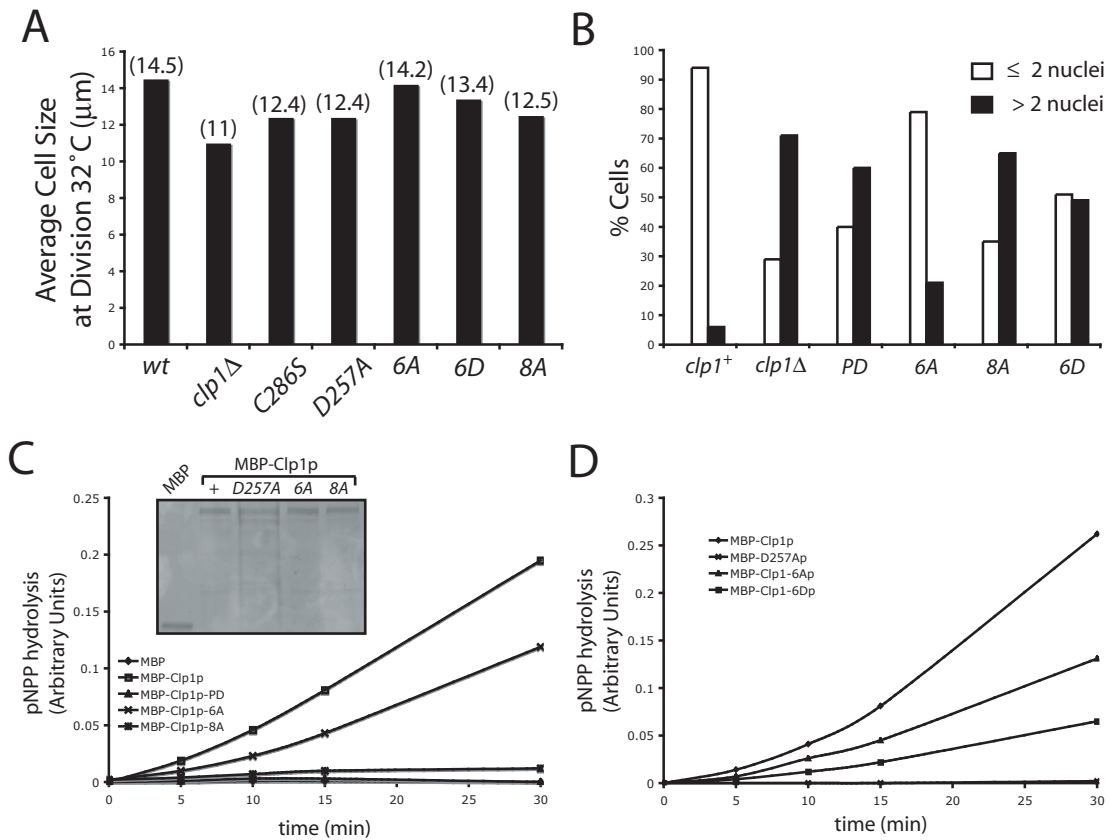
Figure 25. Clp1p phosphosite mutants have reduced activity *in vivo* and *in vitro*. A) *clp1-myc* (KGY 2882), *clp1Δ* (KGY 3381), *clp1::C286S-myc* (KGY 5018), *clp1::D257A-myc* (KGY 4687), *clp1::6A-myc* (KGY 4594), *clp1::6D-myc* (KGY 5184), and *clp1::8A-myc* (KGY 4807) cells were grown at 32ºC and measured live for cell length at division. The number in parenthesis above the bars correspond to the average cell size at division. B) *cdc3-124 clp1-myc* (KGY 5124), *cdc3-124 clp1Δ* (KGY 655), *cdc3-124 clp1::D257A-myc* (KGY 4828), *cdc3-124 clp1::6A-myc* (KGY 4826), *cdc3-124 clp1::8A-myc* (KGY 4827), and *cdc3-124 clp1::6D-myc* (KGY 5366) cells were shifted to the restrictive temperature of 36ºC for 4 hours. Cells were fixed with ethanol and stained with DAPI to visualize nuclei. About 200 cells were counted for each strain and scored for those containing 1 or 2 nuclei, or more than 2 nuclei. C) and D) The indicated MBP-Clp1p mutants were incubated in the presence of *pNPP* at 30ºC for 30 minutes. Sample absorbance was determined at the indicated time points. C)The top panel represents a Coomassie stained SDS PAGE of the reaction inputs.

