The Mitotic Exit Network in *Saccharomyces cerevisiae*:
Regulation of Cyclin Destruction and Exit from Mitosis

by

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DISSERTATION

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Dedicated with love to my parents,
Kenneth and Irene Jaspersen

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When I came to UCSF six years ago, I never imagined that graduate school would be so difficult or so rewarding. There are so many people who have contributed to my graduate career. They have helped me grow as a scientist and as a person and have enriched my life in so many ways.

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Lena Hwang, Julia Charles, Rachel Tinker-Kulberg, and Sue Jaspersen developed the assay for measuring cyclin-ubiquitin ligase activity used throughout this thesis. To make this assay possible, Alex Szidon purified the N-terminus of sea urchin cyclin B and determined the conditions for its iodination. Many of the genetic interactions with *cdc5-1* described in Chapter 2 were done in parallel by Julia Charles. Rachel Tinker-Kulberg constructed the *GAL-PDS1-HA* plasmid used in Chapter 2. Julia Charles performed the experiments shown in Chapter 3, Figures 6 and 7, and provided the purified Cdc14 proteins used in Chapter 4. With these exceptions, the rest of the work was performed by Sue L. Jaspersen under the direction and supervision of David O. Morgan.

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Sue L. Jaspersen

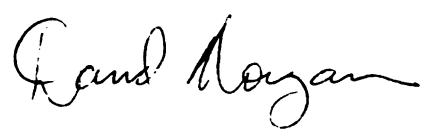
Abstract

Inhibition of mitotic cyclin-dependent kinase (Cdk) activity is required for cells to exit from mitosis and progress into G1. The primary mechanism of mitotic Cdk inactivation is ubiquitin-mediated cyclin proteolysis, which is catalyzed by a multi-subunit ubiquitin ligase known as the anaphase-promoting complex (APC) in association with its substoichiometric activating subunit, Hct1 (also known as Cdh1). In *Saccharomyces cerevisiae*, a second mechanism of mitotic Cdk inactivation involves regulated binding of the Clb-specific Cdk inhibitor, Sic1.

Members of the *S. cerevisiae* late mitotic mutant family, including *cdc14* and *cdc15*, arrest in anaphase with a phenotype similar to cells overexpressing non-degradable forms of mitotic cyclins. Overproduction of Sic1 rescued the growth defect of most of the mutants, indicating that their primary defect is an inability to inactivate mitotic Cdks. Consistent with this possibility, we found that the late mitotic mutants exhibited defects in cyclin-specific APC activation and Sic1 accumulation. A complex array of genetic interactions among the mutants suggests that they form a regulatory network controlling mitotic Cdk inactivation.

Because the mitotic exit network is required specifically for initiation of cyclin proteolysis, it might regulate APC activation by Hct1. We found that Hct1 was phosphorylated at multiple Cdk consensus sites during cell cycle stages when APC activity was low. Recombinant Hct1 activated cyclin-ubiquitin ligase activity of the APC *in vitro*; however, Hct1 phosphorylation by mitotic Cdks inhibited this activity. Inhibitory phosphorylation of Hct1 was removed by the phosphatase Cdc14, suggesting that the requirement for Cdc14 in late mitosis is due, at least in part, to its ability to activate Hct1.

Cdc14 activation in late mitosis requires the function of the protein kinase, Cdc15. We found that Cdc15 was phosphorylated on multiple sites during most of the cell cycle, but was dephosphorylated by Cdc14 for a brief period following anaphase. Genetic and biochemical analysis indicated that phosphorylation negatively regulates the mitotic exit function of Cdc15 without affecting its kinase activity. Dephosphorylation of Cdc15 does not appear to be required for Cdc14 activation, however, raising the possibility that Cdc15 dephosphorylation by Cdc14 might promote a second Cdc15 function in the control of mitotic exit.



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Chapter One

Introduction

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The cell cycle

The cell cycle comprises the ordered set of processes by which one cell grows and divides to become two cells. To ensure that each cell division results in two genetically identical progeny, three basic tasks must be completed once and only once during each cell cycle: DNA must be replicated (S phase), chromosomes must be equally segregated (mitosis), and the cell must divide (cytokinesis). These cell cycle events must be coordinated so that they occur in the proper order and commence only after the previous task is completed. Defects in completing or coordinating any step of the cell cycle can result in the inaccurate transmission of genetic material, leading to cell death or aneuploidy. Cells use elaborate surveillance systems or checkpoint controls to monitor cell cycle events and prevent cell cycle progression when tasks are not successfully completed (Hartwell and Weinert, 1989; Elledge, 1996; Rudner and Murray, 1996).

Sequential passage through the cell cycle is regulated by the activity of cyclin-dependent kinases (Cdks). Cdks are the "engine" that drives the events of the eukaryotic cell cycle. To ensure the proper timing and coordination of cell cycle events, Cdk activity is tightly controlled by several mechanisms (Morgan, 1997). Cdk activity requires binding to cyclins, regulatory subunits whose levels fluctuate during the cell cycle. Cdk activity is also regulated by both activating and inhibitory phosphorylation and through the binding of Cdk inhibitory subunits. These different regulatory mechanisms generate oscillations in Cdk activity that time the different phases of the cell cycle. Cdk phosphorylation of key targets during each cell cycle stage activates specific biochemical pathways that ultimately lead to DNA replication and chromosome segregation.

Cell cycle progression in the budding yeast *Saccharomyces cerevisiae* is driven by a single Cdk, Cdc28, which binds to a series of phase-specific cyclins (Figure 1-1) (Lew et al., 1997). Cyclin-dependent substrate specificity helps different Cdc28 complexes trigger specific events during each cell cycle phase. Oscillations in the levels of the various cyclins

are the primary mechanism involved in timing the yeast cell cycle. Accumulation of cyclins at different points during the cell cycle is the regulated by an elaborate transcriptional program controlled by Cdc28 itself. In addition, cyclin levels within the cell are also regulated post-translationally by cell cycle dependent proteolysis.

The contribution of proteolysis to cell cycle periodicity is an emerging theme in cell cycle research (King et al., 1996; Peters, 1998). Proteolysis is a way to ensure directionality to the cell cycle and to eliminate cyclically acting proteins when they are no longer required. It directly influences Cdk activity since both cyclins and certain Cdk inhibitors are destroyed during defined windows of the cell cycle by ubiquitin-mediated proteolysis. Two E3 ubiquitin ligases catalyze the highly specific proteolytic events of the eukaryotic cell cycle: the SCF (for Skp1/Cullin (or Cdc53)/F-box protein) is essential for destruction of proteins at the G1-S transition, and the anaphase-promoting complex (APC, also known as the cyclosome) promotes the degradation of cell cycle inhibitors at the metaphase-to-anaphase and M-G1 transitions (Figure 1-1).

Mitotic exit

The final tasks of the cell cycle are accomplished in M phase. During mitosis, newly replicated chromosomes are segregated by the mitotic spindle to form two identical sets of genetic material. Mitosis is immediately followed by cytokinesis, the division of cytoplasmic components that results in two daughter cells, each containing a single nucleus. M phase events are initiated in a coordinated and perfectly timed fashion to ensure that each occurs only after the previous one is completed. Exit from mitosis is also tightly regulated so that cell division does not occur prior to completion of chromosome segregation.

Progression through mitosis is driven by the accumulation of B-type cyclins and their associated Cdk activity. In *S. cerevisiae*, cells lacking Cdc28-Clb kinase activity

arrest in G2/M as large budded cells with paired sister chromatids, duplicated spindle pole bodies, and no mitotic spindle (Surana et al., 1991; Fitch et al., 1992; Schwob and Nasmyth, 1993) (J. Ubersax & D. Morgan, personal communication). To exit from mitosis and initiate cytokinesis, mitotic cyclin-Cdk activity must decrease (Ghiara et al., 1991). APC-dependent proteolysis of mitotic cyclins is the primary mechanism of Cdk inactivation at the end of mitosis, although additional Cdk inactivation mechanisms allow mitotic exit in the absence of complete cyclin destruction under some conditions (Toyn et al., 1996; Schwab et al., 1997; Visintin et al., 1997).

The objective of my studies is to understand the mechanisms that control completion of M phase in budding yeast. In this the remainder of this chapter, I will focus my discussion on the regulatory network of proteins that controls the events leading to Cdc28 inactivation, mitotic exit, and cytokinesis in *S. cerevisiae*. The contributions of APC dependent proteolysis, Sic1 accumulation, and the mitotic exit network will all be described.

Cdk inactivation by cyclin proteolysis

Cyclin proteolysis

Cyclin proteolysis is a highly conserved mechanism controlling Cdk inactivation at the end of mitosis. Inhibition of cyclin degradation by overexpression of non-destructible forms of mitotic cyclins results in a late anaphase arrest in a wide range of eukaryotes (Murray et al., 1989; Gallant and Nigg, 1992; Holloway et al., 1993; Surana et al., 1993; Rimmington et al., 1994; Sigrist et al., 1995; Yamano et al., 1996). In *S. cerevisiae*, overproduction of the major mitotic cyclin Clb2 delays spindle disassembly and cytokinesis following anaphase, and overexpression of a non-degradable form of Clb2 causes cells to permanently arrest in anaphase (Ghiara et al., 1991; Stueland et al., 1993; Surana et al., 1993). The defect in mitotic exit observed in these cells is the result of sustained Cdc28-

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Clb kinase activity. Decreasing kinase activity by overexpression of the Cdk-inhibitor Sic1 or by inactivating Cdc28 triggers cytokinesis (Ghiara et al., 1991; Lew and Reed, 1995; Amon, 1997).

Mitotic cyclins are targeted for ubiquitin-mediated proteolysis by a short amino-terminal sequence termed the destruction box (Glotzer et al., 1991). A multi-step reaction is responsible for the covalent attachment of the 76 residue, highly conserved ubiquitin molecule onto lysine side-chains near the cyclin destruction box (Figure 1-2) (Ciechanover, 1994). In the first step of the reaction, the ubiquitin activating enzyme (E1) uses the energy of ATP to from a thioester bond between itself and ubiquitin. Next, activated ubiquitin is transferred to the ubiquitin conjugating enzyme (E2). The E2, together with a ubiquitin ligase (E3), transfers ubiquitin onto the cyclin substrate, forming an isopeptide bond between the activated C-terminal glycine of ubiquitin and a lysine residue in the mitotic cyclin. Following attachment of the first ubiquitin to cyclin, ubiquitin itself becomes a target for further ubiquitination. Repeated rounds of the ubiquitination reaction result in a multi-ubiquitinated cyclin that is recognized and destroyed by the 26S proteasome.

In *S. cerevisiae*, the *UBA1* gene encodes the single major E1 enzyme (Dohmen et al., 1995). At least two different members of the E2 family of enzymes (UBCs) are capable of participating in cyclin ubiquitination reactions. Homologs of the clam E2-C enzyme (UbcX in *Xenopus*, UbcH10 in humans, and UbcP4 in fission yeast) seem to function specifically in mitosis to target mitotic regulatory proteins for destruction (Aristarkhov et al., 1996; Yu et al., 1996; Osaka et al., 1997; Townsley et al., 1997). Ubc4, an E2 enzyme involved in multiple ubiquitination pathways, is also able to function in reconstituted cyclin ubiquitination reactions (King et al., 1995; Yu et al., 1996; Charles et al., 1998). However, in budding yeast, cyclin proteolysis does not seem to require any particular E2 enzyme; yeast cells lacking Ubc4, its homolog Ubc5, or the E2-C homolog Ubc11 do not have any major defects in cyclin ubiquitination and degradation (Zachariae and Nasmyth, 1996; Townsley and Ruderman, 1998a). The E3 required for cyclin

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ubiquitination was first identified in clam and frog egg extracts as a multimeric, 20S complex (Hershko et al., 1994; King et al., 1995; Sudakin et al., 1995). This cyclin ubiquitin ligase complex appears to be highly conserved throughout evolution and is known as the anaphase-promoting complex (APC) or cyclosome (described below and in Table 1-1).

APC-dependent proteolysis is not only required for the destruction of mitotic cyclins, but is also needed for the degradation of other mitotic regulatory proteins. Addition of cyclin B destruction box fragments to *Xenopus* egg extracts blocks both cyclin destruction and chromosome segregation (Holloway et al., 1993). Similarly in *S. cerevisiae*, APC mutants arrest in metaphase prior to sister chromatid separation despite the fact that cyclin destruction is not required until the end of anaphase (Irniger et al., 1995; Zachariae et al., 1996; Hwang and Murray, 1997; Kramer et al., 1998b; Yu et al., 1998; Zachariae et al., 1998b). These experiments suggest that APC-mediated destruction of an anaphase inhibitor is required at the metaphase-to-anaphase transition (Figure 1-1). Members of the functionally related "securin" family of proteins, including Pds1 in *S. cerevisiae* and Cut2 in *Schizosaccharomyces pombe*, bind to and inhibit proteins (separins) that promote sister chromatid separation (Esp1 in *S. cerevisiae* and Cut1 in *S. pombe*) (Ciosk et al., 1998; Kumada et al., 1998). APC-dependent degradation of the securins is required for progression from metaphase to anaphase (Cohen-Fix et al., 1996; Funabiki et al., 1996; Zou et al., 1999). In addition to cyclins and Pds1, other mitotic regulatory proteins also have been shown to be APC substrates in *S. cerevisiae*. Destruction of the microtubule-associated protein Ase1 is necessary for efficient disassembly of the mitotic spindle (Juang et al., 1997). The APC also catalyzes destruction of the WD40 repeat protein Cdc20 and the Polo-related protein kinase Cdc5 (Charles et al., 1998; Prinz et al., 1998; Shirayama et al., 1998).

The anaphase-promoting complex

Through a combination of genetics and biochemistry, components of the APC have been identified in a number of systems (Table 1-1). The vertebrate APC was originally thought to contain eight subunits (Apc1-8), but additional subunits have recently been identified (King et al., 1995; Peters et al., 1996; Yu et al., 1998; Grossberger et al., 1999). Homologs of most of the vertebrate subunits are found in the twelve subunit *S. cerevisiae* APC (Irniger et al., 1995; Zachariae et al., 1996; Hwang and Murray, 1997; Kramer et al., 1998b; Yu et al., 1998; Zachariae et al., 1998b). Sequence analysis of APC subunits has shed little light onto their possible function. Not surprisingly, many of the subunits have motifs thought to mediate protein-protein interactions, including the tetratricopeptide repeats (TPR) found in Cdc16, Cdc23, Cdc27, and the vertebrate subunit, Apc7 (Lamb et al., 1994; Tugendreich et al., 1995; Yu et al., 1998). Apc2 is a distant relative of the cullin family, a class of proteins found in other ubiquitin ligases (Kramer et al., 1998b; Yu et al., 1998; Zachariae et al., 1998b). Based on studies of other E3 enzymes (the SCF in particular), the RING-H2 protein-protein binding domain found in Apc11 has been postulated to interact with the cullin domain of Apc2 to mediate the physical interaction between the E2, the E3, and the substrate (Ohta et al., 1999; Zachariae and Nasmyth, 1999).

Several lines of evidence suggest that the APC mediates the key regulatory step in cyclin destruction. Of the enzymes involved in the cyclin ubiquitination pathway, the APC is the only component whose activity is cell cycle regulated. Early biochemical experiments in clam and frog egg extracts revealed that cyclin ubiquitin ligase activity of the APC was low during interphase and then increased at the end of mitosis, correlating inversely with the stability of B-type cyclins (Hershko et al., 1994; King et al., 1995; Sudakin et al., 1995). Measurements of the cyclin ubiquitin ligase activity *in vitro* in budding yeast and other somatic cells also suggest that the decrease in cyclin stability from metaphase or anaphase until the onset of S phase seen *in vivo* is a consequence of APC activation; APC

activity increases in late mitosis and remains high throughout G1 (Amon et al., 1994; Brandeis and Hunt, 1996; Charles et al., 1998; Fang et al., 1998a; Fang et al., 1998b; Kotani et al., 1998; Kramer et al., 1998a). Inactivation of the APC in yeast cells arrested in G1 results in accumulation of mitotic cyclins as well as other APC substrates.

APC regulation by phosphorylation

Initial studies of cyclin ubiquitin ligase activity *in vitro* indicated that the APC is regulated post-translationally by phosphorylation of a number of subunits (Table 1-1). Mitotically active APC preparations from *Xenopus* are highly phosphorylated on a number of subunits including Apc1, Apc3/Cdc27, Apc6/Cdc16, and Apc8/Cdc23 (King et al., 1995; Peters et al., 1996). Treatment of active APC with phosphatase decreases ubiquitin ligase activity, suggesting that phosphorylation is important for activity (Lahav-Baratz et al., 1995; Peters et al., 1996). Several protein kinases have been implicated in APC regulation: Polo-related kinases (Plk1 in mammals, Plx1 in *Xenopus*, and Cdc5 in budding yeast) promote APC activation, while in mammals and fission yeast Protein Kinase A (PKA) appears to inhibit cyclin-directed APC activity (Yamashita et al., 1996; Charles et al., 1998; Descombes and Nigg, 1998; Kotani et al., 1998; Kotani et al., 1999).

In addition to Polo-related kinases and PKA, a number of studies implicated Cdc2-cyclin B in regulation of APC phosphorylation in higher eukaryotes. In clam extracts, Cdc2-cyclin B treatment is able to partially activate an interphase APC after a lag (Felix et al., 1990; Sudakin et al., 1995). Furthermore, *Xenopus* Cdc2-cyclin B in association with Suc1/Cks1 can directly phosphorylate at least two APC subunits *in vitro* (Apc1 and Apc3/Cdc27) (Patra and Dunphy, 1998). Cdc2 has also been reported to stimulate APC activity indirectly through activation of Polo-related kinases, which may directly phosphorylate Apc1, Apc3/Cdc27, and Apc6/Cdc16 (Kotani et al., 1998; Kotani et al., 1999). Although these observations support the possibility that Cdc2 stimulates APC

activity, the precise mechanism of activation and the relevance of Cdc2 phosphorylation *in vivo* have not been demonstrated.

Our understanding of Cdc2's role in APC activation has been further complicated by observations in budding yeast that Cdc28 activity inhibits APC-dependent cyclin proteolysis *in vivo* (Amon, 1997). Expression of stabilized Clb2 in G1 is capable of activating Cdc28 and repressing the cyclin proteolysis machinery. In addition, inactivation of Cdc28 by induction of two Cdk inhibitors triggers APC-dependent Clb2 proteolysis and sister chromatid separation in mitotically arrested cells. One interpretation of these results is that Cdc28-Clb phosphorylation of the core APC inhibits its activity. An alternative explanation is that Cdc28 blocks APC activation by phosphorylation of the substoichiometric activator, Hct1 (discussed below).

Phosphorylation of the APC core was only recently reported in *S. cerevisiae* (A. Rudner and A. Murray, personal communication). Phosphorylation of several APC subunits, including Cdc16, Cdc27, and Cdc23, increases during mitosis at the time of Pds1 and cyclin destruction. Cdc28 phosphorylates these subunits *in vitro*, and their phosphorylation *in vivo* depends on Cdc28 activity. Mutation of Cdk phosphorylation sites on these subunits has little effect on cell cycle progression and cyclin-specific APC activity; however, several lines of evidence suggest that Cdc28 phosphorylation may have a positive role in regulating APC activity towards other substrates such as Pds1. An intriguing possibility raised by this work, as well as work of others, is that Cdk phosphorylation of the APC promotes its activation by Cdc20 in metaphase (see below), but then is not required to maintain APC activity later in mitosis and in G1 (Shteinberg and Hershko, 1999; A. Rudner and A. Murray, personal communication).