phosphorylation events after prior Cdk1p phosphorylation rendered the phosphatase resistant to auto-dephosphorylation. Such a collaborative effort of two mitotic kinases has been proposed for Cdk1p and the Polo family of protein kinases (Elia et al., 2003). Polo family kinases contain a conserved domain, the Polo Box, which is targeted to phosphoepitopes created by Cdk1p substrate phosphorylation at the consensus of Ser-pSer/Thr-Pro (Figure 26A) (Elia et al., 2003; Barr et al., 2004). In most cases, Polo kinase binding to the phosphoepitope relieves the auto-inhibition of intramolecular bonding within the Polo Box domain and allows phosphorylation of the substrate at the consensus sites Glu/Asp/Gln-X-Ser/Thr-Z. Clp1p primary amino acid sequence contains two such Polo Box recognition motifs, one of which was identified as being phosphorylated *in vivo* (Figure 20C and Figure 26B), as well as several sequences matching the Polo kinase consensus. To examine if the Polo kinase family member in *S. pombe*, Plo1p, played a role in mitotic hyperphosphorylation of Clp1p, we created a mitotic checkpoint arrest through incubation of the *nuf2-2* allele (Nabetani et al., 2001) at the restrictive temperature either in the presence or absence of *plo1* activity. Although *nuf2-2* and *nuf2-2 plo1-24C* both arrested cell division in mitosis with elevated Histone H1 kinase activity, the extent of Clp1p hyperphosphorylation was reduced in the *nuf2-2 plo1-24C* background (Figure 26C). The level of Histone H1 activity obtained from a *nuf2-2* arrest is approximately one third that obtained from *nda3-KM311* arrest, and as a consequence, results in a modest decrease in hyperphosphorylation of Clp1p. Together, these data suggest that Clp1p may be targeted by the Plo1p kinase for mitotic phosphorylation after prior Cdk1p phosphorylation, preventing Clp1p from auto-dephosphorylating at this time. It is not clear if this secondary phosphorylation results in an additional attenuation of Clp1p activity. However, this seems unlikely since measurements of Clp1p activity from mitotic arrests matched so closely those obtained after phosphorylation *in vitro* by Cdk1p. This awaits further experimentation, but Plo1p phosphorylation of Clp1p may act as an additional layer of control to prevent premature Clp1p disruption of Cdk1p phosphorylation events.
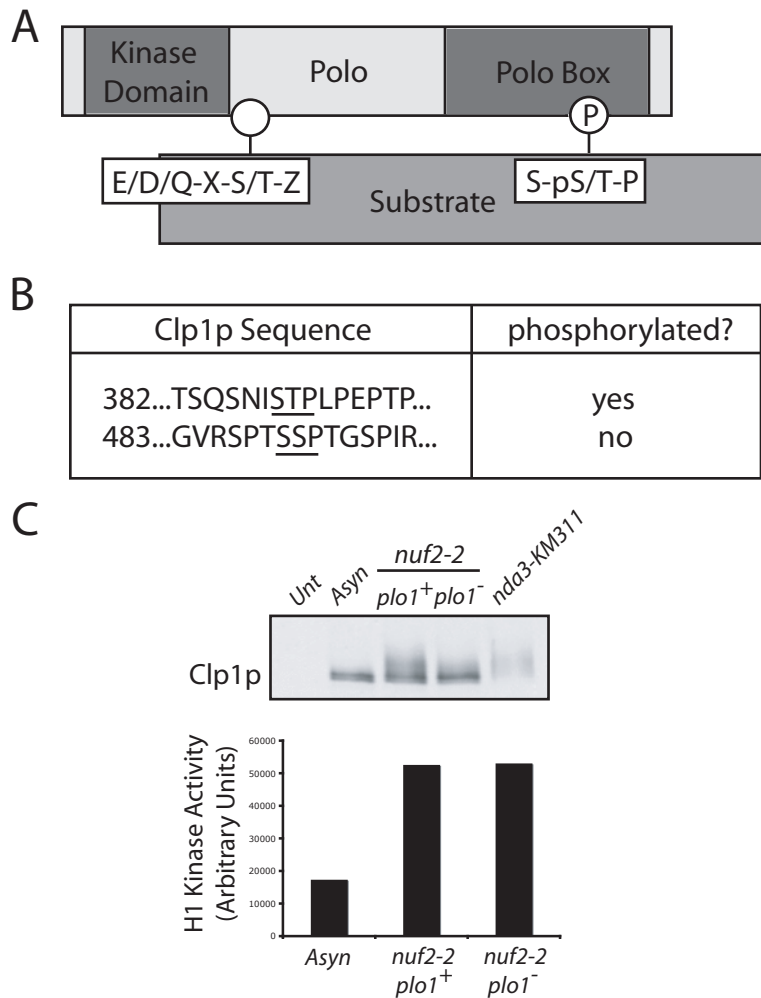
Figure 26. Polo kinase involvement in Clp1p mitotic phosphorylation. A) Model of Polo kinase recruitment of Polo Box Domains to phosphorylated Ser/Thr with the consensus S-S/T-P where S = Ser, T = Thr, and P = Pro. Polo kinase binding frees the kinase domain to phosphorylate substrate with the consensus of E/D/Q-X-S/T-Z, where E = Glu, D = Asp, Q = Gln, X = Any amino acid, S = Ser, T = Thr, and Z = Hydrophobic amino acid. B) Clp1p primary amino acid sequence contains two putative Polo Box Domain binding sites designated by underlining. Site T382 was identified as being phosphorylated *in vivo*, whereas S483 was not identified as a phosphorylation site. C) *wild type* (KGY 246-Unt), *clp1-myc* (KGY 2882-Asyn), *nuf2-2 clp1-myc* (KGY 5364), *nuf2-2 plo1-24C clp1-myc* (KGY 5365), and *nda3-KM311 clp1-myc* (KGY 3388) cells arrested at the respective restrictive temperatures were processed for Clp1p phosphorylation status through immunoblot analysis (upper panel) and for Histone H1 kinase activity (lower panel). Histone H1 activities were quantified by Cherkov counting of radioactive bands.

Summary

Although Clp1p is released from the nucleolus in early mitosis and potentially free to target mitotic substrates for dephosphorylation (Trautmann et al., 2001; Cueille et al., 2001), mitotic substrates still accumulate in the hyperphosphorylated state. Our data suggest that during this time Clp1p phosphatase activity is attenuated through inhibitory phosphorylations by the mitotic kinase, Cdk1p. We have identified mitotic specific phosphorylation events that occur at Cdk1p consensus sites. Cdk1p has the ability to phosphorylate these sites *in vitro*, and Clp1p auto-catalytically reverses them at the exit from mitosis. The resulting effect of this phosphorylation, inhibition of catalytic activity, likely acts to restrain Clp1p targeting of key substrates during early mitosis. This is yet one more example of Cdk1p inhibition of its negative regulators during early mitosis.

We have additionally shown that phosphosite mutants of Clp1p are defective in phosphatase activity both *in vitro* and *in vivo*. It is currently unclear why these mutants are defective, but a plausible explanation is that these residues are important for structural stability of the phosphatase-substrate interaction. Additionally, similar to budding yeast Cdc14p, Clp1p oligomerization may be important for its activity (Taylor et al., 1997). Mutation or phosphorylation of these residues may adversely affect Clp1p oligomerization.

CHAPTER V

DISCUSSION

Summary

Cdc14 proteins reverse mitotic Cdk1p phosphorylation events in a variety of eukaryotes (reviewed by Trautmann and McCollum, 2002). In the budding yeast, *S. cerevisiae*, Cdc14p is essential to bring about complete abrogation of Cdk1p activity during exit from mitosis (Visintin et al., 1998). This study has shown that the Cdc14 family member, *S. pombe* Clp1p, is not essential for mitotic exit, but instead coordinates attenuation of Cdk1p activity with SIN signaling to facilitate proper genomic inheritance of the progeny. *clp1Δ* mutants delay mitotic exit due in part to a failure to disrupt the Cdk1p auto-amplification loop that drives mitotic progression. Clp1p dephosphorylates and inactivates Cdc25p during the exit from mitosis and targets the phosphatase for proteolysis. These events are required for the coordination between exit from mitosis and entry into the subsequent cycle. Clp1p mediated disruption of the Cdk1p auto-amplification loop is temporally regulated to occur only after the onset of anaphase and the initiation of mitotic kinase inactivation in *S. pombe* (Figure 27). The mechanism whereby this occurs involves a positive feedback loop of Clp1p phosphatase activity. Cdk1p mediated phosphorylation of Clp1p during early mitosis attenuates its catalytic activity. Full activity is restored upon the exit from mitosis as Clp1p auto-dephosphorylates and is free to target its substrates for dephosphorylation. Hence, self-sustaining regulation of both Cdk1p and Clp1p drive mitotic progression and exit in *S. pombe*.

Cdc14 mediated inhibition of Cdk1p

Cdc14p in *S. cerevisiae* dephosphorylates several key substrates to achieve complete abrogation of Cdk1p activity, which occurs through stabilization of a Cdk1p inhibitor (Sic1p) and destruction of mitotic B-type cyclins (Visintin et al., 1998). Mitotic Cdk1p inactivation occurs in two successive waves due to the need for budding yeast to delay complete Cdk1p inactivation until the mitotic spindle has elongated through the bud neck. The first phase requires the activities of the APC^{Cdc20} at the metaphase to anaphase transition, while the second phase requires the APC$^{Cdh1/Hct1}$ at mitotic exit (Yeong et al., 2000). *S. cerevisiae* Cdc14p is required for
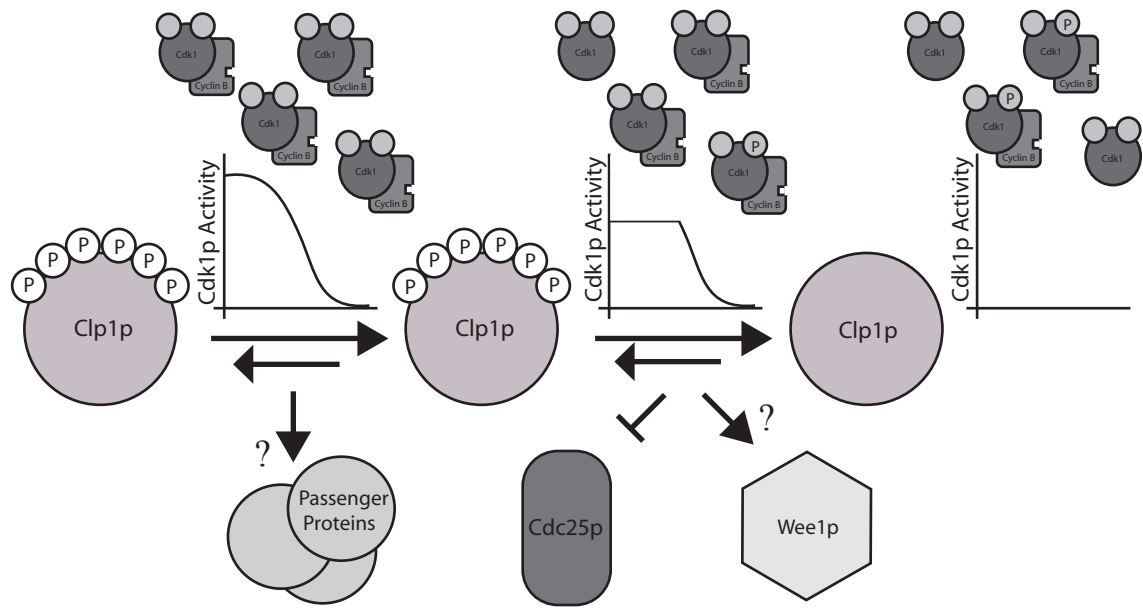
Figure 27. Model for Cdk1p inhibition through Clp1p activation. During mitosis, Clp1p activity is attenuated due to the presence of mitotic phosphorylations. As a consequence, Cdk1p activity remains very high. At this time, Clp1p activity targets the passenger protein complex to promote its kinetochore localization. As Cdk1p activity diminishes due to some cyclin B degradation, Clp1p auto-dephosphorylates, and is then free to dephosphorylate Cdc25p and Wee1p to further inactivate Cdk1p by promoting its Tyr-15 phosphorylation. Note that the forward reaction is always the favored state.