Substoichiometric APC activators

Despite the size and complexity of the APC, enzymatic activity requires an additional activator protein (Table 1-1). Two related versions of this APC activator have

been identified in numerous organisms: these proteins are called Cdc20 and Hct1/Cdh1 in budding yeast, Slp1 and Srw1/Ste9 in fission yeast, Fizzy and Fizzy-related in *Drosphilia* and *Xenopus*, and $p55^{CDC}$/hCDC20 and hCDH1 in humans (I will refer to them by their budding yeast names) (Dawson et al., 1995; Sigrist et al., 1995; Schwab et al., 1997; Sigrist and Lehner, 1997; Visintin et al., 1997; Weinstein, 1997; Yamaguchi et al., 1997; Fang et al., 1998b; Kim et al., 1998; Kitamura et al., 1998; Kramer et al., 1998a; Lorca et al., 1998). Neither Cdc20 nor Hct1 are easily detected in purified preparations of APC, indicating that they are substoichiometric regulators (Peters et al., 1996; Zachariae et al., 1998b). Multiple studies have demonstrated association of the core APC with Cdc20 and Hct1 is a limiting step in APC activation. Cell cycle dependent binding of Cdc20 and Hct1 correlates with increases in APC activity, and loss of function in either of the activators causes defects in mitotic progression (Sethi et al., 1991; Schwab et al., 1997; Visintin et al., 1997; Fang et al., 1998a; Kramer et al., 1998a; Lim et al., 1998; Shirayama et al., 1998; Zachariae et al., 1998a). Addition of Cdc20 or Hct1 to the APC *in vitro* greatly stimulates its cyclin-ubiquitin ligase activity (Fang et al., 1998b; Kramer et al., 1998a; Jaspersen et al., 1999; Kotani et al., 1999). Similarly, overproduction of Cdc20 or Hct1 in mitotically arrested yeast cells causes cyclin levels to decrease in an APC-dependent manner (Schwab et al., 1997; Visintin et al., 1997; Lim et al., 1998; Shirayama et al., 1998). How Cdc20 and Hct1 promote APC-dependent ubiquitination is currently mysterious, although they have been postulated to serve as adaptor proteins that present the substrate to the ubiquitination machinery.

Regulation of Hct1 and Cdc20

As key regulators of APC activity, Cdc20 and Hct1 are critical targets for many mitotic regulatory pathways. Cdc20 function is regulated at multiple levels. In yeast and somatic cells, Cdc20 concentration peaks in late S phase and mitosis, paralleling the increase in binding of Cdc20 to the APC (Weinstein, 1997; Fang et al., 1998b; Fang et al.,

1998a; Kramer et al., 1998a; Prinz et al., 1998; Shirayama et al., 1998). As cells exit mitosis and enter G1, protein levels decrease as a result of APC-dependent proteolysis of Cdc20 (Prinz et al., 1998; Shirayama et al., 1998). Cdc20 is also phosphorylated *in vivo*, perhaps by mitotic Cdks (Weinstein, 1997; Kotani et al., 1999). Phosphorylation of Cdc20 does not appear to affect binding to the APC, but may instead enhance its ability to interact with APC substrates or regulate its own proteolysis by the APC (Kotani et al., 1999). Finally, Cdc20 function is inhibited by association with the checkpoint signaling protein Mad2 in cells arrested in metaphase as a result of spindle defects (Fang et al., 1998a; Hwang et al., 1998; Kallio et al., 1998; Kim et al., 1998).

Unlike Cdc20, Hct1 levels do not change during the cell cycle in yeast or vertebrate somatic cells (Fang et al., 1998b; Fang et al., 1998a; Kramer et al., 1998a; Prinz et al., 1998). However, Hct1-dependent APC activity is confined to late mitosis and G1 by the regulated association of Hct1 with the APC (Kramer et al., 1998a; Zachariae et al., 1998a). Chapter 3 examines how Hct1 activity is regulated. Briefly, phosphorylation of Hct1 during most of the cell cycle by Cdc28 blocks its association with the APC (Zachariae et al., 1998a; Jaspersen et al., 1999). Dephosphorylation of Hct1 at the end of mitosis triggers APC activation and mitotic exit. The ability of Cdc28 to phosphorylate Hct1 and block APC activation explains previous results that Cdc28 inhibits cyclin destruction (Amon, 1997). We also found that overproduction of an Hct1 mutant lacking the Cdk phosphorylation sites causes premature cyclin destruction, indicating that Hct1 phosphorylation is involved in coordinating the timing of cyclin destruction. Regulation of Hct1 function by phosphorylation is likely used not only in yeast, but also in higher eukaryotes to prevent premature APC activation (Kramer et al., 1998a; Kotani et al., 1999).

Timing of cyclin destruction

Despite sequence similarities, Cdc20 and Hct1 do not play identical roles in regulating APC activity. As a result of differences in the cell cycle regulation of the two proteins, binding of Cdc20 to the APC increases during mitosis, preceding Hct1 binding which is maximal in late mitosis and during G1 (Fang et al., 1998a; Kramer et al., 1998a). This provides a temporal regulation to APC activation, as well as alters the responsiveness of the APC to certain regulatory inputs. Based primarily on genetic evidence in budding yeast, Cdc20 and Hct1 are also thought to confer substrate specificity to the APC *in vivo* (Schwab et al., 1997; Visintin et al., 1997; Lim et al., 1998; Shirayama et al., 1998). Pds1 degradation at the metaphase-to-anaphase transition is promoted by Cdc20, after which Hct1 stimulates APC-dependent proteolysis of Clb2 (Figure 1-1). Part of this apparent substrate preference may be simply attributable to differences in the timing of APC activation by Cdc20 and Hct1, or it may reflect accessibility of substrates within the cell. However, additional data in both yeast and vertebrates indicates that Cdc20 and Hct1 contribute to substrate recognition (Fang et al., 1998a; Kramer et al., 1998a; Prinz et al., 1998; Pfleger and Kirschner, 2000).

Sequential activation of the APC by Cdc20 then Hct1 is important in timing mitotic events. In yeast cells, APC activation by Hct1 depends on previous activation by Cdc20, apparently because Cdc20 triggers destruction of proteins that inhibit Hct1 (Figure 1-4) (Lim et al., 1998; Shirayama et al., 1999). One obvious candidate is the anaphase inhibitor, Pds1, which is required to prevent cyclin destruction in cells arrested in mitosis as a result of DNA or spindle damage (Yamamoto et al., 1996; Alexandru et al., 1999; Tinker-Kulberg and Morgan, 1999). Through an unknown mechanism, proteolysis of Pds1 promotes activation of the phosphatase Cdc14 (Cdc14 is required for Hct1 dephosphorylation, see below) (Shirayama et al., 1999). However, the fact that cells lacking both Pds1 and Cdc20 arrest in anaphase with high levels of Clb2 clearly indicates

that a second protein must be degraded by APC-Cdc20 to trigger Hct1 activation (Lim et al., 1998; Shirayama et al., 1999). Searching for mutations that allow *pds1Δ cdc20Δ* cells to live, Shirayama and colleagues determined that the S phase cyclin Clb5 is a second link between APC activation by Cdc20 and Hct1 (Figure 1-4) (Shirayama et al., 1999). They speculated that decreases in Cdc28 kinase activity, as the result of APC-Cdc20 dependent Clb5 destruction and increases in Cdc14 activity, is sufficient to reduce Hct1 phosphorylation below some critical threshold to trigger activation of the APC by Hct1. Their results do not exclude the possibility that APC-Cdc20 mediated destruction of other mitotic cyclins, including Clb2 and Clb3, also may contribute to APC-Hct1 activation during a normal cell cycle (Yeong et al., 2000).

Cdk inactivation by Sic1

Complete cyclin destruction is not absolutely required for mitotic exit due to additional mechanisms of Cdk inactivation. In budding yeast, late mitotic Cdk inactivation is also promoted by the Clb-specific Cdk inhibitor Sic1 (Mendenhall, 1993; Donovan et al., 1994; Schwob et al., 1994). Cells in which APC-dependent proteolysis has been compromised or cells lacking Sic1 are viable, indicating that neither Cdk inactivation pathway is essential for mitotic exit. However, at least one pathway is required since double mutants between the APC and Sic1 are dead (*hct1Δ sic1Δ* or *cdc23-1 sic1Δ*, for example), presumably due to an inability to inactivate Cdc28 (Toyn et al., 1996; Schwab et al., 1997; Visintin et al., 1997). It is not clear if Sic1 homologs exist in other eukaryotes, but other Cdk inactivation mechanisms may also contribute to mitotic exit in these organisms (Minshull et al., 1996; Jin et al., 1998).

Regulation of Sic1

The level of Sic1 protein increases in late mitosis as a result of both transcriptional and post-translational mechanisms. Nuclear accumulation of the *SIC1* transcription factor Swi5 is inhibited until the end of mitosis by Cdc28-Clb phosphorylation of its nuclear localization sequence (Moll et al., 1991). A decrease in Cdc28 phosphorylation at the end of mitosis results in Swi5 translocation into the nucleus and a burst in *SIC1* transcription (Knapp et al., 1996; Toyn et al., 1996). Cellular levels of Sic1 are also regulated by ubiquitin-dependent destruction mediated by the SCF ubiquitin ligase (Figure 1-1) (Schwob et al., 1994). Phosphorylation of Sic1 by both Cdc28-Clb and Cdc28-Cln kinases targets the protein for SCF-dependent proteolysis from the onset of S phase until mitosis (Schneider et al., 1996; Feldman et al., 1997; Verma et al., 1997). As a result of mitotic cyclin destruction, Sic1 phosphorylation is hypothesized to decrease in late mitosis, resulting in increased Sic1 stability.

The phosphatase Cdc14

Reversing the effects of Cdc28

Cdc28-Clb kinases inhibit their own inactivation until the end of mitosis by at least three mechanisms (Figure 1-3). Phosphorylation of Hct1 renders it unable to associate with the APC to trigger cyclin proteolysis. In addition, phosphorylation of Sic1 targets it for destruction by the SCF, and phosphorylation of Swi5 reduces *SIC1* transcription, keeping cellular levels of the Cdk inhibitor low. The ability of Cdc28 to inhibit its own inactivation by these mechanisms allows the accumulation of Cdc28-Clb activity from late G1 through mitosis. The mutual antagonism between Cdc28 and its inhibitors, Hct1 and Sic1, leads to the potential for a regulatory loop that could enhance the abrupt, all-or-none kinetics of Cdc28 inactivation in late mitosis.

A key issue remains unresolved, however. What initiating event is responsible for reducing Cdc28 activity (or increasing APC or Sic1 activity) to some threshold where these regulatory relationships bring on rapid and complete Cdc28 inactivation? Part of the answer may be that Cdc20 triggers APC-dependent destruction of Clb2, Clb3, and Clb5 (Alexandru et al., 1999; Shirayama et al., 1999; Yeong et al., 2000). Degradation of a population of mitotic cyclins presumably decreases Cdc28 kinase activity and reduces Hct1, Sic1, and Swi5 phosphorylation. However, reversal of Cdc28 action in late mitosis cannot be accomplished solely by Cdk inactivation: dephosphorylation of its substrates is also required. In *S. cerevisiae*, Cdk inactivation and mitotic exit require the function of the protein phosphatase Cdc14 to reverse the effects of Cdc28 by catalyzing dephosphorylation of Hct1, Sic1, and Swi5 (Figure 1-3).

Functions of Cdc14

CDC14 encodes an essential protein phosphatase required for mitotic exit in *S. cerevisiae* (Hartwell et al., 1973; Wan et al., 1992). Analysis of Cdc14 enzymatic activity *in vitro* showed that it is an oligomeric dual-specificity phosphatase able to dephosphorylate both phospho-serine/threonine and phospho-tyrosine residues (Taylor et al., 1997). Loss of Cdc14 function causes cells to arrest in anaphase with low levels of Sic1 and cyclin ubiquitin ligase activity (Jaspersen et al., 1998), making it an excellent candidate to be the phosphatase which reverses the effects of Cdc28 on Sic1, Swi5, and Hct1 to trigger mitotic exit.

Recent work, described in part in Chapter 3, indicates that Cdc14 directly acts on these three cell cycle regulators to promote Cdk inactivation and mitotic exit (Visintin et al., 1998; Jaspersen et al., 1999). Overexpression of Cdc14 in yeast cells results in a G1-like state due to inactivation of Cdc28. In these cells, phosphorylation of Hct1 is abolished, APC-Hct1 activity is stimulated, and levels of the mitotic cyclins decrease in an APC-dependent manner. Sic1 protein also accumulates in cells overproducing Cdc14, leading to

further inhibition of Cdc28-Clb activity. The effects of Cdc14 observed *in vivo* appear to reflect a direct catalytic role for Cdc14 on Cdc28 antagonists, since recombinant Cdc14 is able to remove phosphate from purified Swi5, Sic1, and Hct1 that have been treated with Cdc28. Therefore, Cdc14 triggers mitotic exit by at least three parallel mechanisms, each of which inhibits Cdc28 activity (Figure 1-3).

Several lines of evidence indicate that Cdc14 may have additional functions during the cell cycle. Cdc14 genetically interacts with the origin-recognition complex protein, Orc2, suggesting that Cdc14 is involved in regulation of S-phase initiation (Hardy, 1996). However, Cdc14 may only play an indirect role in DNA replication as a result of its effects on Cdc28 activity (Fitzpatrick et al., 1998). High rates of plasmid loss and defects in nucleolar segregation in *cdc14-1* mutants may reflect a role for Cdc14 in chromosome segregation (Hartwell and Smith, 1985; Palmer et al., 1990; Granot and Snyder, 1991). Finally, in Chapter 4, I provide evidence that Cdc14 promotes cytokinesis by regulating the phosphorylation state of the protein kinase Cdc15. A role for Cdc14 in cell division was also previously suggested following genetic analysis of an inactive *CDC14* allele (Shirayama et al., 1996).

Regulation of Cdc14

Amounts of Cdc14 protein do not fluctuate during the cell cycle although overexpression studies suggest that Cdc14 is the rate-limiting regulator of Cdk inactivation at the end of mitosis (Visintin et al., 1998; Jaspersen et al., 1999; Shou et al., 1999; Visintin et al., 1999). Several recent reports indicate that Cdc14 function is restrained during most of the cell cycle by an association with the nucleolar protein Net1/Cfi1, a part of the RENT complex (regulator of nucleolar silencing and telophase) (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999). Binding to Net1 sequesters Cdc14 in the nucleolus and may also inhibit Cdc14 phosphatase activity. Shortly after anaphase, Cdc14 is transiently released from the complex into the nucleus and the cytoplasm where it is

thought to dephosphorylate its targets and trigger mitotic exit (Figure 1-3). The pathways leading to Cdc14 release are not understood, although Cdc14 redistribution requires the activities of the mitotic exit network of proteins (Figure 1-4) (Shou et al., 1999; Visintin et al., 1999). Currently, localization is the only mechanism predicted to regulate Cdc14 activity; however, other levels of Cdc14 regulation have not been rigorously excluded.

The mitotic exit network

In *S. cerevisiae*, exit from mitosis is regulated by a collection of proteins known as the mitotic exit network or the late mitotic family. Grouped together on the basis of their terminal arrest phenotype, the late mitotic mutants were identified through various genetic screens as cells that arrest in late anaphase with large buds, an elongated spindle, and separated DNA (Hartwell et al., 1973; Johnston and Thomas, 1982; Johnston et al., 1990; Shirayama et al., 1994a; Shirayama et al., 1994b; Luca and Winey, 1998). At the mutant arrest point, anaphase has occurred, but spindle disassembly and cytokinesis are blocked. Interestingly, many of the late mitotic mutants encode potential regulatory proteins, including the protein kinases Cdc15 and Dbf2, the Polo-like kinase Cdc5, the protein phosphatase Cdc14, the guanine-nucleotide exchange factor Lte1, the Ras-like GTPase Tem1, and a protein of unknown function Mob1 (Johnston et al., 1990; Schweitzer and Philippsen, 1991; Wan et al., 1992; Kitada et al., 1993; Shirayama et al., 1994a; Shirayama et al., 1994b; Luca and Winey, 1998). Multiple genetic interactions between members of the late mitotic family, presented in Chapter 2, suggest that the late mitotic genes encode members of a regulatory network controlling mitotic exit.

Cdc28 inactivation through Cdc14

The late mitotic arrest phenotype is similar to that observed in yeast cells overproducing a non-degradable form of Clb2 (Surana et al., 1993), raising the possibility

that the late mitotic proteins are required for the inactivation of Cdc28-Clb complexes. Consistent with this possibility, mutants in the mitotic exit network arrest with high levels of Clb2, negligible levels of APC activity toward cyclins, and low levels of Sic1 (Surana et al., 1993; Shirayama et al., 1994b; Toyn and Johnston, 1994; Charles et al., 1998; Jaspersen et al., 1998). Overexpression of *CDC5*, *CDC14*, or a non-phosphorylatable form of *CDC15* causes Sic1 accumulation and premature activation of the APC, resulting in cyclin proteolysis and Cdk inactivation (Charles et al., 1998; Shirayama et al., 1998; Visintin et al., 1998; Jaspersen et al., 1999; Jaspersen and Morgan, 2000). Overproduction of the Cdk inhibitor Sic1 suppresses the growth arrest of most of the late mitotic mutants, providing further evidence that an inability to inactivate Cdc28 is their primary defect (Donovan et al., 1994; Toyn et al., 1996; Charles et al., 1998; Jaspersen et al., 1998).

Only one of the late mitotic gene products, the phosphatase Cdc14, has been implicated convincingly in direct control of Cdk inactivation (discussed previously, Figure 1-3). The functions of the other members of the network are less clear. One possibility is that the rest of the late mitotic proteins trigger Cdk inactivation indirectly through activation of Cdc14 (Figure 1-4). The idea that Cdc14 is the downstream effector of the mitotic exit network is supported by several lines of evidence. First, overexpression of *CDC14* suppress the growth defect of almost all mitotic exit mutants, but overproduction of any other component cannot suppress the growth defect of *cdc14-1* cells (Shirayama et al., 1996; Grandin et al., 1998; Jaspersen et al., 1998). Second, the ability of *CDC14* overexpression to drive Clb2 destruction and Sic1 accumulation does not depend on the functions of many members of the pathway (Visintin et al., 1998). Finally, redistribution of Cdc14 following anaphase requires the actions of Cdc15, Cdc5, Dbf2, and Tem1 (Shou et al., 1999; Visintin et al., 1999). When Cdc14 is released from the nucleolus by deletion of Net1, Cdc15 and Tem1 function are no longer required for Cdc28 inactivation. The

signaling pathways used by the mitotic exit network to trigger Cdc14 activation are unclear, but may involve phosphorylation of Net1 (Shou et al., 1999).

Other roles in mitotic exit

It is not clear if the sole function of the mitotic exit network is to trigger Cdc14 release from the nucleolus, or whether it has additional functions required for mitotic exit. In mammals, the Cdc5 homolog Plk1 directly phosphorylates APC, and studies in yeast also suggest that Cdc5 may directly activate the APC (Figure 1-4) (Charles et al., 1998; Kotani et al., 1998; Kotani et al., 1999). In addition, components of the late mitotic network have been proposed to play roles in licensing duplication of the spindle pole body and in regulating transcription of a variety of genes, including those involved in cell wall integrity (Liu et al., 1997; Komarnitsky et al., 1998; Luca and Winey, 1998).

Characterization of *S. pombe* homologs of the late mitotic genes has also supported the possibility that the mitotic exit network has addition functions in *S. cerevisiae* (Figure 1-5A). Despite conservation at the level of primary sequence, analysis of the late mitotic proteins in *S. pombe* has revealed no clear role for the fission yeast homologs in mitotic cyclin-Cdk regulation (Gould and Simanis, 1997; LeGoff et al., 1999). Instead, Cdc7, Spg1, Plo1, Sid2, and other proteins form a regulatory network that controls septum formation and cytokinesis (Figure 1-5B). Mutants in these genes do not form a division septum, but cell growth, DNA replication, and mitosis continue in the absence of cytokinesis. Based on extensive genetic analysis, the localization pattern of the proteins *in vivo*, and biochemical data *in vitro*, a speculative signaling pathway leading to the onset of septum formation has been proposed in *S. pombe* (Figure 1-5B) (Schmidt et al., 1997; Furge et al., 1998; Jwa and Song, 1998; Sohrmann et al., 1998; Cerutti and Simanis, 1999; Sparks et al., 1999).

Whether or not regulation of cytokinesis is a conserved function of the late mitotic network is currently under intense investigation. Because Cdc28 inactivation and

cytokinesis are linked in *S. cerevisiae* (Ghiara et al., 1991; Lew and Reed, 1995), it has been difficult to determine if the mitotic exit network directly controls cytokinesis, or is merely indirectly involved as a result of a role in the regulation of mitotic Cdk activity. However, a detailed characterization of the *cdc15-lyt1* mutant phenotype in budding yeast suggests that Cdc15 function is critical for formation of the active division septum required for cytokinesis (Jiménez et al., 1998). Further evidence for a role in cytokinesis comes from observations that Cdc15 and Tem1 function are required for correct localization of the actin cytoskeleton at the site of cleavage (Lippincott and Li, 1998; Shannon and Li, 1999). In these experiments, Tem1 was shown to physically interact with Cyk1, a protein required for formation of the actomyosin ring (Shannon and Li, 1999). Finally, despite the fact that Cdk inactivation occurs normally in *tem1Δ net1-1* mutants, these cells are still defective in cytokinesis (Shou et al., 1999). Taken together, these results support the possibility that the late mitotic network directly regulates cytokinesis in budding yeast.