activation of the latter APC complex through dephosphorylation of Cdh1p/Hct1p, and therefore controls the complete attenuation of Cdk1p at mitotic exit (Visintin et al., 1998; Jaspersen et al., 1999). hCdc14A also possesses the ability to activate the APC$^{Cdh1/Hct1}$ in reconstituted preparations through dephosphorylation of hCdh1p (Bembenek and Yu, 2001). However, it remains unclear whether all Cdc14 family members will prove to regulate mitotic exit and if so, whether this occurs through APC/C activation. In fact, Cdc14 family members are not essential for mitotic exit in many eukaryotes examined, but instead are required to regulate cytokinesis and the centrosome cycle (Kaiser et al., 2002; Mailand et al., 2002a; Kaiser et al., 2004; Gruneberg et al., 2002; Trautmann et al., 2001; Cueille et al., 2001). This work and that of others have shown that *S. pombe* cells lacking the Clp1p phosphatase display only a delay in exit from mitosis, and seem to regulate the inactivation of Cdk1p dramatically different from *S. cerevisiae* Cdc14p (Cueille et al., 2001; Wolfe and Gould, 2004; Esteban et al., 2004).

Our data indicate that Clp1p reverses Cdk1p dependent phosphorylation events present on Cdc25p at mitotic exit, resulting in its inactivation. These dephosphorylation events play a key role in disrupting the Cdk1p auto-amplification loop and attenuating Cdk1p activity in late mitosis. Our data further suggest that these phosphorylation events are necessary for a timely progression through mitosis, as their elimination reduces Cdc25p ability to promote mitotic entry. Cdc25 proteins from other eukaryotes are similarly hyperphosphorylated during mitosis, and these events have been predicted to affect the timing of mitotic entry (Hoffmann et al., 1993; Izumi and Maller, 1993). In the case of *Sp*Cdc25, *X*Cdc25, and *h*Cdc25C, this mitotic phosphorylation is dependent upon Cdk1p activity and stimulates its catalytic activity (Kovelman and Russell, 1996; Izumi and Maller, 1993; Hoffmann et al., 1993). As the hyperphosphorylation of Cdc25 homologs represents a conserved element of mitotic control, it will be interesting in the future to determine if their inactivation by Cdc14 homologs is also conserved.

In a mechanism unrelated to the dephosphorylation events, Clp1p also targets Cdc25p for ubiquitin-mediated proteolysis during the exit from mitosis. Previous data has implicated the HECT-domain containing ubiquitin ligase, Pub1p, in the *in vivo* ubiquitination of Cdc25p (Nefsky and Beach, 1996). However, this study did not address the temporal aspect of Cdc25p destabilization. We have demonstrated that Cdc25p late mitotic turnover does not require Pub1p, but instead, requires the function of Clp1p in addition to the APC/C ubiquitin ligase. The N-

terminal regulatory domain of Cdc25p contains intrinsic elements capable of promoting its instability. This same region contains 3 putative destruction boxes and is sufficient for Clp1p binding. It is not clear at this time whether stabilization of Cdc25p truncated at the N-terminus results from the lack of signals present or from a lack of Clp1p binding. Again, Cdc25 turnover by APC/C represents a conserved feature of its regulation. *h*Cdc25A is a target of the APC/C in late mitosis following the removal of Cdk1p sites of phosphorylation (Mailand et al., 2002b). Whether or not one of the human Cdc14 homologs initiates proteolysis of *h*Cdc25A through its dephosphorylation has not been determined. In *S. pombe*, Clp1p plays a more active role in Cdc25p proteolysis than merely reversal of Cdk1p phosphorylation events, as elimination of sites of phosphorylation does not lead to its rapid turnover in G_1 in the absence of Clp1p function. The exact mechanism of how Clp1p functions with the APC/C to regulate stability of Cdc25p remains obscure. We speculate that this could involve Clp1p activation of the APC/C or involve its role as an accessory factor for Cdc25p destruction.