Regulation of the mitotic exit network

As positive regulators of Cdc28 inactivation and mitotic exit, the activities of the late mitotic proteins should be cell cycle regulated. The kinase activity of Cdc5 rises during mitosis and declines as cells enter G1 as a result of both transcription and post-translational controls (Kitada et al., 1993; Charles et al., 1998; Cheng et al., 1998; Shirayama et al., 1998). Dbf2 kinase activity also increases during mitosis at the time of Cdk inactivation: a dephosphorylated form of Dbf2 appears coincidentally with the increase in its kinase activity (Johnston et al., 1990; Toyn and Johnston, 1994). In contrast, Cdc15 protein levels and kinase activity are not cell cycle regulated (Jaspersen et al., 1998). Analysis of Cdc15 regulation is the subject of Chapter 4 (Jaspersen and Morgan, 2000). I show that the mitotic exit functions of Cdc15 are negatively regulated by phosphorylation, which might control the subcellular localization of Cdc15 (R. Menssen & W. Seufert, personal communication). Mob1 localization during the cell cycle is also regulated by changes in its

phosphorylation state (F. Luca & M. Winey, personal communication). Characterization of the protein levels and activity of Tem1 have not been reported, but Lte1 has been recently been shown to localize to the bud throughout most of the cell cycle (A. Amon, personal communication). How these regulatory influences are integrated to provide the correct timing of Cdk inactivation is not understood.

Several lines of evidence suggest that the two component GTPase activating protein, Bub2-Byr4/Bfa1, negatively regulates the function of the mitotic exit network. In cells arrested in mitosis as the result of spindle damage, Bub2 and Byr4 are required to prevent Dbf2 activation, cyclin destruction, and Sic1 accumulation in the absence of Mad2 function (Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999). The effects of *bub2Δ* require Cdc14 function and can be mimicked by overexpression of *TEM1* (Alexandru et al., 1999). In addition, overexpression of Byr4 causes an arrest phenotype similar to that of the late mitotic mutants (Li, 1999). These results argue that Bub2-Byr4 acts as an upstream inhibitor of the late mitotic network (Figure 1-4).

Analysis of mutants in dynein heavy chain, as well as other nuclear migration mutants, suggest that yeast cells have a checkpoint to ensure that the anaphase spindle has successfully elongated and is correctly positioned between the mother and daughter cell before proceeding with cytokinesis (Yeh et al., 1995; Muhua et al., 1998). Based on genetic interactions between Bub2 and proteins that affect various aspects of spindle and microtubule function and the localization of Bub2 and Byr4 to the spindle pole body, it is tempting to speculate that Bub2-Byr4 might monitor some aspect of mitotic spindle function, such as spindle positioning or elongation (Fraschini et al., 1999; Li, 1999) (D. Thompson & A. Murray, personal communication). Inhibition of the mitotic exit network by a Bub2 checkpoint might ensure that cells delay Cdk inactivation and spindle disassembly until anaphase is completed.

Figure 1-1. Progression through the *S. cerevisiae* cell cycle is regulated by Cdc28 and proteolysis.

Oscillations in the activity Cdc28 drive progression through the budding yeast cell cycle (inner circle). During G1, Cdc28 associates with the G1 cyclins (Cln1-3) to control bud emergence, spindle pole body duplication, and activation of S phase cyclins. Initiation of DNA replication at S phase requires Cdc28 activity associated with S phase cyclins (Clb5 and Clb6). Activity of Cdc28 in association with the mitotic cyclins (Clb1-4) prevents the re-replication of DNA, promotes formation of the mitotic spindle, and regulates progression through mitosis. Inactivation of Cdc28 is required for cells to complete mitosis, initiate cytokinesis, and enter the next cell cycle.

Proteolysis of cell cycle regulators is required for at least three transitions in the yeast cell cycle (gray boxes). Activation of Cdc28-Clb5/6 kinases at the onset of S phase occurs following destruction of the Cdk inhibitor Sic1 by the SCF. Sister chromatid separation at the metaphase-to-anaphase transition is controlled by the destruction of the anaphase inhibitor Pds1 by the Cdc20-dependent APC. APC-Cdc20 also catalyzes the degradation of Clb5, which inhibits activation of the Hct1-dependent APC and accumulation of Sic1. Following anaphase, APC-Hct1 ubiquitinates the Clbs, as well as other mitotic regulatory proteins, to promote the transition from mitosis to G1.

Figure 1-1

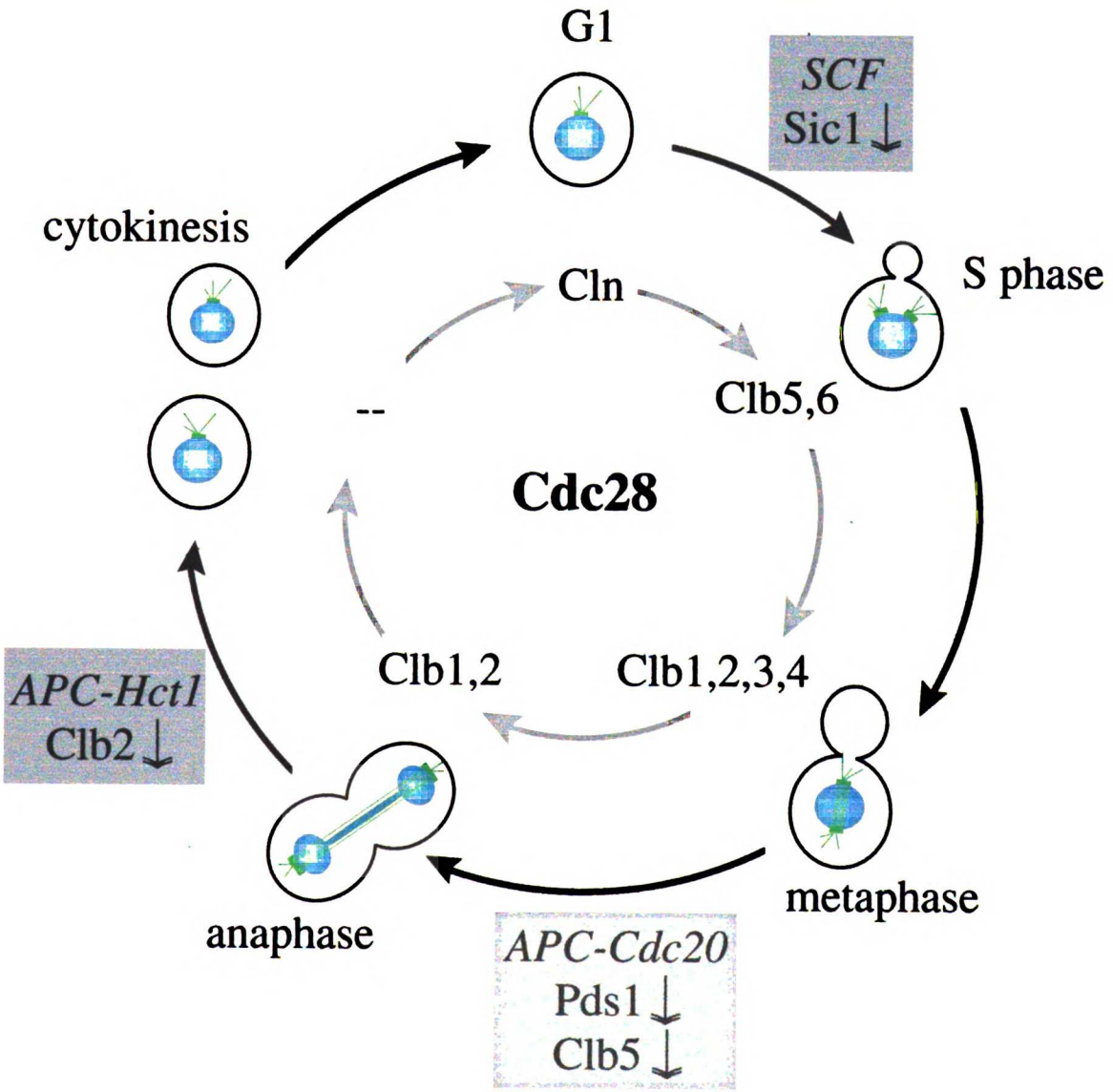
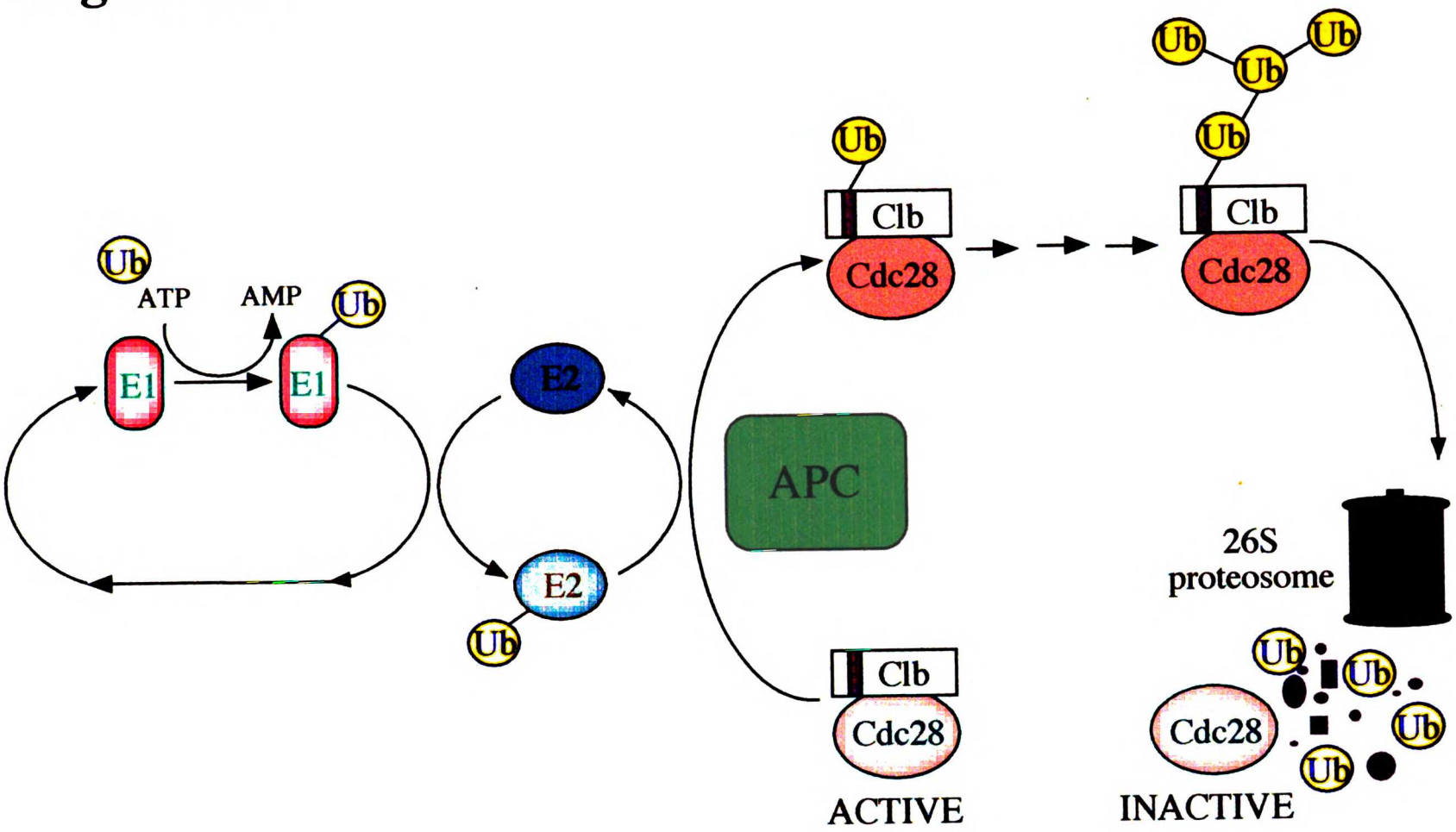


Figure 1-2. Degradation of mitotic cyclins by the ubiquitin-proteasome pathway.

A destruction box sequence (purple bar) in the amino-terminal of cyclin (Clb) targets it for degradation by the ubiquitin-proteasome pathway. Ubiquitin (Ub, yellow circles) is activated by the ubiquitin activating enzyme (E1) through the hydrolysis of ATP. It then forms an isopeptide bond with the ubiquitin conjugating enzyme (E2). Transfer of ubiquitin from the E2 to the cyclin is catalyzed by the ubiquitin ligase activity of the APC. The process is reiterated, resulting in a multi-ubiquitinated cyclin that is recognized and proteolyzed by the 26S proteasome. Destruction of cyclin inactivates the mitotic Cdk.

Figure 1-2


Ub
ATP
AMP
Ub
E1
E1
E2
E2
Ub
APC
Clb
Cdc28
ACTIVE
Ub
Ub
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proteosome
Ub
Ub
Ub
Ub
Cdc28
INACTIVE

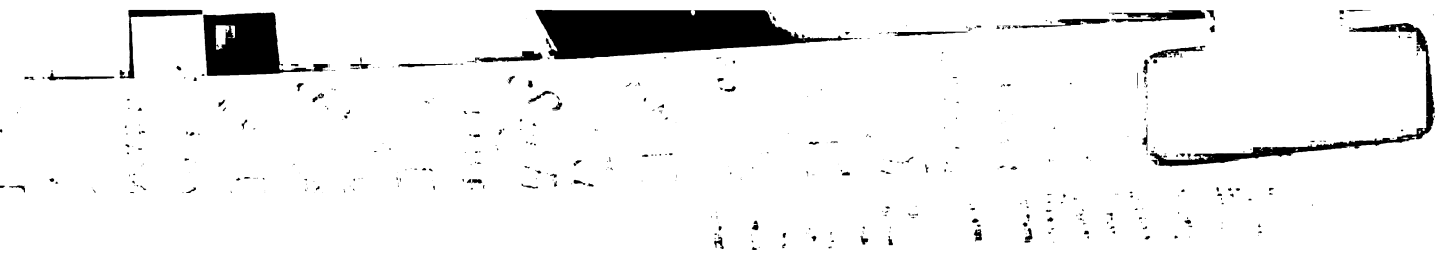


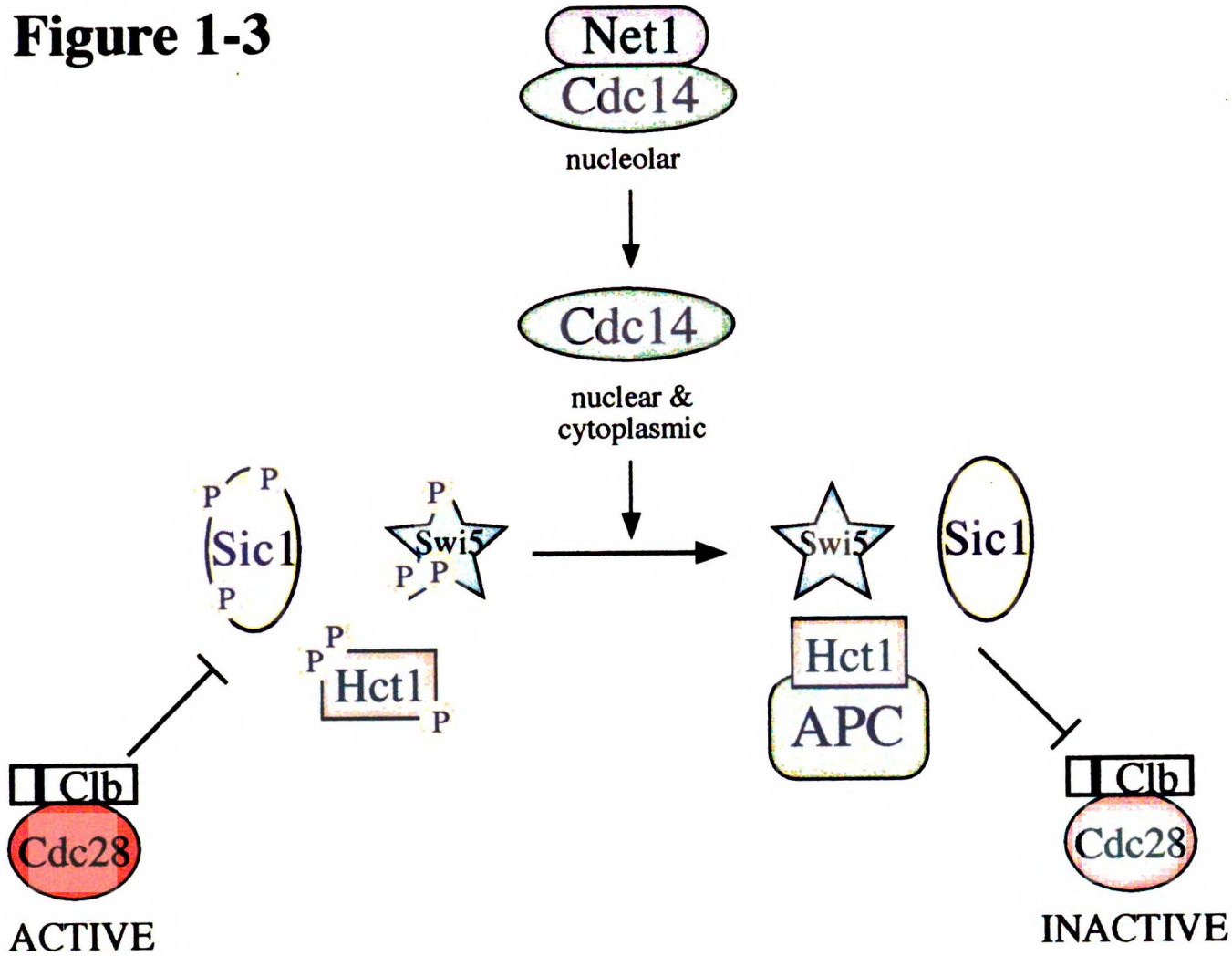
Table 1-1. Subunits of the APC

	S. cerevisiae	*S. pombe*	Mammals	Motifs	Modifications
Core subunits					
Apc1	Apc1	Cut4	Apc1/Tsg24	Rpn1/2 repeats	phosphorylated
Apc2	Apc2/Rsi2		Apc2	Cullin domain	
Apc3	Cdc27	Nuc2	Cdc27	TPR repeats	phosphorylated
Apc4	Apc4	Lid1	Apc4		
Apc5	Apc5		Apc5		
Apc6	Cdc16	Cut9	Cdc16	TPR repeats	phosphorylated
Apc7	not present		Apc7	TPR repeats	
Apc8	Cdc23	Cut23	Cdc23	TPR repeats	phosphorylated
Apc9	Apc9				
Apc10	Apc10/Doc1	Apc10	Apc10	Doc domain	
Apc11	Apc11		Apc11	RING-H2 domain	
Apc12	Cdc26/Apc12	Hcn1	Cdc26		
Apc13	p19				
Activating subunits					
Cdc20	Cdc20	Slp1	Cdc20	WD repeats	phosphorylated
Cdh1	Hct1/Cdh1	Srw1/Ste9	Cdh1	WD repeats	phosphorylated

Figure 1-3. Cdc14 dephosphorylates Cdc28 substrates to promote mitotic exit.

Phosphorylation of Hct1, Sic1, and Swi5 by Cdc28 allows accumulation of mitotic cyclins and Cdc28-Clb kinase activity from S phase until mitosis. Following anaphase, Cdc14 is released into the nucleus and cytoplasm where it dephosphorylates Hct1 to promote APC activation and Clb destruction. Cdc14 also removes Cdk-dependent phosphates from Sic1 and Swi5, leading to Sic1 accumulation and inhibition of Cdc28-Clb kinase activity. By alleviating the mutual antagonism between Cdc28 and its inhibitors, Cdc14 triggers a decrease in Cdc28 kinase activity that allows cells to exit from mitosis.

Figure 1-3



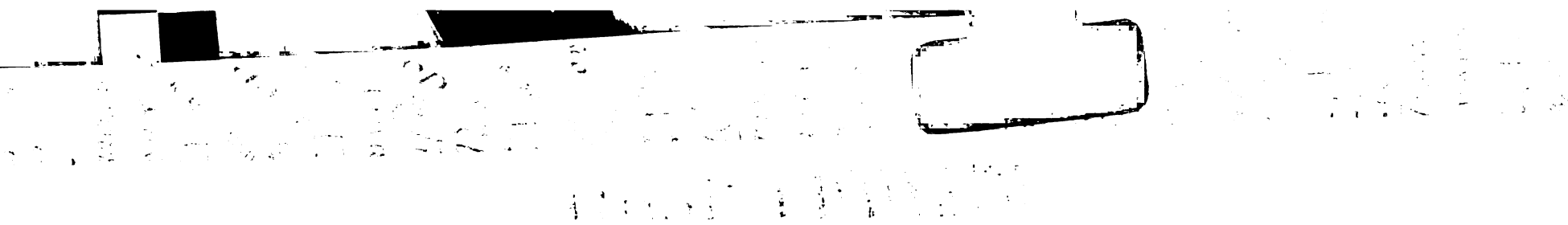


Figure 1-4. A speculative model of the regulatory network controlling exit from mitosis.

Phosphorylation of the APC and Cdc20 by Cdc28-Clb and/or Cdc5 leads to APC-Cdc20 activation, through a direct or indirect mechanism. APC-Cdc20 mediates sister chromatid separation by degrading the anaphase inhibitor Pds1; it also targets Clb5 (as well as a population of Clb2) for destruction, partially relieving inhibition of Hct1 and Sic1. Complete activation of Hct1 and Sic1 requires dephosphorylation of Cdk sites by Cdc14, whose release from the nucleolus following anaphase is signaled by the mitotic exit network. Inactivation of Cdc28 is required for disassembly of the mitotic spindle and for initiation of cytokinesis, which may also be regulated by the late mitotic proteins. By analogy with *S. pombe*, signaling by the late mitotic network may involve regulation of Cdc15 binding to Tem1 by Lte1 and Bub2-Byr4.

Figure 1-4

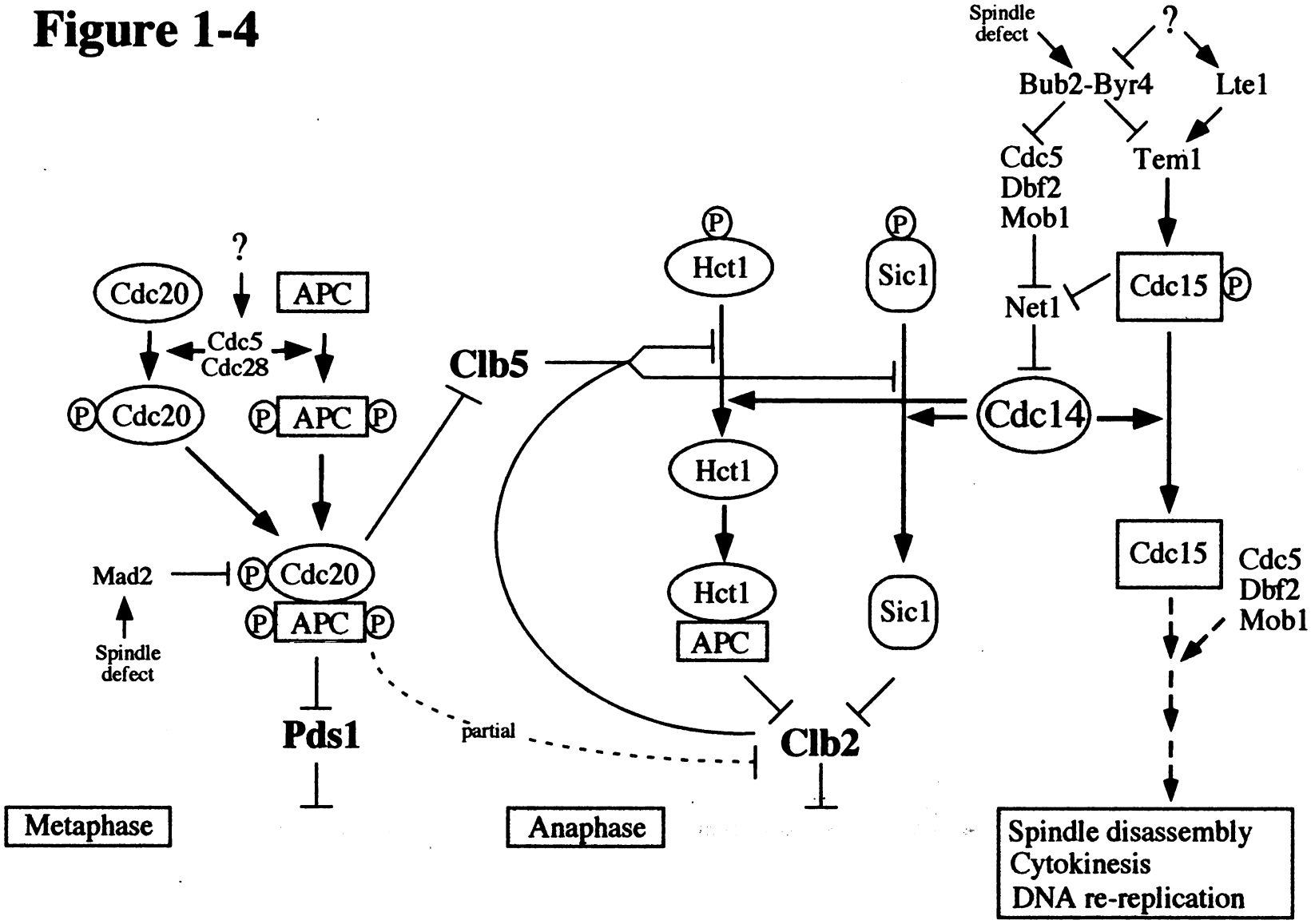

Spindle defect
?
Bub2-Byr4
Lte1
Cdc5
Dbf2
Mob1
Tem1
Hct1
Sic1
Net1
Cdc15
P
Cdc20
APC
Cdc5
Cdc28
Clb5
Cdc14
Mad2
Spindle defect
Pds1
partial
Clb2
Metaphase
Anaphase
Spindle disassembly
Cytokinesis
DNA re-replication

Figure 1-5. Regulation of cytokinesis in *S. pombe.*

(A) The mitotic exit network is conserved between *S. cerevisiae* and *S. pombe*. Names of the late mitotic proteins in budding and fission yeast and their predicted biochemical function are shown. Homologs of Cdc14 and Lte1 have not been identified in *S. pombe*. **(B)** In fission yeast, homologs of the late mitotic family regulate the onset of septum formation. The details of signal transduction are not well understood, but part of the pathway likely involves changes in the localization of Cdc7 and the nucleotide state of Spg1. As cells enter mitosis, Spg1-GTP and Cdc7 are found on both spindle pole bodies. Following anaphase, the two component GAP, Cdc16-Byr4, catalyzes GTP hydrolysis by Spg1 at one of the spindle pole bodies, decreasing Cdc7 binding to Spg1 at that pole. Asymmetric localization of Cdc7 is thought to send a signal from the spindle pole body to the medial ring to initiate synthesis of the division septum required to complete cytokinesis. This signal may involve activation of Sid2, which localizes to both the spindle pole bodies and to the division site.

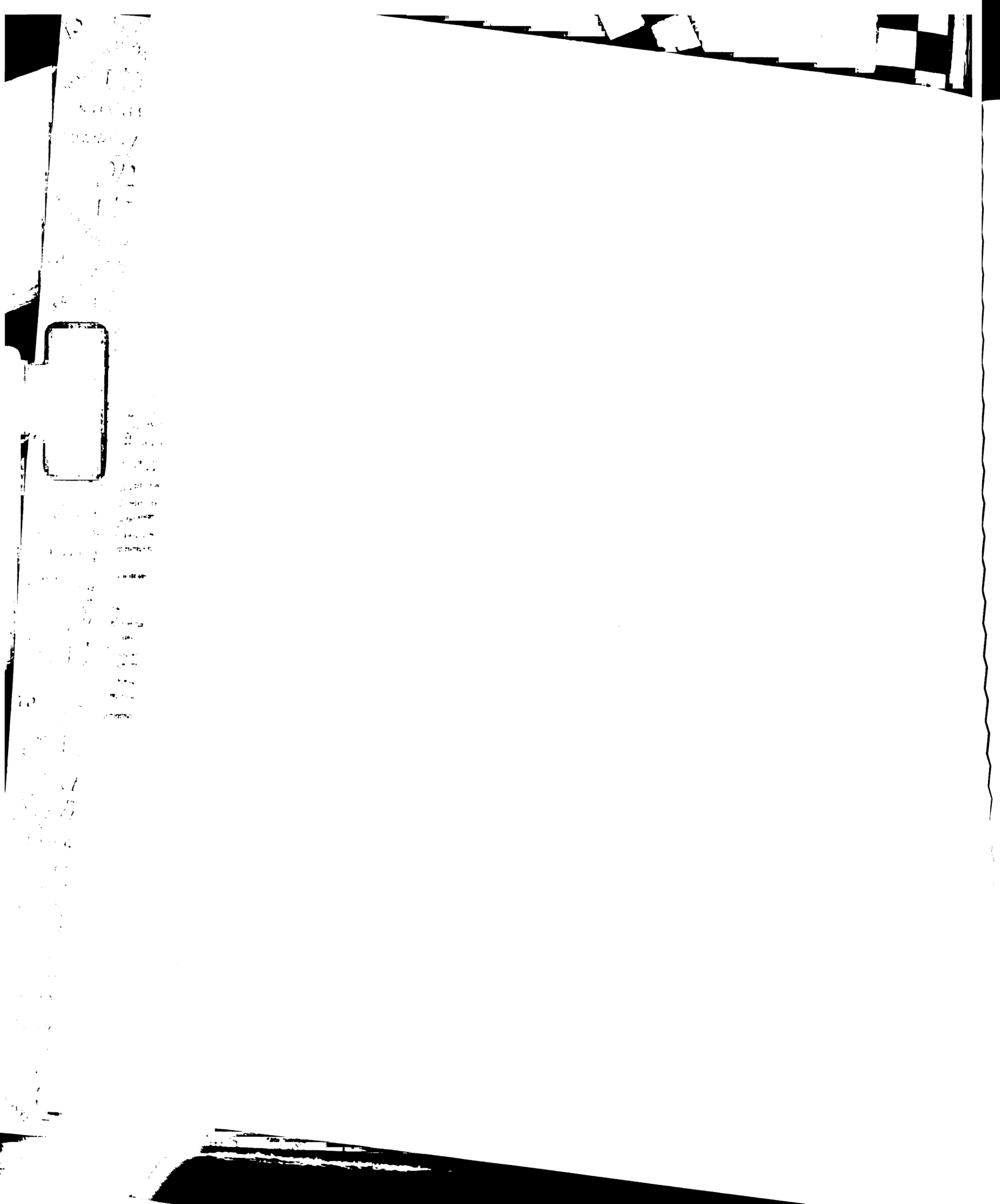
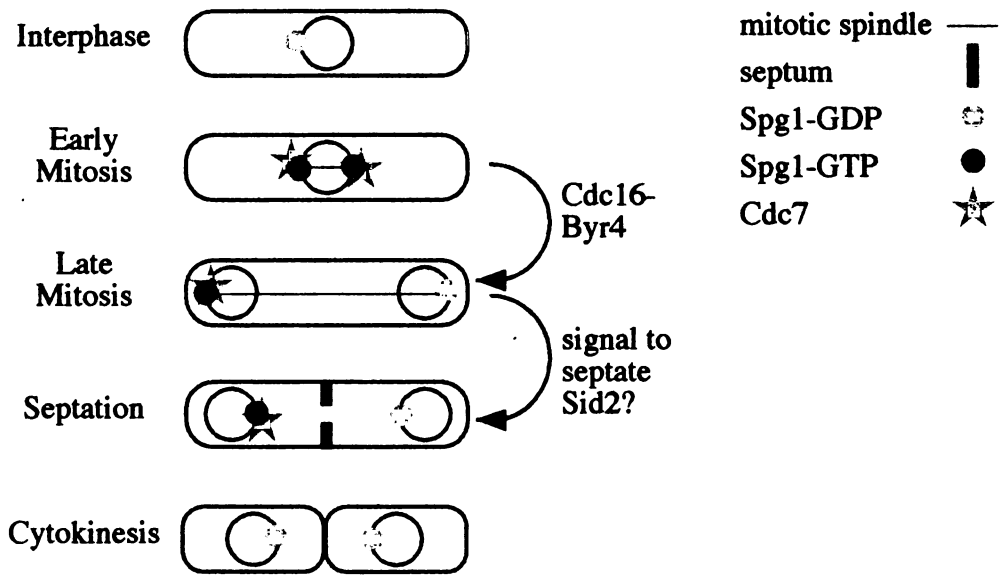


Figure 1-5

A

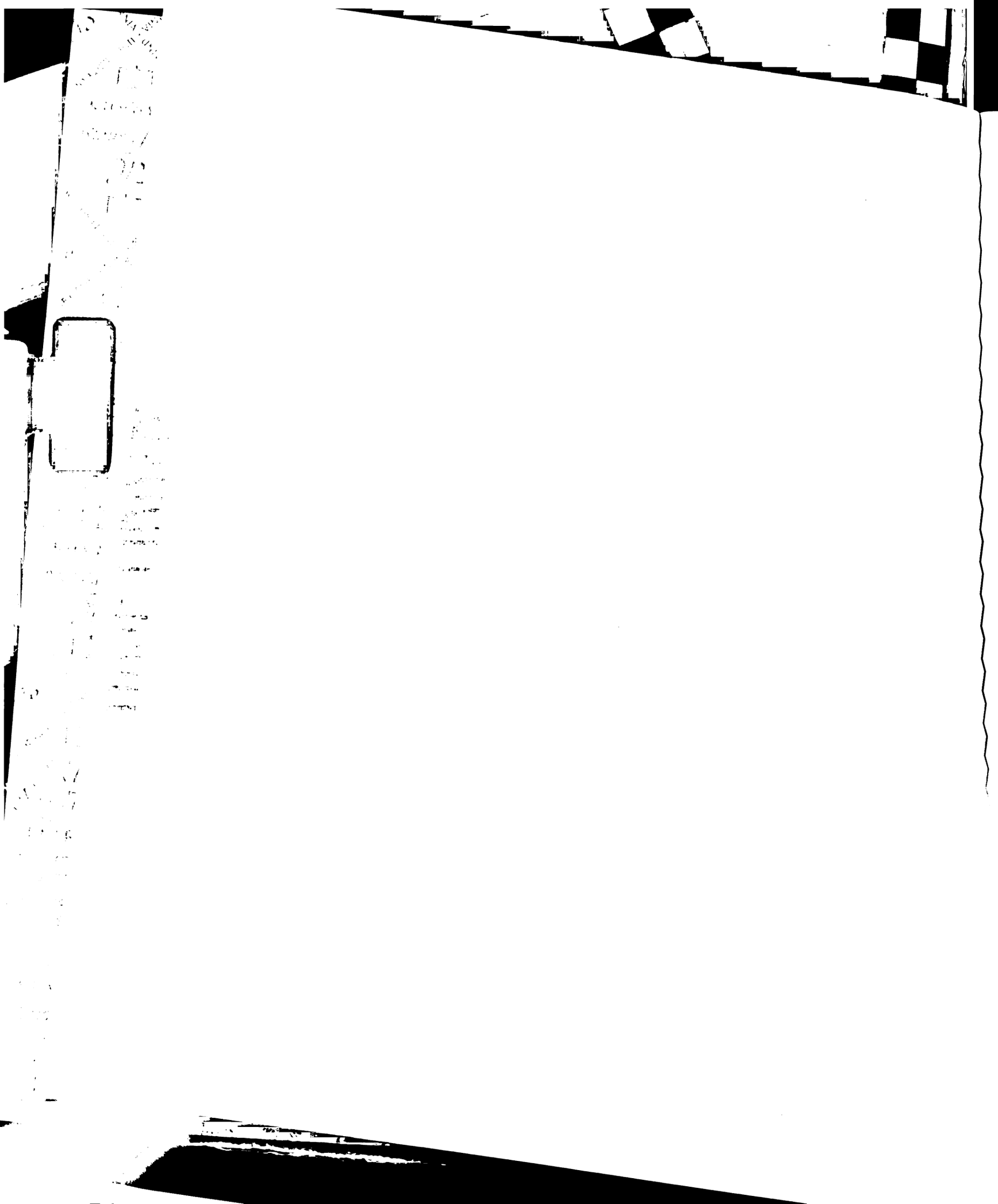
S. cerevisiae	*S. pombe*	Biochemical function
Cdc15	Cdc7	protein kinase
Cdc5	Plo1	protein kinase
Cdc14	---	protein phosphatase
Dbf2	Sid2	protein kinase
Tem1	Spg1	GTPase
Lte1	---	GNEF
Bub2	Cdc16	GAP with Byr4
Byr4/Bfa1	Byr4	GAP with Bub2
Mob1	Mob1	unknown

B



Chapter Two

A late mitotic regulatory network controlling cyclin destruction in *Saccharomyces cerevisiae*



A late mitotic regulatory network controlling cyclin destruction in *Saccharomyces cerevisiae*

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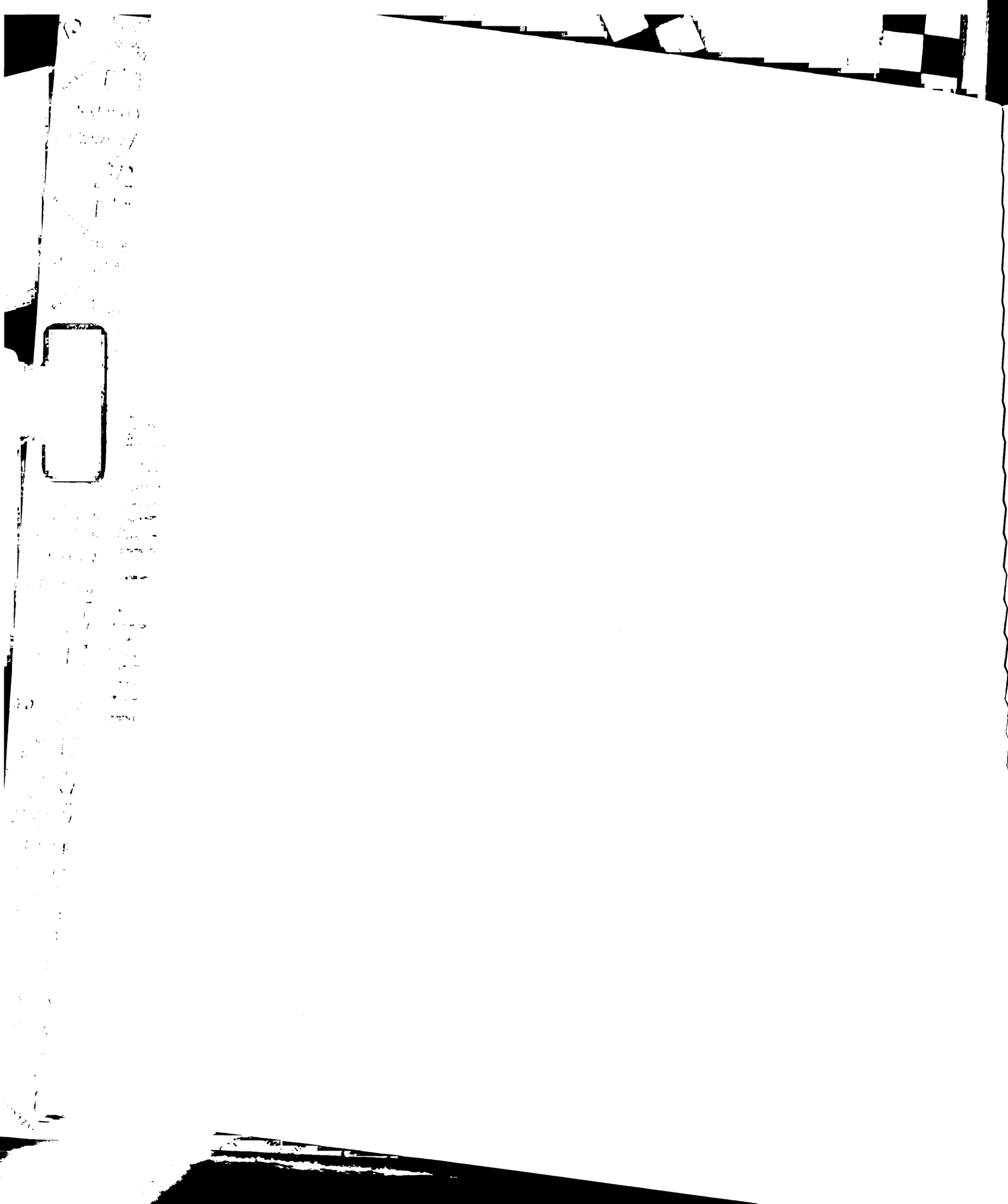