Exit from mitosis is also coincident with the binding of Cdc25p to 14-3-3 proteins and its subsequent nuclear exclusion in *S. pombe* (Lopez-Girona et al., 1999). Phosphorylation of Cdc25p by response site kinases promotes its affinity for 14-3-3 molecules (Zeng and Piwnica-Worms, 1999; Lopez-Aviles et al., 2005). In *Xenopus* Cdc25 and *h*Cdc25C, Cdk1p phosphorylation at the -2 site relative to the response site interferes with response site phosphorylation (Bulavin et al., 2003). A similar mechanism may be operating within *S. pombe* Cdc25p in which mitotic phosphorylation events drive nuclear accumulation of Cdc25p by precluding response site phosphorylation. This model suggests that the resumption of Cdc25p and 14-3-3 binding could only occur after reversal of the mitotic Cdk1p phosphorylation events by Clp1p. Consistent with this notion, we have found an increased ability to detect Cdc25p in late mitotic nuclei in the absence of Clp1p. Whether this results from the increased abundance of Cdc25p in *clp1Δ* cells or from Clp1p regulating rates of Cdc25p nuclear export through 14-3-3 binding has not been determined. This model also suggests that a late mitotic response site kinase would play similar inhibitory roles to both Cdc25p function and Cdk1p activity. In *S. pombe*, the checkpoint kinases Chk1p and Cds1p phosphorylate the response sites in Cdc25p upon checkpoint activation (Zeng et al., 1998; Zeng and Piwnica-Worms, 1999; Furnari et al., 1999). Additionally, these sites are phosphorylated during G_2 by the stress-activated kinase, Srk1p, in

the absence of checkpoint signaling (Lopez-Aviles et al., 2005). Thus, one or more of these kinases may collaborate with Clp1p to remove remaining nuclear Cdc25p.

Our results suggest that Clp1p functions in late mitosis to combat Cdk1p activity by disrupting the Cdk1p auto-amplification loop. Activation of Cdc25 family members and inactivation of the Wee1 and Myt1 family of kinases at the G_2/M transition by Cdk1p drives mitotic initiation in a switch-like fashion (reviewed by O'Farrell, 2001). Our data suggest that disruption of this amplification loop is an active mechanism whereby Cdc14 phosphatases aid in Cdk1p inactivation. *S. cerevisiae* rely on full destruction of mitotic cyclins and accumulation of a Cdk1p inhibitor (Sic1p) for abrogation of Cdk1p activity (Visintin et al., 1998). Because full Cdc13p (Cyclin B) destruction is never observed in late mitosis of wild type *S. pombe* cells (Chang et al., 2001), and the Cdk1p inhibitor Rum1p plays no obvious roles in Cdk1p inactivation (Blanco et al., 2000), *S. pombe* cells may utilize Tyr-15 phosphorylation of Cdk1p as a mechanism to further inactivate Cdk1p. Evidence from *S. cerevisiae* points to the fact that in times of prolonged spindle checkpoint activation, cells can adapt and inactivate Cdk1p through inhibitory phosphorylation, as occurs in *cdc55Δ* mutants (Minshull et al., 1996). Bypass of this arrest does not affect proteolysis of the cyclin B, Clb2p, but instead relies on Tyr-19 phosphorylation to inhibit Cdk1p activity (Minshull et al., 1996).

There are two plausible mechanisms that could account for the disruption of the Cdk1p positive feedback loop in late mitosis. Downregulation of Cdk1p through degradation of small pools of cyclin B could reduce Cdk1p activity to levels such that more cyclin B is targeted for degradation. Disruption of the positive feedback loop would therefore occur passively as the amount of Cdk1p activity required to sustain it would be eliminated. Second, active disruption of the positive feedback loop through activation of specific phosphatase(s) could reduce Cdk1p activity to levels that are competent to stimulate APC/C activity. This would subsequently lead to more cyclin B destruction and a concomitant drop in kinase activity. Our evidence suggests that disruption of the positive feedback loop stems from a combination of the two possibilities. While elimination of the amplification loop most likely does not act as a trigger for Cdk1p inactivation, disruption of it is an active process, requiring the Clp1p phosphatase in *S. pombe*. We speculate that the concerted efforts of protein phosphatases (*i.e.* Cdc14 family members) working with response site kinases, together with the APC/C, play important roles in shutting off

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this amplification loop. It will be important in the future to determine the temporal order of such late mitotic events that catalyze the abrogation of Cdk1p activity.

This model also suggests that the inhibitory Thr-14/Tyr-15 kinases (Wee1 and Myt1) are similarly dephosphorylated and activated during late mitosis to aid in the inactivation of Cdk1p. Wee1 proteins in *Xenopus* and human cells are inactivated through phosphorylation in mitosis, possibly due to the concerted efforts of Cdk1p and Polo kinases (Mueller et al., 1995a; Watanabe et al., 2004). Although it is not clear if Wee1 proteins are re-activated by dephosphorylation in late mitosis, *S. cerevisiae* Swe1p is transiently dephosphorylated during the exit from mitosis, just prior to its degradation and degradation of the major cyclin B, Clb2 (Harvey and Kellogg, 2003). While activity assays have not been reported for Swe1p, its mitotic hyperphosphorylation may inhibit its catalytic activity. Therefore it is possible that the dephosphorylated form of Swe1p in late mitosis plays a role in inactivating Cdk1p. Should this be the case, then the feed-forward amplification loop which rapidly stimulates activation of Cdk1p at mitotic onset, additionally provides a rapid switch to inactivate mitotic Cdk1p at mitotic exit.

Clp1p Mitotic Phosphorylation - Analogous to FEAR?