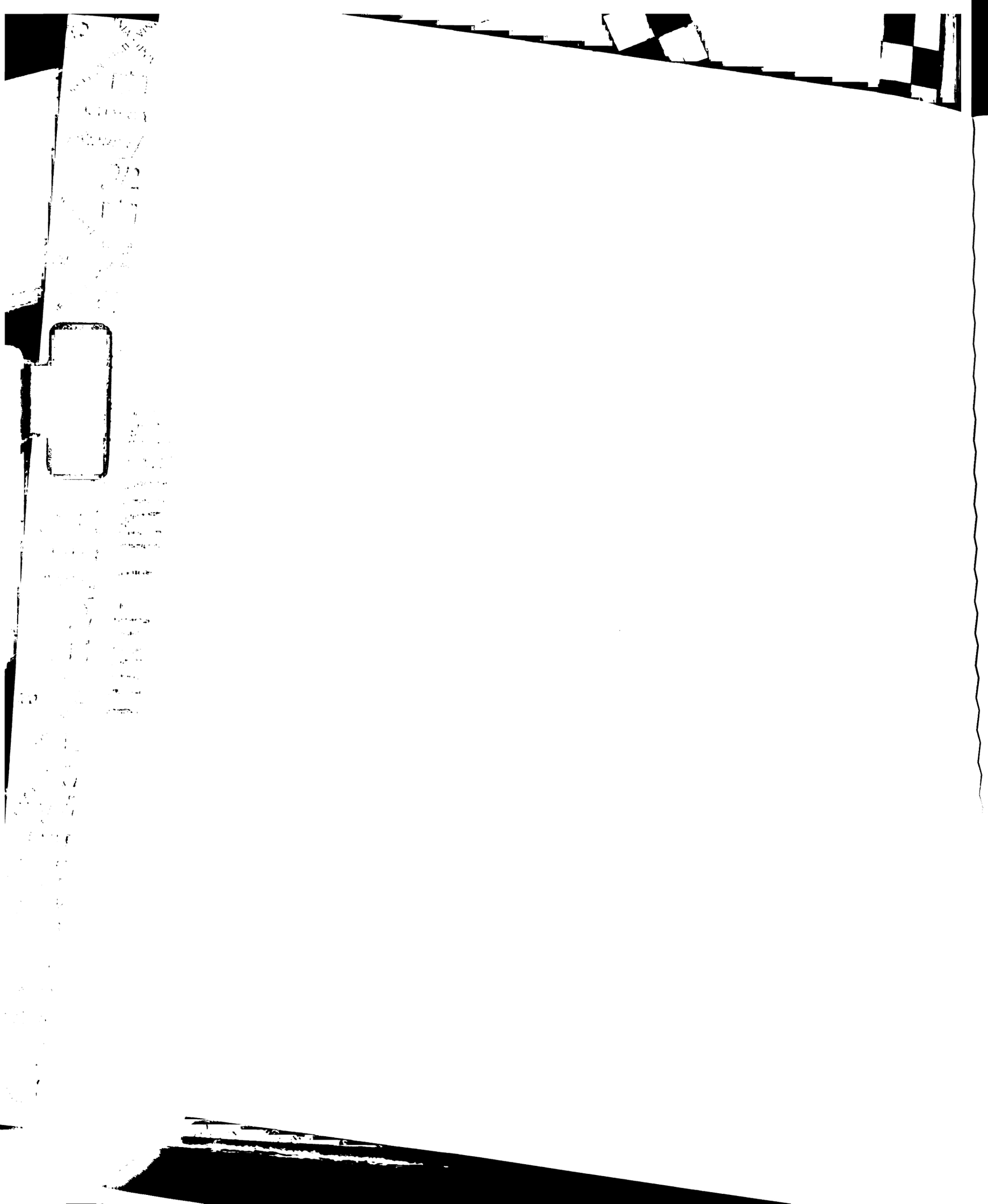
Abstract

Exit from mitosis requires the inactivation of mitotic cyclin-dependent kinase-cyclin complexes, primarily by ubiquitin-dependent cyclin proteolysis. Cyclin destruction is regulated by a ubiquitin ligase known as the anaphase-promoting complex (APC). In the budding yeast *Saccharomyces cerevisiae*, members of a large class of late mitotic mutants, including *cdc15*, *cdc5*, *cdc14*, *dbf2*, and *tem1*, arrest in anaphase with a phenotype similar to that of cells expressing non-degradable forms of mitotic cyclins. We addressed the possibility that the products of these genes are components of a regulatory network that governs cyclin proteolysis. We identified a complex array of genetic interactions among these mutants, and found that the growth defect in most of the mutants is suppressed by overexpression of *SPO12*, *YAK1*, and *SIC1*, and is exacerbated by overproduction of the mitotic cyclin Clb2. When arrested in late mitosis, the mutants exhibit a defect in cyclin-specific APC activity that is accompanied by high Clb2 levels and low levels of the anaphase inhibitor Pds1. Mutant cells arrested in G1 contain normal APC activity. We conclude that Cdc15, Cdc5, Cdc14, Dbf2, and Tem1 cooperate in the activation of the APC in late mitosis, but are not required for maintenance of that activity in G1.

Introduction

Progression through the eukaryotic cell division cycle is governed by oscillations in the activities of cyclin-dependent kinases (CDKs). Entry into mitosis is initiated by mitotic CDK-cyclin complexes, including the Cdc2-cyclin B complex in vertebrates and the Cdc28-Clb complex of *Saccharomyces cerevisiae* (King et al., 1994; Nasmyth, 1996; Morgan, 1997). Exit from mitosis requires CDK inactivation, which is accomplished primarily by ubiquitin-dependent destruction of the cyclin subunit (Murray, 1995; King et al., 1996; Hoyt, 1997). The importance of cyclin destruction for exit from mitosis is underscored by the observation in a wide range of eukaryotes that overexpression of non-destructible forms of mitotic cyclin causes cells to arrest in anaphase (Murray et al., 1989; Gallant and Nigg, 1992; Holloway et al., 1993; Surana et al., 1993; Rimmington et al., 1994; Sigrist et al., 1995; Yamano et al., 1996). Under some conditions, however, additional CDK inactivation mechanisms allow mitotic exit in the absence of complete cyclin destruction (Minshull et al., 1996; Toyn et al., 1996; Schwab et al., 1997; Visintin et al., 1997; Jin et al., 1998).

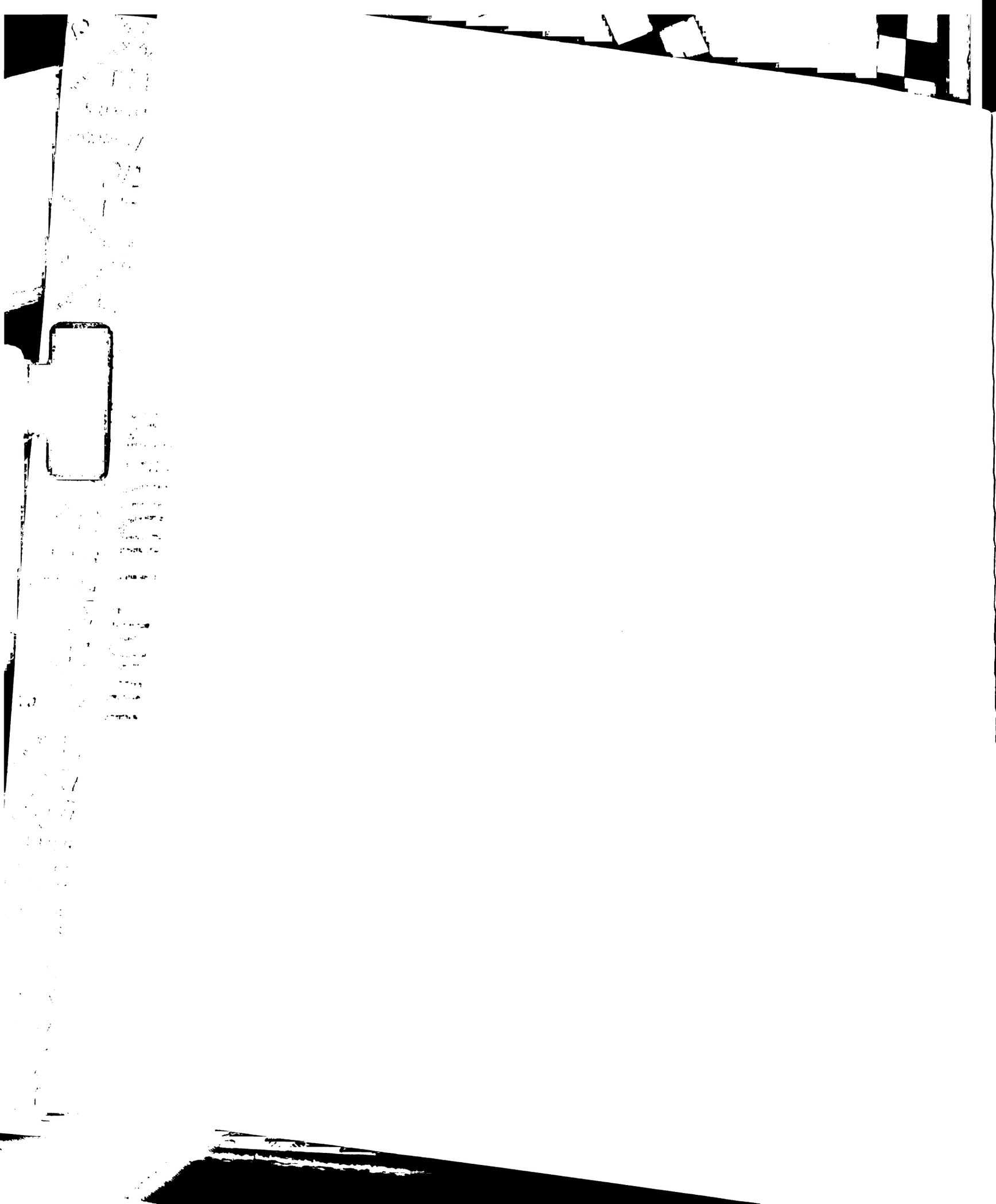
Mitotic cyclin destruction requires the covalent attachment of a chain of ubiquitin molecules to a region near the amino-terminus of the cyclin protein (Glotzer et al., 1991). Ubiquitination of cyclin, like that of other proteins, begins with the transfer of ubiquitin from the ubiquitin activating enzyme (E1) to a ubiquitin conjugating enzyme (E2) (Hershko et al., 1994; King et al., 1995; Hochstrasser, 1996). The E2, together with a ubiquitin ligase (E3), transfers the ubiquitin onto the cyclin substrate. The E3 required for cyclin ubiquitination is a multi-subunit protein complex known as the anaphase promoting complex (APC) or cyclosome (Irniger et al., 1995; King et al., 1995; Sudakin et al., 1995; Peters et al., 1996; Zachariae et al., 1996; Hwang and Murray, 1997; Kramer et al., 1998b; Yu et al., 1998; Zachariae et al., 1998b). Several lines of evidence suggest that the APC



mediates the key regulatory step in cyclin destruction (Hershko et al., 1994; King et al., 1995; Sudakin et al., 1995).

In addition to being required for the ubiquitination of mitotic cyclins, the APC also catalyzes the ubiquitination of other mitotic regulatory proteins. APC-dependent degradation of the Pds1 protein of *S. cerevisiae* (or Cut2 of *S. pombe*) is required for progression from metaphase to anaphase (Cohen-Fix et al., 1996; Funabiki et al., 1996); thus, mutation or inhibition of the APC causes a metaphase arrest and not the anaphase arrest that results from overexpression of non-degradable cyclin (Holloway et al., 1993; Irniger et al., 1995; Cohen-Fix et al., 1996; Zachariae et al., 1998b). Other APC substrates have also been identified in *S. cerevisiae*, including the microtubule-associated protein Ase1, whose destruction is necessary for efficient disassembly of the mitotic spindle (Juang et al., 1997). The APC is also required for the destruction of the WD40 repeat protein Cdc20 and the Polo-related protein kinase Cdc5 (Charles et al., 1998; Prinz et al., 1998; Shirayama et al., 1998).

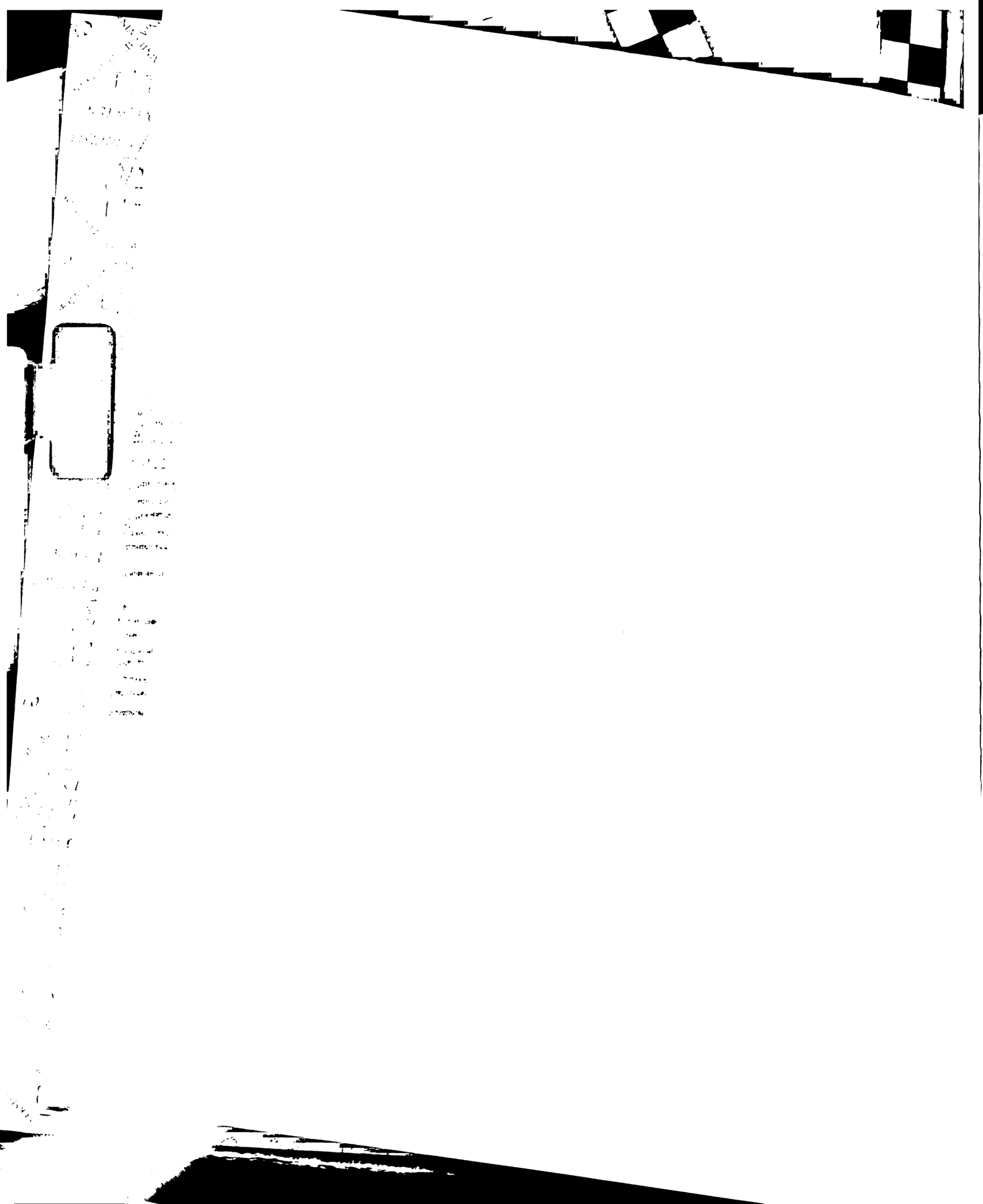
Studies of APC regulation have focused almost exclusively on its cyclin-ubiquitin ligase activity, which increases in metaphase or anaphase and remains high throughout G1 (Amon et al., 1994; King et al., 1995; Lahav-Baratz et al., 1995; Sudakin et al., 1995; Brandeis and Hunt, 1996; Zachariae and Nasmyth, 1996; Charles et al., 1998). In higher eukaryotes, activation of the APC towards cyclin substrates is initiated by Cdc2-cyclin B (Felix et al., 1990; Lahav-Baratz et al., 1995; Sudakin et al., 1995), whereas in budding yeast there is evidence that Cdc28-associated kinase activity inhibits cyclin ubiquitination by the APC (Amon, 1997). Recent studies have also implicated other protein kinases in APC regulation: Polo-related kinases (Plk1 in mammals, Plx1 in Xenopus, and Cdc5 in budding yeast) promote APC activation, while in mammals and fission yeast Protein Kinase A (PKA) appears to inhibit cyclin-directed APC activity (Yamashita et al., 1996; Charles et al., 1998; Descombes and Nigg, 1998; Kotani et al., 1998; Shirayama et al.,



1998). Little is known about how these various regulatory influences are integrated to provide the correct timing of cyclin destruction.

To ensure the proper order of mitotic events, the APC may also be regulated at the level of substrate specificity. APC-dependent ubiquitination of proteins involved in sister chromatid cohesion (Pds1) occurs at the metaphase to anaphase transition, while mitotic cyclins (e.g. Clb2), Cdc20, and Ase1 remain stable until the end of anaphase (Pellman et al., 1995; Cohen-Fix et al., 1996; Zachariae et al., 1996; Shirayama et al., 1998). Recent work suggests that this additional level of regulation may be conferred in *S. cerevisiae* by Cdc20 and Hct1/Cdh1 (Schwab et al., 1997; Visintin et al., 1997; Lim et al., 1998; Shirayama et al., 1998). Overexpression of *CDC20* results in APC-dependent destabilization of Pds1 but has little effect on the destruction of Ase1 and Clb2; *cdc20* mutants arrest in metaphase with stable Pds1 (Sethi et al., 1991; Visintin et al., 1997; Shirayama et al., 1998). Similar evidence suggests that *HCT1* promotes the destruction of Clb2 and Ase1 but not that of Pds1 (Schwab et al., 1997; Visintin et al., 1997). The regulation of these putative specificity factors is not well understood, although recent studies suggest that Cdc20 may be regulated by multiple mechanisms: its levels increase during mitosis, and its function may be negatively regulated in response to spindle damage (Hwang et al., 1998; Kim et al., 1998; Prinz et al., 1998; Shirayama et al., 1998).