Whereas Clp1p is released from the nucleolus in early mitosis (Trautmann et al., 2001; Cueille et al., 2001), its ability to target Cdk1p mitotic substrates for dephosphorylation is limited. We have shown that mitotic phosphorylation of Clp1p by Cdk1p attenuates its catalytic activity during early mitosis. Clp1p auto-catalytically reverses these phosphorylation events at mitotic exit. This not only provides a switch-like activation of Clp1p in late mitosis, but also a mechanism to prevent reversal of mitotic Cdk1p phosphorylation events prior to sister chromatid separation when Cdk1p activity is maximal. We propose that full Clp1p activation may occur in two steps. The first occurs upon release from the nucleolus as cells enter mitosis when Clp1p becomes concentrated at the contractile ring and at the kinetochore (Trautmann et al., 2001; Cueille et al., 2001; Trautmann et al., 2004). Although its activity is attenuated due to mitotic phosphorylation, Clp1p activity is required at the kinetochore for monitoring chromosome bi-orientation (Trautmann et al., 2004). The second step involves autocatalytic reversal of these inhibitory phosphorylation events at mitotic exit, resulting in full Clp1p activation. During this phase, Clp1p is able to target Cdc25p for dephosphorylation and disrupt the Cdk1p auto-amplification loop.

The regulation of Clp1p in *S. pombe* resembles the stepwise activation of *S. cerevisiae* Cdc14p. The FEAR and MEN networks collaborate to promote the full release of Cdc14p from its nucleolar inhibitor (reviewed by Stegmeier and Amon, 2004). Whereas the FEAR network causes the early anaphase release of Cdc14p, the MEN promotes full activation and release of Cdc14p during the exit from mitosis (Pereira et al., 2002; Stegmeier et al., 2002; Shou et al., 1999; Visintin et al., 1999). Upon release from Net1p/Cfi1p by the FEAR, Cdc14 targets substrates involved in a number of mitotic processes. Cdc14p aids stability of the anaphase B spindle through dephosphorylation of the INCENP-Aurora-Survivin passenger protein complex and other MAPs that localize to the spindle midzone (Higuchi and Uhlmann, 2005; Pereira and Schiebel, 2003). Cdc14p also enters into a positive feedback loop for MEN activation as it dephosphorylates and activates the effector kinase Cdc15p (Stegmeier et al., 2002). Strikingly, FEAR released Cdc14p also interacts with the MEN GAP component, Bfa1p, but fails to dephosphorylate it until the exit from mitosis (Pereira et al., 2002). These data suggest that while Cdc14 may be targeted to distinct substrates by the FEAR, an additional layer of specificity controlling the temporal order of substrate dephosphorylation remains that we do not yet understand.

The preference of Cdc14p to target only a subset of its substrates during early anaphase raises the intriguing question of how selectivity is achieved. Why are some substrates preferentially targeted prior to others? The involvement of a single Cdk1p capable of driving both the budding and fission yeast cell cycles also raises the same important question. As for Cdk1p, it is thought that both substrate accessibility and the requirement of distinct substrates for varying levels of Cdk1p activity guide the temporal regulation of substrate phosphorylation (Hayles et al., 1994; Loog and Morgan, 2005). The same argument could be made for FEAR released Cdc14p: its access to some substrates is either limited by accessibility or requires a higher degree of Cdc14p activity that only becomes present upon MEN activation.

Most likely, both mechanisms operate to control Cdc14p substrate dephosphorylation. FEAR released Cdc14p is distributed throughout the nucleus, and concentrates at the spindle pole body, the mitotic spindle, and at the kinetochores, but fails to localize to the cytoplasm (Pereira et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2002). This localization pattern precludes its ability to dephosphorylate the Sic1p transcription factor, Swi5p, which resides in the cytoplasm when phosphorylated by Cdk1p (Moll et al., 1991). Thus, Cdc14p fails to

inactivate Cdk1p through Sic1p accumulation during early anaphase. With regard to Bfa1p, Cdc14p overexpression in metaphase-arrested cells is capable of driving its dephosphorylation (Pereira et al., 2002). Yet, despite their interaction at the spindle pole body during early anaphase, Cdc14p does not dephosphorylate Bfa1p until after MEN activation (Pereira et al., 2002). This suggests that perhaps MEN activation, or Cdk1p inactivation, aids Cdc14p-mediated Bfa1p dephosphorylation.

One key function of Clp1p prior to anaphase occurs at the kinetochore and involves the maintenance of the INCENP-Aurora-Survivin complex at these sites to monitor chromosome bi-orientation (Trautmann et al., 2004). *clp1Δ* mutants display frequent sister chromatid segregation defects and high rates of minichromosome loss (Trautmann et al., 2004). Clp1p phosphatase activity is presumably required at the kinetochore at this time as a phosphatase dead mutant shows similar levels of minichromosome loss as the *clp1Δ* mutant (Trautmann et al., 2004). Clp1p likely targets one or more of these passenger proteins for dephosphorylation in the presence of elevated Cdk1p activity prior to the initiation of anaphase. Similar to Cdc14p dephosphorylation of FEAR substrates, Clp1p could require a lower level of activity to target the passenger protein complex. It is additionally possible that because Clp1p is not stoichiometrically phosphorylated, that the fraction of Clp1p localized to the kinetochore is not inhibited through phosphorylation. Determining the key substrates in this complex that are targeted by Clp1p and analysis of their relative affinities for Clp1p should also distinguish between substrate specificity and requirements for varying levels of Clp1p activity. Whatever the mechanism involved, we propose that similar to budding yeast Cdc14p, *S. pombe* Clp1p activation involves a two-step process, one in which only a subset of Clp1p targets are dephosphorylated when Cdk1p activity remains high, followed by one in which Cdk1p activity is attenuated and Clp1p is free to dephosphorylate the remainder of its substrates. As *h*Cdc14A and *X*Cdc14 are similarly hyperphosphorylated during early mitosis, this may represent a conserved mechanism whereby Cdc14 protein substrate selectivity is temporally determined (Kaiser et al., 2002; Kaiser et al., 2004).