In *S. cerevisiae*, various genetic screens have led to the identification of a group of mutants that arrest in late anaphase with large buds, an elongated spindle, and separated DNA (Hartwell et al., 1973; Johnston and Thomas, 1982; Johnston et al., 1990; Molero et al., 1993; Shirayama et al., 1994a; Shirayama et al., 1994b; Luca and Winey, 1998). This arrest phenotype is similar to that observed in yeast overexpressing a non-degradable form of Clb2, raising the possibility that the late mitotic gene products are required for the inactivation of Cdc28-Clb complexes (Surana et al., 1993). Interestingly, the late mitotic mutants all encode potential regulatory proteins, including the protein kinases Cdc15 and Dbf2, the Polo-like kinase Cdc5, the protein phosphatase Cdc14, and the Ras-like GTPase



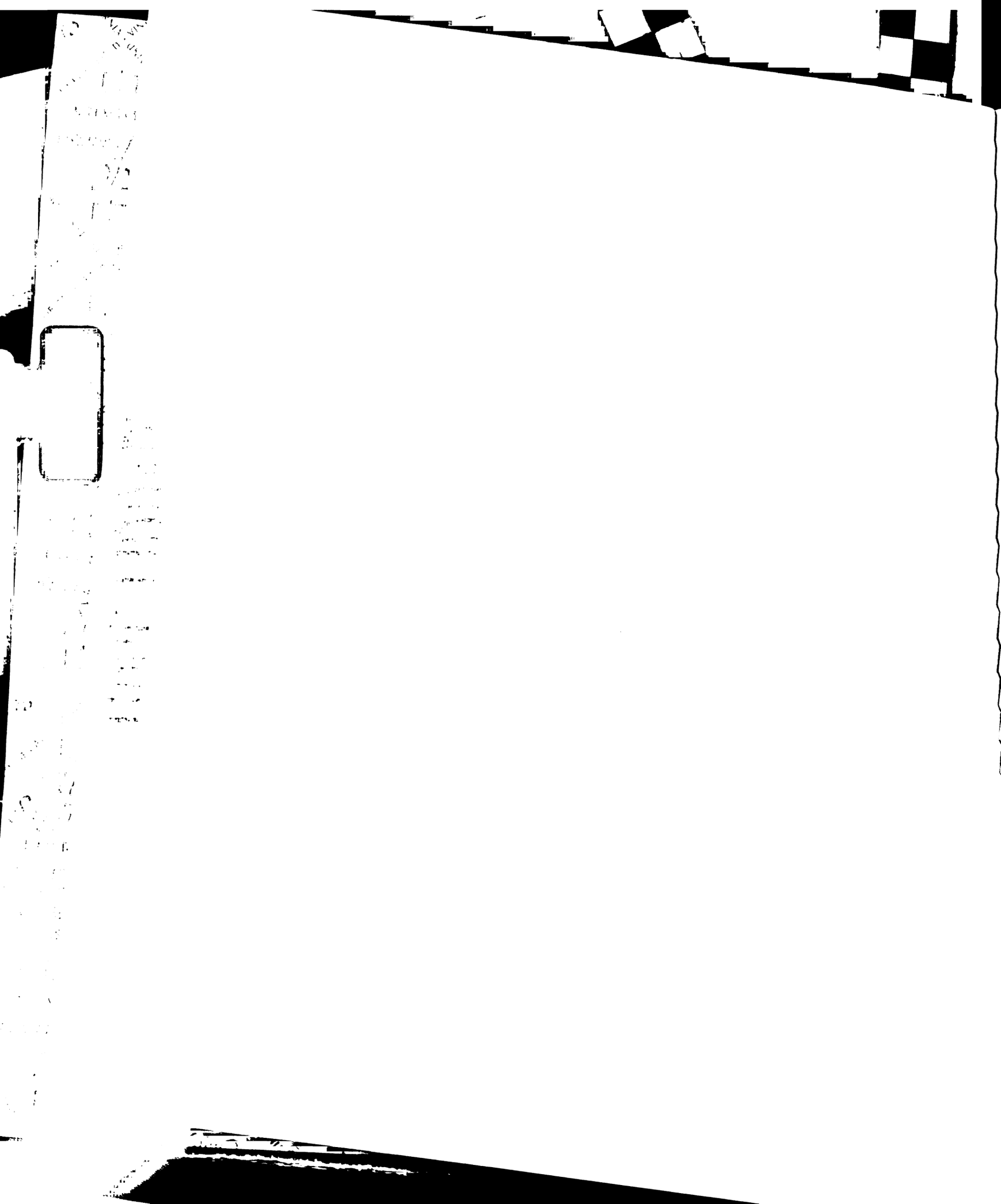
Tem1 (Johnston et al., 1990; Schweitzer and Philippsen, 1991; Wan et al., 1992; Kitada et al., 1993; Shirayama et al., 1994b). Recent studies suggest that Cdc5 promotes mitotic exit by stimulating APC activity toward cyclins (Charles et al., 1998; Shirayama et al., 1998), and it seems likely that the other late mitotic proteins also contribute to the control of cyclin destruction.

In the present work, we address the hypothesis that the proteins encoded by the late mitotic gene family form a regulatory network governing Cdc28 inactivation in late mitosis. In support of this hypothesis, we find that several late mitotic mutants display an extensive array of genetic interactions. These mutants arrest with elevated levels of Clb2, decreased amounts of Pds1, and negligible cyclin-specific APC activity. We therefore conclude that the proteins encoded by the late mitotic genes promote mitotic exit by activating the cyclin-ubiquitin ligase activity of the APC.

Results

Genetic interactions among late mitotic mutants

The similar anaphase arrest phenotype of *cdc15-2*, *cdc5-1*, *cdc14-1*, *dbf2-2*, and *tem1-3* mutants suggests that the proteins encoded by these genes may have overlapping functions in the control of mitotic exit. Consistent with this possibility, a variety of previous studies have revealed that overexpression of some late mitotic genes results in growth of other late mitotic mutants at the non-permissive temperature (Kitada et al., 1993; Shirayama et al., 1994b; Shirayama et al., 1996). We extended these studies by carrying out a systematic high copy suppression analysis of the major late mitotic mutants in a common strain background. Multicopy plasmids carrying *CDC15*, *CDC5*, *CDC14*, *DBF2*, and *TEM1* were each sufficient to rescue the temperature-sensitive growth defects of many of the late mitotic mutants (Table 2-2). The *tem1-3* mutant was suppressed by all of the late mitotic genes except *DBF2*, while *cdc14-1* and *dbf2-2* grew only when their



wild type genes were supplied. Interestingly, *CDC14* was unique in its ability to restore growth to the majority of mutants at 37°C.

Further evidence that the late mitotic mutants are functionally linked is that many double mutants are inviable (Table 2-3). In addition, most of the viable double mutants exhibited growth defects and reduced viability at semi-permissive temperatures (Table 2-3). In particular, the *cdc5-1* and *tem1-3* mutants exhibited synthetic interactions with all other late mitotic family members examined. In contrast, the *cdc14-1* mutant had no obvious synthetic interaction with *cdc15-2* and *dbf2-2* mutants and only minor interactions with *cdc5-1* and *tem1-3*. These genetic interactions suggest that the proteins encoded by the late mitotic mutants work together to coordinate exit from mitosis.

High copy suppressors of cdc15-2

To identify additional genes involved in control of exit from mitosis, we performed a screen for *GAL*-driven cDNAs that allowed growth of a *cdc15-2* strain at 37°C (Figure 2-1). Other than *GAL-CDC15*, the most robust suppressor of *cdc15-2* was *GAL-SPO12*, which also suppressed the growth arrest of a complete deletion of *CDC15* (our unpublished data) and has previously been shown to suppress the growth defect in *dbf2* and dominant *CDC15* mutants (Parkes and Johnston, 1992; Shirayama et al., 1996). The *SPO12* locus encodes a protein of unknown function; mutation or deletion of this gene causes diploid cells to skip a meiotic division and produce dyad spores (Klapholz and Esposito, 1980; Malavasic and Elder, 1990). Disruption of *SPO12* has minor effects on progression through mitosis (Malavasic and Elder, 1990; Parkes and Johnston, 1992). We also found that growth of *cdc15-2* was restored at 37°C upon overexpression of a putative open reading frame, YGR230W, that encodes a protein with homology to Spo12. The function of this protein is unknown.

A fourth suppressor contained a 3' fragment of the *YAK1* gene. *YAK1* encodes a non-essential protein with homology to protein kinases (Garrett and Broach, 1989), and

our suppressor encoded an amino-terminally truncated version of Yak1 that is initiated at methionine 233, several residues before the beginning of the kinase domain. Mutants in *YAK1* were originally identified as extragenic suppressors of *ras2* mutants (Garrett and Broach, 1989). *RAS2* encodes a GTPase involved in activating adenylate cyclase, the enzyme responsible for cAMP production in yeast (Toda et al., 1985). Subsequent genetic studies suggested that Yak1 antagonizes the effects of the cAMP-dependent kinase PKA (Garrett et al., 1991; Hartley et al., 1994; Ward and Garrett, 1994). Its ability to suppress *cdc15-2* is therefore consistent with previous studies showing that the anaphase arrest in *cdc15* mutants is accompanied by high levels of cAMP, and decreasing cAMP levels alleviates the *cdc15-2* defect at 37°C (Spevak et al., 1993).

Finally, growth of *cdc15-2* cells was partially restored at 37°C by *GAL*-driven overexpression of *SIC1* (Figure 2-1), which encodes an inhibitor of Cdc28-Clb kinases and has previously been reported to suppress *cdc15* mutants when overexpressed (Mendenhall, 1993; Schwob et al., 1994; Toyn et al., 1996). Growth of *cdc15-2* cells was rescued even more effectively by *SIC1* on a 2μ plasmid (our unpublished data). The ability of *SIC1* to suppress the growth defect in the *cdc15* mutant is of particular interest because it suggests that the primary defect in this mutant is an inability to inactivate Cdc28.

Overexpression of SIC1, SPO12, and truncated YAK1 allows growth of late mitotic mutants

If Cdc15 cooperates with the other late mitotic proteins to regulate exit from mitosis, then high copy suppressors of *cdc15-2* should also allow growth of the other mutants at 37°C. Indeed, overexpression of *SIC1* partially restored growth to all of the late mitotic mutants at 37°C (Table 2-4) (Donovan et al., 1994; Toyn et al., 1996; Charles et al., 1998). *SPO12* overexpression resulted in robust growth of *cdc15-2*, *cdc5-1*, *dbf2-2*, and *tem1-3* at 37°C but did not restore growth to *cdc14-1* cells (Table 2-4) (Parkes and Johnston, 1992; Toyn and Johnston, 1993; Shirayama et al., 1996). Similarly,

overproduction of truncated Yak1 partially rescued the temperature-sensitive growth defect of all late mitotic mutants except *cdc14-1* (Table 2-4).

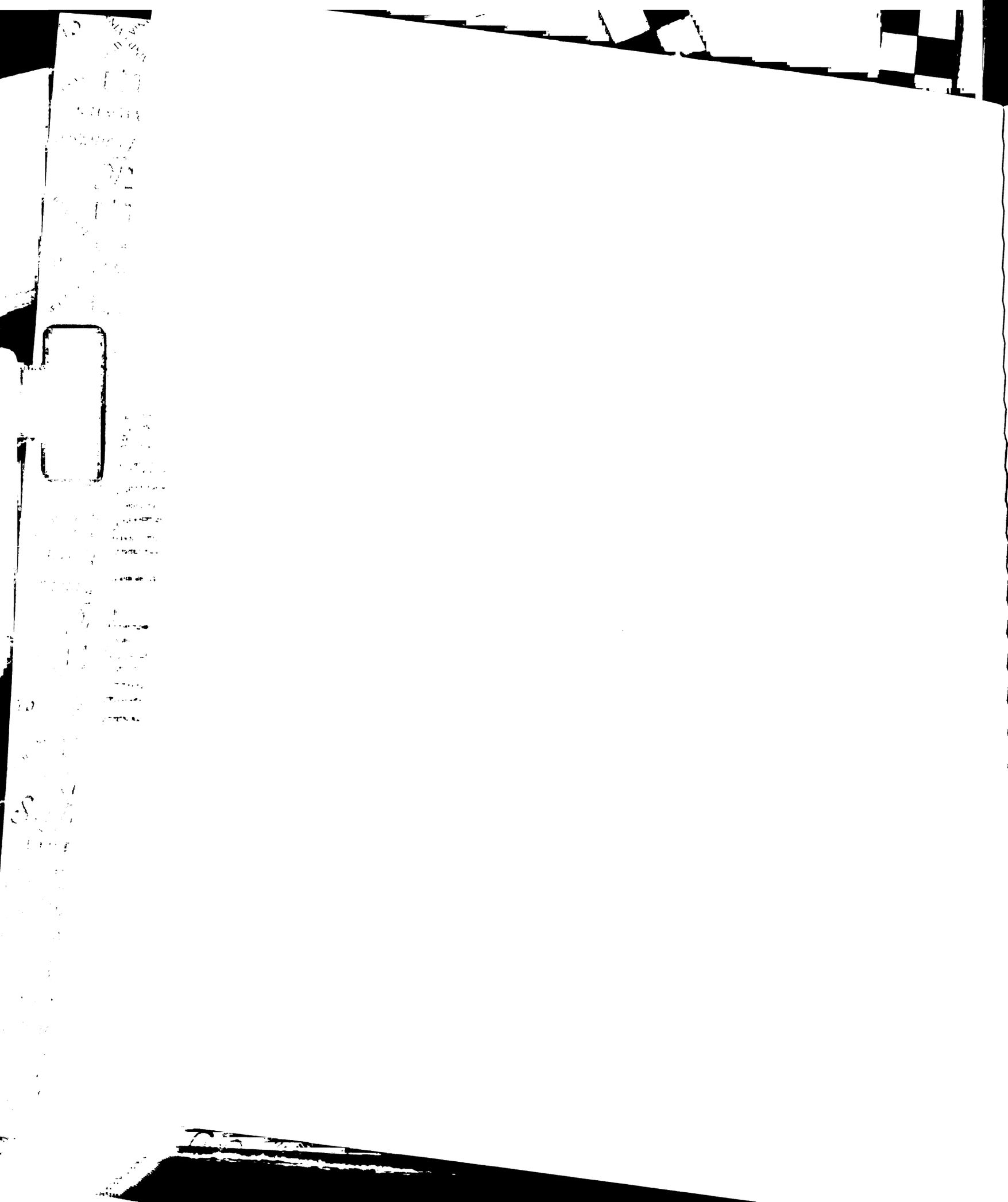
Overexpression of CLB2 is toxic in late mitotic mutants

Mutants defective in cyclin destruction should be sensitive to increased production of cyclin protein. Overproduction of Clb2 is known to be toxic in *cdc5-1* and *tem1-3* mutants at the permissive temperature, but has no effect on growth of wild type strains (Shirayama et al., 1994b; Charles et al., 1998). In the present work, we found that overexpression of *CLB2* also prevents growth of *cdc14-1* and *dbf2-2* mutants at 23°C (Figure 2-2). Although a *cdc15-2* strain overexpressing *CLB2* was able to grow at the permissive temperature, the excess *CLB2* was lethal in this mutant at a semi-permissive temperature (Figure 2-2). These synthetic interactions are consistent with the possibility that the late mitotic proteins act as positive regulators of cyclin destruction.

Clb2 destruction is reduced in late mitotic mutants

The late mitotic mutants arrest in anaphase with separated chromosomes, suggesting that mutants in these genes may be defective in the destruction of cyclins but not that of Pds1 (Hartwell et al., 1973; Kitada et al., 1993; Surana et al., 1993; Shirayama et al., 1994b; Toyn and Johnston, 1994). We therefore compared Clb2 and Pds1 protein levels in the late mitotic mutants at their arrest point. As previously reported, *cdc15-2* and *cdc5-1* mutants arrest with high Clb2 levels, while only a small fraction of the Pds1 protein remains (Figure 2-3A) (Cohen-Fix et al., 1996; Charles et al., 1998; Shirayama et al., 1998). Similarly, *cdc14-1*, *dbf2-2*, and *tem1-3* mutants all arrest with mitotic levels of Clb2 and low levels of Pds1 (Figure 2-3A), supporting the notion that the late mitotic mutants are defective specifically in the destruction of mitotic cyclins.

To directly measure the stability of Clb2 and Pds1, we constructed *cdc15-2* mutant strains containing an integrated copy of *CLB2* or *PDS1* under the control of the *GAL*



promoter. Each protein was fused to a single copy of a hemagglutinin (HA) epitope tag at its carboxy-terminus. The half-lives of both proteins at various points in the cell cycle were determined by inducing their expression with galactose and then repressing transcription and translation with dextrose and cycloheximide, respectively. Clb2HA and Pds1HA were competent for destruction, as both were highly unstable in a G1 arrest (Figure 2-3B). The rapid degradation of both proteins in G1 was dependent on APC function (our unpublished data). In *cdc15-2* cells arrested in metaphase with the microtubule-depolymerizing drug nocodazole, Pds1 and Clb2 proteins were both stable (Figure 2-3B). In *cdc15-2* cells arrested in late anaphase, Clb2 was greatly stabilized relative to G1 cells (Figure 2-3B). In contrast, the majority of the Pds1 protein was rapidly degraded at the mutant arrest point (Figure 2-3B), although a significant fraction of the protein remained stable. This pool of stable Pds1 was larger than that observed in our studies of endogenous Pds1 (Figure 2-3A), suggesting that it represents an artifact of Pds1 overproduction in late mitotic cells.

Cyclin ubiquitination by the APC is defective in late mitotic mutants

To determine if decreased Clb2 destruction in the late mitotic mutants is due to a defect in the cyclin-specific proteolysis machinery, we measured the cyclin-ubiquitin ligase activity of the APC *in vitro*. We used a recently described assay (Charles et al., 1998) in which the APC is immunoprecipitated from yeast extracts with antibodies against an epitope-tagged APC subunit, in this case Cdc27HA expressed on a plasmid under the control of its own promoter (Lamb et al., 1994). The immunoprecipitated APC is incubated with purified yeast E1 (Uba1), E2 (Ubc4), bovine ubiquitin, ATP, and ^{125}I-labelled N-terminus of sea urchin cyclin B1 (Glotzer et al., 1991; Holloway et al., 1993). The conjugation of ubiquitin to the cyclin N-terminus is assessed by polyacrylamide gel electrophoresis of reaction products.

Mutant strains were arrested in late anaphase by shifting asynchronous cultures to 37°C until 80-95% of the cells were arrested as large budded cells. The APC isolated from the late mitotic mutants at 37°C had negligible cyclin-ubiquitin ligase activity, except in the case of *cdc14-1* mutants, which reproducibly contained a small amount of activity (Figure 2-4A). The level of APC activity measured *in vitro* was reflective of the amount of Clb2 protein and Clb2-associated kinase activity (Figure 2-4B and 4C, respectively). Thus, the late mitotic proteins are required for activation of the APC toward mitotic cyclins.

When mutant cells were arrested in G1 with α-factor and then shifted to the restrictive temperature in the continued presence of α-factor, the APC activity from *cdc15-2*, *cdc14-1*, *dbf2-2*, and *tem1-3* cells was equivalent to that of wild type cells arrested in α-factor (Figure 2-4A). *cdc5-1* cells displayed low APC activity in G1, probably because this mutation results in a severe defect in APC activation even at the permissive temperature (Charles et al., 1998). We conclude that the late mitotic gene products are required for initiation but not maintenance of APC activity towards cyclin.

All of the late mitotic mutants arrest with negligible levels of the Cdk inhibitor Sic1 (Figure 2-4D). This is consistent with previous evidence that Cdc28-dependent kinase activity inhibits Swi5-dependent *SIC1* transcription and also inhibits Sic1 stabilization (Moll et al., 1991; Donovan et al., 1994; Toyn et al., 1996; Verma et al., 1997).

CDC15 encodes a protein kinase whose activity is not regulated in the cell cycle

If the products of the late mitotic genes are activators of the APC, their activity might be expected to increase in mitosis. Indeed, the expression of *CDC5*, *CDC14*, and *DBF2* is known to peak during mitosis (Johnston et al., 1990; Wan et al., 1992; Kitada et al., 1993); in addition, the levels and kinase activities of the Cdc5 and Dbf2 proteins rise during mitosis and decline as cells enter G1 (Toyn and Johnston, 1994; Hardy and Pautz,

1996; Charles et al., 1998; Shirayama et al., 1998). Studies of Cdc15 protein levels or activity during the cell cycle have not been reported.

CDC15 is predicted to encode a 110 kDa protein kinase (Schweitzer and Philippsen, 1991). To verify this prediction, we constructed a version of Cdc15 with three copies of the HA epitope tag at its carboxy-terminus, and either expressed the gene from its own promoter on a 2μ plasmid or replaced the endogenous gene with the epitope-tagged copy. Cells expressing Cdc15HA3 but not those expressing untagged Cdc15 contained a 110 kDa protein that was recognized by the anti-HA monoclonal antibody 12CA5 (Figure 2-5A). Immunoprecipitates from cells expressing Cdc15HA3 contained an associated kinase activity that phosphorylated myelin basic protein (MBP) *in vitro* (Figure 2-5B). Kinase activity was abolished by a point mutation at a conserved lysine in the ATP binding site of the Cdc15 kinase domain (K54L; Figure 2-5B). In addition to phosphorylating MBP, Cdc15HA3 also phosphorylated itself (Figure 2-5B and our unpublished data).

To analyze Cdc15 protein levels across the cell cycle, cells in which the endogenous *CDC15* was replaced with *CDC15HA3* were arrested in G1 with mating pheromone and then released. Whereas Clb2 protein levels oscillated as cells progressed through the cell cycle, levels of Cdc15 protein remained constant (Figure 2-6A). We also measured Cdc15-associated kinase activity across the cell cycle, using a strain expressing Cdc15HA3 from its own promoter on a multicopy plasmid. As before, Cdc15 protein levels did not fluctuate as cells were released from a G1 arrest and allowed to proceed through the cell cycle (Figure 2-6B). Furthermore, neither Cdc15 autophosphorylation nor Cdc15-associated MBP kinase activity appeared to change across the cell cycle (Figure 2-6B).

Discussion

Several lines of genetic evidence, presented here and in previous work, reveal extensive overlaps in the functions of Cdc15, Cdc5, Cdc14, Dbf2, and Tem1 (Kitada et

al., 1993; Donovan et al., 1994; Shirayama et al., 1994b; Shirayama et al., 1996). First, mutants in these genes arrest at the restrictive temperature with remarkably similar phenotypes, including large buds, extended spindles, separated DNA masses, high levels of Clb2, low levels of Pds1 and Sic1, and low cyclin-directed APC activity. Second, the temperature-sensitive growth defect in many late mitotic mutants can be suppressed by overexpression of other genes in the family. Third, the growth defect in all of the mutants is enhanced by *CLB2* overexpression, and suppressed in all but one mutant by overexpression of *SIC1*, *SPO12*, and truncated *YAK1*. Finally, we have found an extensive array of synthetic lethal interactions in strains bearing two late mitotic mutations. These results are all consistent with the possibility that the late mitotic genes promote overlapping functions required for the exit from mitosis.

The functions of the late mitotic genes appear to converge on the cyclin destruction machinery. All five of the genes we studied are required for the activation of cyclin-ubiquitin ligase activity of the APC in late mitosis, while none are required for the maintenance of that activity in G1. We suspect that the products of the late mitotic genes directly promote cyclin-specific APC activation, rather than controlling it indirectly by promoting an essential mitotic process whose completion is required to allow cyclin destruction. The latter possibility does not seem consistent with the ability of *SIC1* overexpression to suppress the growth defects in these mutants. Good evidence for a direct regulatory role exists for Cdc5, whose overproduction at any cell cycle stage triggers APC activation (Charles et al., 1998; Shirayama et al., 1998); in addition, the mammalian homolog of Cdc5, Plk1, is able to directly phosphorylate and activate the APC (Kotani et al., 1998).

Previous work showed that overexpression of genes that antagonize the cAMP pathway suppresses the growth defect in the *cdc15-2* mutant (Spevak et al., 1993). Similarly, we found that many of the late mitotic mutants are suppressed by overexpression of truncated *YAK1*, which may, like full-length *YAK1*, oppose the actions of PKA

(Garrett and Broach, 1989; Garrett et al., 1991; Hartley et al., 1994; Ward and Garrett, 1994). Considering recent evidence that PKA acts as an inhibitor of the APC *in vitro* (Kotani et al., 1998), it might be predicted that inhibition of the PKA pathway by *YAK1* could increase APC activity and thereby allow late mitotic mutants to exit mitosis.

The late mitotic mutants are defective primarily in the degradation of cyclin and not that of Pds1, suggesting that these genes activate the Hct1-dependent pathway that is thought to specify the ubiquitination of late mitotic substrates such as Clb2, Ase1, and Cdc5 (Schwab et al., 1997; Visintin et al., 1997; Charles et al., 1998; Shirayama et al., 1998). The destruction of the majority of Pds1 in *cdc15-2*, *cdc5-1*, *cdc14-1*, *dbf2-2*, and *tem1-3* is consistent with the fact that these mutants complete chromosome segregation. Interestingly, late mitotic mutants arrested in anaphase still contain a small amount of stable Pds1 protein, which may represent an inactive pool of the protein whose destruction is not required for chromosome segregation.

The products of the late mitotic genes may also contribute to Cdc28 inactivation by mechanisms other than cyclin destruction. Recent studies suggest that cyclin destruction is not essential for mitotic exit under some conditions (Minshull et al., 1996; Toyn et al., 1996; Schwab et al., 1997; Visintin et al., 1997; Jin et al., 1998). Cells lacking *HCT1* are able to exit mitosis despite a severe defect in cyclin destruction, possibly because Cdc28 is inactivated in these cells by the inhibitor Sic1 (Schwab et al., 1997; Visintin et al., 1997). The fact that the late mitotic genes are essential for mitotic exit implies that they may have functions in addition to the activation of cyclin destruction. For example, they may stimulate the synthesis or stabilization of Sic1 (Figure 2-7).

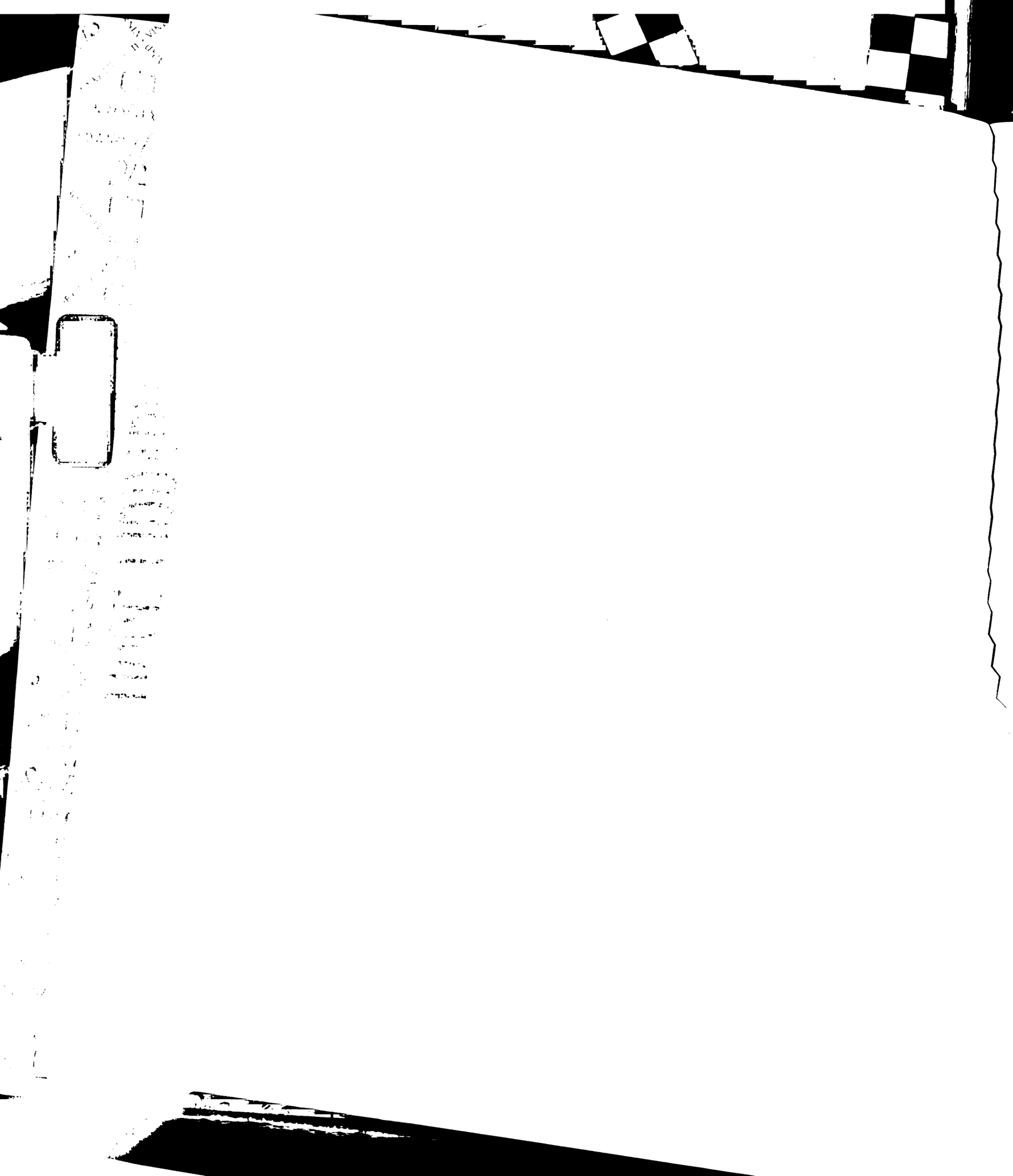
In light of previous evidence that APC-dependent proteolysis is inhibited by Cdc28 activity (Amon, 1997), it is conceivable that late mitotic gene products act entirely through the upregulation of Sic1, which would lead indirectly to APC activation. This seems unlikely, however, given the fact that the late mitotic genes are essential for viability and

SIC1 is not, and given the biochemical evidence that at least one late mitotic gene product, Cdc5, acts directly on the APC (Kotani et al., 1998).

The reversal of Cdc28 action in late mitosis cannot be accomplished solely by Cdc28 inactivation: dephosphorylation of its substrates is presumably required. Thus, defects in the dephosphorylation of Cdc28 substrates would also be expected to result in a late mitotic arrest. Interestingly, Cdc14 is homologous to protein phosphatases and possesses phosphatase activity *in vitro* (Wan et al., 1992; Taylor et al., 1997), raising the possibility that it is responsible for dephosphorylating Cdc28 substrates. Interestingly, the *cdc14-1* mutant displayed unique behaviors in our experiments that are consistent with this possibility: the *cdc14-1* mutant defect was not rescued effectively by any of the suppressors, and overexpressed *CDC14* was the most effective suppressor of the other mutants.

To understand how the products of the late mitotic genes fit into the complex pathways that trigger Cdc28 inactivation after chromosome segregation, we will need a better understanding of the regulation of these proteins. Production of three of the late mitotic gene products (Cdc5, Cdc14, and Dbf2) is increased during mitosis at the time when APC activation occurs, but the mechanisms underlying this regulation remain obscure (Johnston et al., 1990; Wan et al., 1992; Kitada et al., 1993; Toyn and Johnston, 1994; Hardy and Pautz, 1996; Charles et al., 1998; Shirayama et al., 1998). We found that bulk Cdc15 protein and activity does not appear to be regulated during the cell cycle, but this does not exclude cell cycle-dependent changes in Cdc15 localization or accessibility of Cdc15 substrates. Alternatively, constant Cdc15 activity may act through a regulated component of the pathway (such as Cdc5) to specifically activate cyclin proteolysis at the end of mitosis.

The five genes studied in the present work are members of a growing family of genes with overlapping functions in the completion of mitosis. Additional genes in this family include *LTE1*, which interacts genetically with *CDC15* and *TEM1* and encodes a



putative guanine-nucleotide exchange factor (Shirayama et al., 1994a; Shirayama et al., 1994b; Shirayama et al., 1996). *MOB1* encodes a protein that physically associates with Dbf2 and is required for the completion of anaphase; *mob1* mutants display genetic interactions with *DBF2*, *CDC15*, *CDC5*, and *LTE1* (Komarnitsky et al., 1998; Luca and Winey, 1998). Dbf2 also interacts physically with the CCR4 transcription complex and might thereby exert effects on gene expression in late mitosis (Liu et al., 1997). The existence of this complex network of late mitotic regulatory proteins implies that progression from anaphase to G1 is a key regulatory transition in the cell cycle. It seems likely that the late mitotic regulators serve as components in signaling pathways that monitor mitotic events and promote Cdc28 inactivation and mitotic exit only upon successful completion of anaphase and preparation for cytokinesis.

Materials and Methods

Yeast Strains and Plasmids

All strains (Table 2-1) were derivatives of W303 (*MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-1 can1-100*). Strains were made cogenic by backcrossing at least four times to AFS34 and were made *bar1* by a subsequent cross to AFS92 (gift of A. Straight, UCSF), or were constructed in AFS92 using a pop-in, pop-out strategy (Guthrie and Fink, 1991).

Multicopy plasmids carrying the genes encoded by the late mitotic mutants were cloned as follows. pSJ107 (pRS426-*CDC15HA*) was made by cloning the hemagglutinin (HA) epitope into a PstI site generated by oligonucleotide mutagenesis at the stop codon of a 4 kb genomic *CDC15* fragment. pJC29 (pRS426-*HACDC5*) was created by inserting the HA epitope into an NcoI-EcoRI site generated at the start codon of *CDC5*. The construct contains 300 bp of 5' sequence and 500 bp of 3' sequence in addition to the *CDC5* open reading frame. pPD.2 (pRS426-*CDC14HA3*) contains 564 bp of promoter sequence and the open reading frame of *CDC14* ligated in frame to a triple HA (HA3) tag in pRS426. To construct pSJ57 (pRS426-*HA3DBF2*), the *DBF2* open reading frame and 380 bp of 3' sequence were ligated in frame into a 2μ plasmid containing the *DBF2* promoter sequence and a triple HA tag. Finally, pSJ56 (pRS426-*TEM1HA3*) was generated by fusing the 3' end of the *TEM1* open reading frame (accompanied by 300 bp of 5' sequence) to a HA3 tag in pRS426. All of these constructs were shown to complement the appropriate temperature-sensitive mutant in single copy and on the multicopy plasmid.

Strains containing *GAL-CLB2-URA3* were obtained from crosses to ADR58 (gift from A. Rudner, UCSF; (Hwang and Murray, 1997)). Wild type and mutant strains containing *PDS1HA-URA3* were obtained from crosses to ADR1002, a wild type strain containing *PDS1HA-URA3* integrated at the *PDS1* locus (a gift from D. Koshland, Carnegie Institute; (Cohen-Fix et al., 1996)). To construct pSJ50 (*GAL-CLB2HA*) and

pRTK-C1 (*GAL-PDS1HA*), the open reading frames of *CLB2* and *PDS1* were amplified from genomic DNA by PCR and cloned into a pRS304-based plasmid (Sikorski and Hieter, 1989) containing the *GAL1/10* promoter and a single C-terminal HA tag. Strains containing *GAL-CLB2HA* or *GAL-PDS1HA* were made by digesting pSJ50 and pRTK-C1 with Bsu36I for integration at *TRP1*.

All *CDC15* constructs were derived from a 4 kb PvuII genomic fragment containing the *CDC15* gene (gift of A. Rudner; (Schweitzer and Philippsen, 1991)). To create SLJ23, *CDC15* was tagged at the C-terminus with a HA3 tag and integrated into AFS92 at the *CDC15* locus using a pop-in, pop-out strategy (Guthrie and Fink, 1991). pSJ103 (pRS426-*CDC15HA3*) was made by subcloning the *CDC15HA3* genomic fragment into pRS426. A kinase-deficient mutant *CDC15* (pSJ59) was generated by site-directed mutagenesis of pSJ103, using the following oligonucleotide to change lysine 54 to a leucine (K54L): 5'-GTACACGACCTCTAGAATTGCCACGAC-3'. The wild-type HA3-tagged *CDC15* constructs fully complement the growth defects of *cdc15-2* and *cdc15Δ*. The K54L mutant does not complement either strain (our unpublished data).

Yeast Methods

Standard protocols were used for yeast transformation, genetic analysis, and cell propagation (Guthrie and Fink, 1991). To arrest temperature-sensitive strains, cells were grown at 23°C to mid-log phase and arrested with 1 μg/ml α-factor or 15 μg/ml nocodazole at 23°C for 3.5 hours or by shifting cells to 37°C for 3.5 hours. During the last 30 minutes of the arrests, α-factor- and nocodazole-arrested cultures were shifted to 37°C in the continued presence of the arresting agent. To measure the turnover of Pds1 and Clb2, cells were grown in YP/2% raffinose to an OD_{600} of 0.3 and arrested. Expression from the *GAL* promoter was induced by the addition of galactose to 2% for 30 minutes. Transcription and translation were then repressed with 2% dextrose and 10 μg/ml cycloheximide, and cells were harvested at the indicated times. Arrest and release from

α-factor was done by growing cells at 30°C to an OD_{600} of 0.3. α-factor (1 μg/ml) was added for 3 hours, cells were pelleted, washed three times in fresh media, and released in fresh media at 30°C.

High Copy Suppressor Screen

To screen for high copy suppressors of *cdc15-2*, SLJ02 was transformed with a *URA3*-marked *GAL*-cDNA library (a gift from A. Straight, UCSF; (Liu et al., 1992)). Transformants were selected on SC-ura/dextrose plates at 23°C. Cells were washed off the plates and resuspended in SC-ura/galactose-raffinose media and allowed to grow for 6 hours at 23°C. The culture was then diluted and plated onto YP/galactose-raffinose plates at 37°C to select for suppressors. From approximately 25,000 SC-ura/dextrose transformants, 312 colonies formed on YP/galactose-raffinose at 37°C. The putative suppressors were retested for growth at 37°C. Growth at 37°C was then shown to be plasmid- and galactose-dependent for 189 of the suppressors.

Ninety-two suppressors were chosen for further analysis. Restriction mapping and sequence analysis of 12 cDNAs revealed that *SPO12* or *SIC1* were responsible for suppression. To allow rapid analysis of the remaining suppressors, whole colony PCR was done using a primer complementary to the *GAL*-promoter and a primer in the *SPO12* gene or the *SIC1* gene. In two independent PCR analyses, 71 of the suppressors were found to be *SPO12* and 15 of the suppressors were *SIC1*. Sequencing of plasmids rescued from the 6 remaining suppressors revealed that three were an identical fusion with the kinase domain of *YAK1* (594 bp downstream of the start codon), one was *CDC15*, and two were YGR230W, an opening reading frame with homology to *SPO12* on chromosome VII. All of these plasmids retested in their ability to restore growth to *cdc15-2* at 37°C.

Lysate Preparation and Immunoblotting

Yeast lysates were prepared by resuspending cells in 3-5 pellet volumes of ice cold LLB (50 mM Hepes-NaOH, pH 7.4, 75 mM KCl, 50 mM NaF, 50 mM ß-glycerophosphate, 1 mM EGTA, 0.1% NP40, 1 mM DTT, 1 mM phenylmethyl-sulfonylfluoride, 2 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin) and lysing by mechanical disruption in a Beadbeater (Biospec). Lysates were clarified by centrifugation at 14,000 x g for 10 minutes at 4°C. Protein concentrations of extracts were determined with the BioRad Protein Assay (BioRad), using BSA as a standard.

For immunoblots, equal amounts of total protein were loaded on SDS-PAGE gels and proteins were electrophoretically transferred to nitrocellulose. Clb2 and Cdc28 proteins were detected with affinity-purified polyclonal antibodies as described (Gerber et al., 1995; Charles et al., 1998). For detection of HA-tagged proteins, the mouse monoclonal antibody 12CA5 was used as previously described (Gerber et al., 1995). Sic1 immunoblots were performed with a 1:1000 dilution of α-Sic1 polyclonal antibodies (a gift from M. Tyers, University of Toronto (Skowyra et al., 1997))

Kinase Assays

To measure Cdc15-associated kinase activity, cell lysates (250 µg-1 mg) were incubated with 3 µg of 12CA5 and protein A-sepharose (Sigma) for 2 hours at 4°C. Immune complexes were washed three times in LLB and once in kinase buffer (50 mM Hepes-NaOH, pH 7.4, 5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 5 mM ß-glycerophosphate, and 1 mM DTT), and incubated for 10 minutes at 23°C in a 20 µl reaction mixture containing 20 µM ATP, 2 µg myelin basic protein, and 5 µCi [γ-^{32}P]ATP (3000 mCi/mmol) in kinase buffer. Reaction products were analyzed on 12% SDS-PAGE gels followed by autoradiography. Clb2-associated kinase activity was measured as described (Gerber et al., 1995) by immunoprecipitating Clb2 from 100 µg of yeast lysate with 0.3 µg affinity-purified anti-Clb2 antibody and protein A-sepharose for 2 hours at 4°C.

In Vitro Ubiquitination Assay

Ubiquitin-ligase activity of the APC was measured as described (Charles et al., 1998). Briefly, the APC was immunoprecipitated with 12CA5 monoclonal antibodies from 500 μg of yeast lysate (containing Cdc27HA, a gift from P. Hieter, University of British Columbia (Lamb et al., 1994)). Immune complexes were washed three times in LLB, once in High Salt QA (20 mM Tris-HCl pH 7.6, 250 mM KCl, 1 mM $MgCl_2$, 1 mM DTT), twice in Buffer QA (20 mM Tris-HCl pH 7.6, 100 mM KCl, 1 mM $MgCl_2$, 1 mM DTT), and were then incubated for 15 minutes at 23°C in a 15 μl reaction containing 3.5 pmol Uba1, 47 pmol Ubc4, 1 mM ATP, 20 μg bovine ubiquitin (Sigma), and 0.25 μl ^{125}I-labeled sea urchin (13-91) cyclin B1 in Buffer QA. Reaction products were electrophoresed on 7.5-15% gradient gels and analyzed for ubiquitin conjugates by autoradiography with the BioMaxMS System (Kodak).