Cdk1p ensures that its activity is not prematurely attenuated prior to anaphase initiation by inhibiting the activities of its negative regulators. In the absence of such a mechanism, precocious mitotic exit and cytokinesis could occur in the absence of sister chromatid segregation, leading to incomplete genome inheritance of the resulting progeny. This mechanism

of inhibition is a conserved feature of mitotic control. The Cdk1p inhibitor Sic1p in budding yeast acts as a Cdk1p sensor, responding to a threshold level of Cdk1p activity. While phosphorylation by Cdk1p at multiple sites leads to rapid Sic1p turnover, a reduction in Cdk1p activity during the exit from mitosis prevents its phosphorylation, and as a consequence Sic1p accumulates (Nash et al., 2001). Whether or not all Cdk1p inhibitors respond to a threshold level of Cdk1p activity has not been determined. However, our data suggest that Clp1p may also respond differently to varying levels of Cdk1p activity. Clp1p phosphatase activity is inhibited when Cdk1p activity is maximal, and Clp1p only regains its full strength as it auto-dephosphorylates upon Cdk1p attenuation. Thus, the removal of Cdk1p mediated inhibition from its negative regulators is likely to play key roles in stimulating mitotic exit in eukaryotes.

Future Directions

We have shown that the Cdc14p family member, *S. pombe* Clp1p, disrupts the Cdk1p auto-amplification loop during the exit from mitosis, thereby attenuating Cdk1p activity. We have additionally shown that the cessation of the feedback loop is temporally regulated to only occur after sister chromatid separation, once Cdk1p activity begins to diminish. Many questions remain as to how these events are achieved in a coordinated manner to couple Cdk1p inactivation with other late mitotic events.

While these data have demonstrated that disruption of the Cdk1p auto-amplification loop in late mitosis through Cdc25p inactivation plays an important role in Clp1p-mediated Cdk1p activity attenuation, they also implicate an activation of the Wee1p inhibitory kinase for Cdk1p-mediated inhibition. Esteban, *et al* reported that Wee1p is not regulated by the Clp1p/Flp1p phosphatase during mitotic exit and suggested that perhaps Cdk1p feedback loop inactivation involved the related Mik1p kinase (Esteban et al., 2004). Mik1p primarily is thought to function during S phase (Christensen et al., 2000), and its involvement in the Cdk1p auto-amplification loop has not been reported. The activities of Wee1p homologs in a variety of eukaryotes are attenuated upon mitotic entry due to phosphorylation. *S. cerevisiae* Swe1p is hyperphosphorylated in mitosis, which abolishes its ability to delay mitotic progression. The kinetics of Swe1p phosphorylation and dephosphorylation have been monitored in detail and involve several mitotic specific hyperphosphorylation events (Harvey and Kellogg, 2003). Strikingly, Swe1p becomes hypophosphorylated during the exit from mitosis, just prior to

cytokinesis (Harvey and Kellogg, 2003). Whether this hypophosphorylated state requires the activity of Cdc14p has not been determined. *In vitro*, *S. pombe* Wee1p is an excellent substrate for Cdk1p (our unpublished observations). We have also determined that Clp1p and Wee1p interact using a yeast-2 hybrid assay, requiring the presence of the Wee1p N-terminus (our unpublished observations). It will be interesting to determine whether Clp1p targets Wee1p for dephosphorylation at the end of mitosis, and whether this dephosphorylation leads to its activation. Immunoprecipitation kinase assays of Wee1p in the presence or absence of $clp1^+$ from cells synchronously exiting mitosis will be used to determine the effect of Clp1p on functional Wee1p activity in late mitosis.

This work additionally shows a potential involvement by the polo family of kinases in Clp1p mitotic phosphorylation. Activation of polo kinases upon Polo Box recruitment to Cdk1p sites of phosphorylation is quickly becoming the rule rather than the exception (Elia et al., 2003; Watanabe et al., 2004). This collaboration between Cdk1p and Polo family kinases may be operating within *S. pombe* Clp1p. Secondary phosphorylation events at non-Cdk1p consensus sites may preclude Clp1p's ability to auto-dephosphorylate or further debilitate its activity. Because Clp1p *in vitro* phosphatase activity subsequent to Cdk1p phosphorylation was nearly identical to that obtained from immunoprecipitates of mitotic cells, it seems unlikely that Plo1p phosphorylation of Clp1p would additionally attenuate its catalytic activity. However, we need to examine whether or not Plo1p phosphorylates Clp1p, whether this requires prior phosphorylation by Cdk1p, and whether this precludes Clp1p from auto-catalytically removing these phosphorylation events. If Plo1p phosphorylation of Clp1p does prevent Clp1p auto-phosphatase activity, this would suggest then that an activating phosphatase is required to dephosphorylate Clp1p prior to Clp1p reversing the Cdk1p phosphorylation events. We would then expect that in its absence, Clp1p would remain in the hyperphosphorylated state as cells exit mitosis. As a consequence, mutation of this phosphatase would yield similar mitotic exit defects displayed by a *clp1*Δ mutant, and Cdk1p substrates should remain in the hyperphosphorylated state. In the event that these predictions of Plo1p phosphorylation of Clp1p are correct, we will take a candidate approach to identify potential phosphatase(s) involved in reversing these Plo1p phosphorylation events.

Our data also suggest a differential requirement of various substrates for Clp1p activity. In early mitosis, when Clp1p is in the hyperphosphorylated state, it presumably targets the

passenger protein complex (INCENP-Aurora-Survivin) for dephosphorylation (Trautmann et al., 2004). However, other Clp1p substrates (Cdc25p, itself) accumulate in the hyperphosphorylated state at this time. These data suggest that lower amounts of Clp1p phosphatase activity are sufficient to target some substrates, but not others, for dephosphorylation. Because we lack evidence that the Clp1p fraction localized to the kinetochore is hyperphosphorylated, we will generate phosphospecific antibodies to mitotic Clp1p phosphorylation sites and, using indirect immunofluorescence, determine whether the kinetochore Clp1p fraction is phosphorylated. If there is a different level of activity required by some substrates, then we will determine the mechanism of how this selectivity is achieved. We will determine the relative Clp1p binding affinities and specificities for early versus late substrates in order to understand Clp1p temporal selectivity.

While our data suggest that Cdk1p mediated mitotic phosphorylation of Clp1p inhibits its ability to dephosphorylate its substrates, we do not understand the mechanism of how this inhibition is achieved. We plan to use kinetic analysis comparing hyper versus hypophosphorylated Clp1p to understand whether phosphorylation inhibits Clp1p ability to bind or dissociate substrate. Our work has demonstrated that Clp1p has the ability to self-associate *in vivo*. Because this study was not performed in the absence of other *S. pombe* proteins, we do not know whether this is indicative of an oligomeric association. This merits further examination because recombinant *Sc*Cdc14p forms oligomers that constitute the active fraction (Taylor et al., 1997), and *X*Cdc14 runs as a multimer on gel filtration analysis (Kaiser et al., 2004). Clp1p oligomerization may be required for functional activity *in vivo*, and this may vary across the cell cycle. Co-immunoprecipitation studies from synchronous samples will be used to determine whether this self-association varies across the cell cycle. Analysis of the Clp1p phosphosite mutants demonstrated that they possessed reduced activity both *in vitro* and *in vivo*. We will determine whether this reduced activity stems from an altered pattern of oligomerization by using a bacterial dual expression system to co-express Clp1p and associated phosphosite mutants with different affinity tags. This should allow us to determine whether Clp1p forms oligomers, whether phosphorylation by Cdk1p influences this ability, and whether the phosphosite mutants are varied in their abilities to oligomerize.

Along with the effect of Cdk1p phosphorylation, we would also like to determine whether Clp1p responds to a threshold of Cdk1p activity, similar to the mechanism of Sic1p

turnover from *S. cerevisiae* (Nash et al., 2001). By adding back sites to the phosphosite mutants of Clp1p, we will be able to address whether increasing phosphorylation events leads to a concomitant attenuation of Clp1p activity. By equipping a sensor for Cdk1p activity within its negative regulators, mitotic exit can be delayed until Cdk1p activity is attenuated. Addressing these key questions should increase our ability to describe the ordered progression of late mitotic events that culminate in Cdk1p inactivation and a cycle reset.

CONCLUDING REMARKS

The recent years have shed increasing light as to how the events at the G_2/M transition and mitotic exit are regulated. Mitotic initiation is driven by activation of the Cdk1-cyclin B complex, and its inactivation, as well as a reversal of its mitotic phosphorylation events, is required for a timely exit from mitosis and the initiation of cytokinesis. In *S. cerevisiae*, the conserved Cdc14p family phosphatase regulates the inactivation of Cdk1p during the exit from mitosis by promoting the ubiquitin-mediated proteolysis of cyclin B, and the stabilization of a Cdk1p inhibitor.

Our studies into the mechanism of how the Cdc14 family member, *S. pombe* Clp1p, inhibits Cdk1p suggest that all Cdc14 family members may not regulate Cdk1p in the same manner as does *Sc*Cdc14p. Our data suggest that Clp1p promotes a resumption of Tyr-15 phosphorylation of Cdk1p in late mitosis by eradicating the Cdk1p positive amplification loop through Cdc25p inactivation (and most likely, Wee1p activation). Furthermore, our data support a model in which Clp1p activity in early mitosis is attenuated by, and responsive to, elevations in Cdk1p activity. We hypothesize that this inhibition is required to prevent premature mitotic Cdk1p inactivation prior to anaphase, and this inhibition represents a conserved feature of the regulation of different Cdc14 family members. In conclusion, our studies have underscored the importance of communication between positive and negative regulators of mitotic progression to ensure the fidelity of the cell division process.

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