Acknowledgments

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Table 2-1. Yeast Strains

Strain	Relevant Genotype	Source
AFS34[a]	*MATa ade2-1 can1-100 ura3-1 leu2-3,112, his3-11,15, trp1-1*	A. Straight
AFS92	*MATa bar1*	A. Straight
AFS80	*MATa bar1 cdc16-1*	A. Straight
SLJ02	*MATa cdc15-2*	A. Rudner
SLJ127	*MATa bar1 cdc15-2*	This work
JC34	*MATa bar1 cdc5-1*	J. Charles
SLJ250	*MATa bar1 cdc14-1*	This work
SLJ256	*MATa bar1 dbf2-2*	This work
SLJ200[b]	*MATa bar1 tem1-3*	This work
ADR58	*MATa bar1 ura3::GAL-CLB2-URA3 (pDK27)*	A. Rudner
ADR1002	*MATa pds1::PDS1HA-URA3*	A. Rudner
SLJ423	*MATa bar1 pds1::PDS1HA-URA3*	This work
SLJ424	*MATa bar1 cdc16-1 pds1::PDS1HA-URA3*	This work
SLJ425	*MATa bar1 cdc15-2 pds1::PDS1HA-URA3*	This work
SLJ426	*MATa bar1 cdc5-1 pds1::PDS1HA-URA3 trp1::LacO-TRP1*	This work
SLJ427	*MATa bar1 cdc14-1 pds1::PDS1HA-URA3*	This work
SLJ428	*MATa bar1 dbf2-2 pds1::PDS1HA-URA3*	This work
SLJ429	*MATa bar1 tem1-3 pds1::PDS1HA-URA3*	This work
SLJ269	*MATa bar1 cdc15-2 trp1::GAL-CLB2HA-TRP1 (pSJ50)*	This work
SLJ272	*MATa bar1 cdc15-2 trp1::GAL-PDS1HA-TRP1 (pRTK-C1)*	This work
SLJ23	*MATa bar1 cdc15::CDC15HA3*	This work

[a] All strains are in a W303 background.

[b] Constructed in AFS92 as described (Shirayama et al., 1994b).

Table 2-2. Multicopy Suppression of Late Mitotic Mutants

	Effect[a] of multicopy plasmid on colony growth at 37°C					
	vector	*CDC15HA*	*HACDC5*	*CDC14HA3*	*HA3DBF2*	*TEM1HA3*
WT	+	+	+	+	+	+
cdc15-2	-	+	-[b*]	+[c]	-[b]	-
cdc5-1	-	-[b*]	+	+/-	-[b]	+
cdc14-1	-	-	-[b]	+	-	-
dbf2-2	-	-[b,d*]	-[b*]	-	+	-[d]
tem1-3	-	+[d]	+	+	-	+

[a] Wild type and mutant strains were transformed with the indicated gene on a 2μ plasmid carrying a *URA3* marker (pRS426; (Sikorski and Hieter, 1989)) and transformants were selected on SC-ura plates at 23°C. Suppression of each mutant arrest was analyzed by streaking for single colonies on SC-ura plates at 37°C. + indicates robust growth on SC-ura plates at 37°C; +/-, weak growth on SC-ura plates at 37°C; -, no growth on SC-ura plates at 37°C. In some cases, similar results have been obtained in previous studies, as indicated by the superscripts.

[b] (Kitada et al., 1993)

[c] (Shirayama et al., 1996)

[d] (Shirayama et al., 1994b)

[*] Result differs from that previously reported; previous work used the *cdc15-1* and *dbf2-1* alleles in a different strain background.

Table 2-3. Synthetic Interactions between Late Mitotic Mutants

	Maximum permissive temp (°C)	Maximum permissive temperature (°C) of double mutants				
		cdc15-2	*cdc5-1*	*cdc14-1*	*dbf2-2*	*tem1-3*
cdc15-2	33	--	dead	30	30	dead
cdc5-1	33	dead	--	23	dead	dead
cdc14-1	30	30	23	--	30	23
dbf2-2	33	30	dead	30	--	dead
tem1-3	33	dead	dead	23	dead	--

Heterozygous diploids were generated by crossing single haploid mutant strains. Diploids were sporulated for 4 days at 23°C. For each cross, 20-40 tetrads were dissected and analyzed. 'Dead' indicates that no viable double mutant spores were obtained from nonparental ditype tetrads at 23°C. The maximum permissive temperature for growth of viable double mutants was determined by comparing growth to that of the single mutant parents at 23°, 30°, 33°, and 37°C. The data is presented twice for clarity.

Figure 2-1. High copy suppressors of *cdc15-2*.

Four of the *cdc15-2* high copy suppressors (*GAL-CDC15*, *GAL-SIC1*, *GAL-SPO12*, and *GAL-YAK1ΔN*) were retransformed into a *cdc15-2* strain (SLJ02), grown in YP/raffinose to mid log phase, serially diluted 5-fold, and spotted onto YP/dextrose or YP/galactose-raffinose plates. Plates were incubated at 23°C or 37°C for 2.5 days.

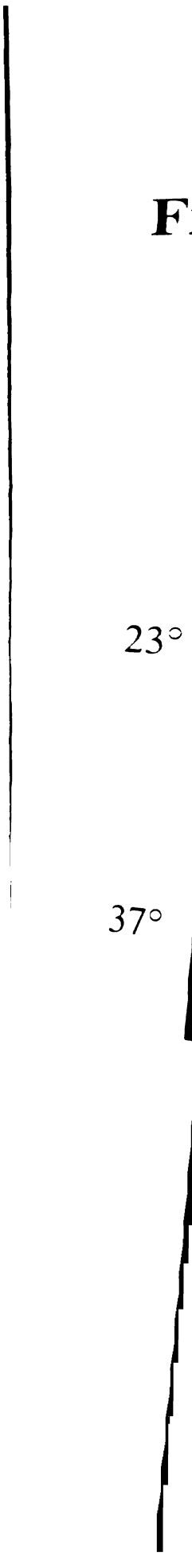

F
23°
37°

Figure 2-1

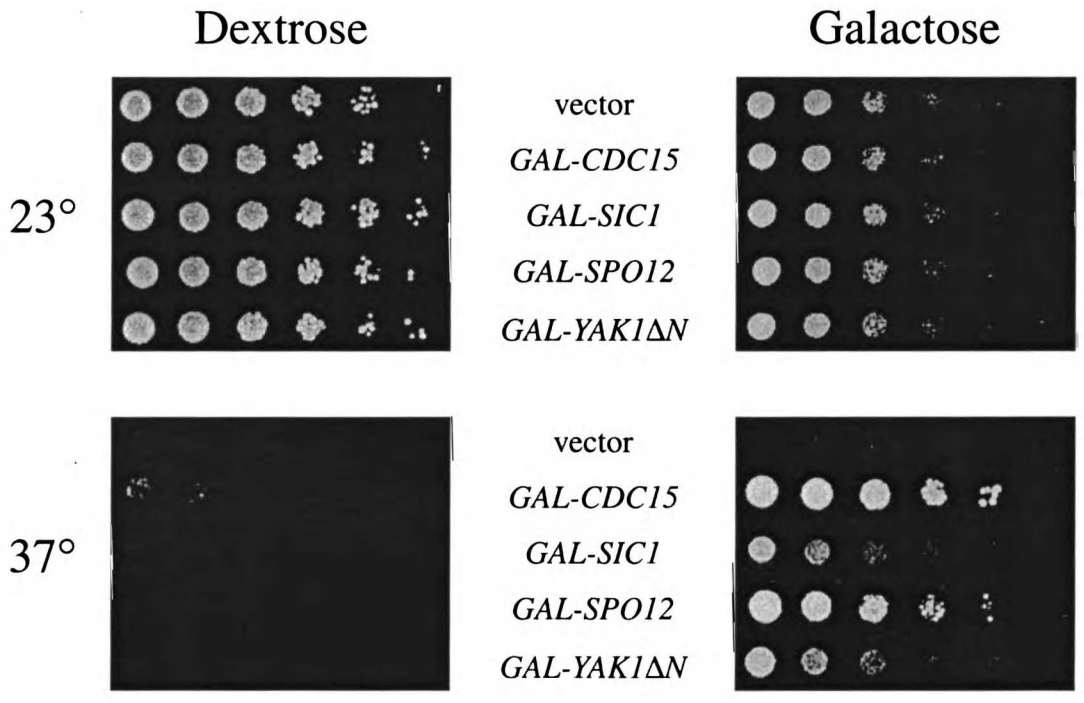

Dextrose
Galactose
23°
37°
vector
GAL-CDC15
GAL-SIC1
GAL-SPO12
GAL-YAK1ΔN
vector
GAL-CDC15
GAL-SIC1
GAL-SPO12
GAL-YAK1ΔN

Table 2-4. Suppression of Late Mitotic Mutants by *GAL*-cDNAs

	Effect[a] of *GAL*-cDNA on colony growth at 37°C				
	vector	*CDC15*	*SIC1*	*SPO12*	*YAK1ΔN*[b]
WT	+++	+++	+++	+++	+++
cdc15-2	-	+++	+	+++	++
cdc5-1	-	-	+	++	+/-
cdc14-1	-	-	+/-[c]	-	-
dbf2-2	-	+/-	++	+++	++
tem1-3	-	+++	+	+++	+

[a] Wild type and mutant strains were transformed with the indicated *GAL*-cDNA plasmids and transformants were selected on SC-ura/dextrose at 23°C. To analyze the ability of the *GAL*-cDNA to restore growth of the mutant at 37°C, cultures were grown overnight to OD_{600} = 1.0 and spotted onto YP/galactose-raffinose plates in a series of six 5-fold dilutions. After 3 days at 37°C, growth on the plates was scored as follows: +++ indicates robust growth (at the fourth dilution and beyond); ++, growth at the third dilution; +, growth at the first and second dilution; +/-, growth of only the first dilution; -, no growth above background.

[b] The *YAK1* clone contains only the 3' end of the gene (see Materials and Methods for details).

[c] Growth was very poor, mainly microcolonies.

Figure 2-2. *CLB2* overexpression enhances the growth defect in *cdc15-2*, *cdc14-1*, *dbf2-2*, and *tem1-3* mutants.

Mutant strains containing *GAL-CLB2* were generated by crossing to ADR58. Progeny from tetratype spores were grown to mid-log phase in YP/raffinose at 23°C, serially diluted 5-fold, and spotted onto YP/dextrose or YP/galactose-raffinose plates. Plates were incubated for 2 days at 30°C or 3 days at 23°C.

Figure 2-2

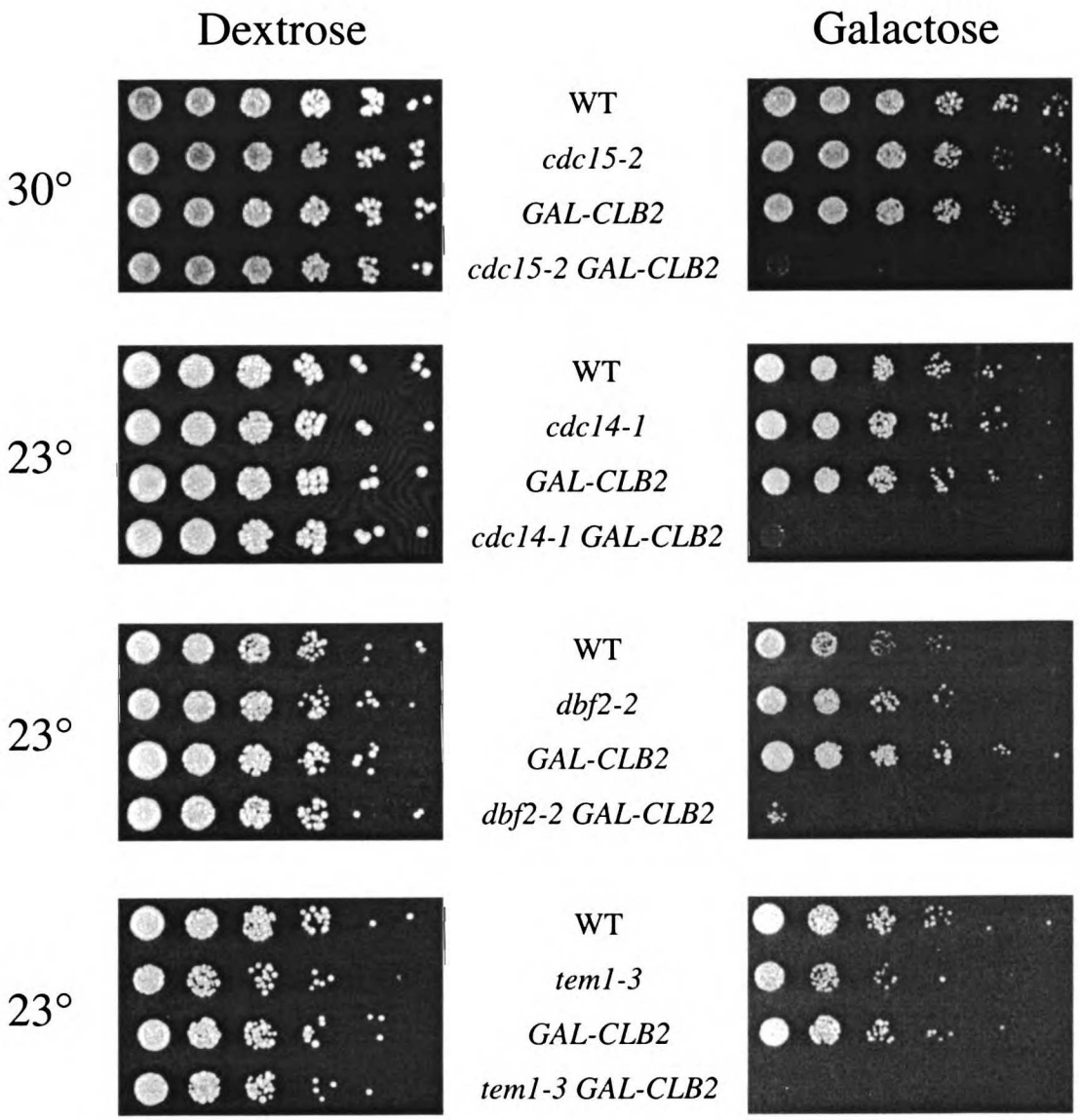

Dextrose
Galactose
30°
WT
cdc15-2
GAL-CLB2
cdc15-2 GAL-CLB2
23°
WT
cdc14-1
GAL-CLB2
cdc14-1 GAL-CLB2
23°
WT
dbf2-2
GAL-CLB2
dbf2-2 GAL-CLB2
23°
WT
tem1-3
GAL-CLB2
tem1-3 GAL-CLB2

Figure 2-3. Clb2, but not Pds1, is stabilized in late mitotic mutants.

(A) Wild type or mutant strains in which the endogenous copy of Pds1 was replaced with Pds1HA (SLJ423-429) were grown in YPD to mid-log phase at 23°C. Cultures were divided and either grown as asynchronous cultures or arrested as indicated for 3.5 hours. During the last 30 minutes of the nocodazole and α-factor arrests, these cultures were also shifted to 37°C. Cell lysates were subjected to immunoblotting with the anti-HA antibody 12CA5 to detect Pds1 (which normally migrates as a doublet; left panels) or with anti-Clb2 antibodies (right panels).

(B) *GAL-PDS1HA* or *GAL-CLB2HA* was integrated at the *TRP1* locus of *cdc15-2* to create SLJ272 and SLJ269, respectively. Mid-log phase YP/raffinose cultures were arrested in 1 μg/ml α-factor, in 15 μg/ml nocodazole, or at 37°C for 3.5 hours. During the last 30 minutes, α-factor- and nocodazole-arrested cultures were shifted to 37°C. Galactose was added to a final concentration of 2% to induce expression of Pds1HA or Clb2HA. After 30 minutes of induction, transcription and translation were repressed by addition at time zero of 2% dextrose and 10 μg/ml cycloheximide, and cells were harvested at the indicated times. Cell lysates were subjected to western blotting with 12CA5 antibodies.

Figure 2-3

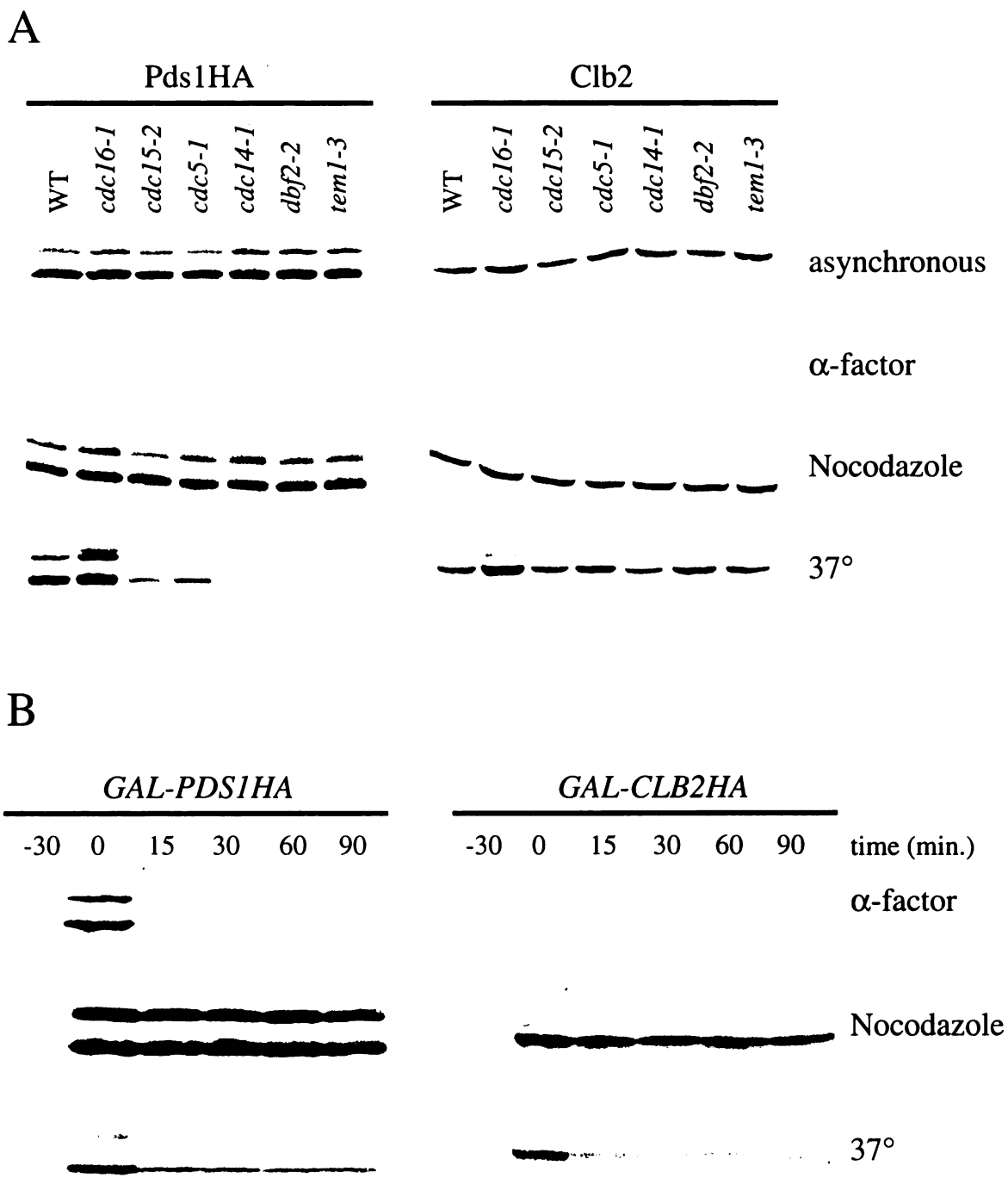

A
Pds1HA
Clb2
WT
cdc16-1
cdc15-2
cdc5-1
cdc14-1
dbf2-2
tem1-3
asynchronous
α-factor
Nocodazole
37°
B
GAL-PDS1HA
GAL-CLB2HA
-30
0
15
30
60
90
time (min.)
α-factor
Nocodazole
37°

Figure 2-4. The late mitotic mutants arrest with low APC activity toward cyclin.

(A) Wild type and mutant strains were transformed with a plasmid carrying *CDC27HA* under the control of its own promoter. Cells were grown to mid-log phase at 23°C, cultures were divided, and half were shifted to 37°C for 4 hours. The second set of cultures were arrested for 3.5 hours at 23°C with 1 μg/ml α-factor, and then shifted to 37°C in the presence of α-factor for an additional hour. The Cdc27HA subunit of the APC was immunoprecipitated from 500 μg of cell lysate with 12CA5, and conjugation of ubiquitin to the ^{125}I-labeled N-terminus of sea urchin cyclin B1 was assessed as described in Materials and Methods. Ubiquitin conjugates were observed at approximately 8 kDa intervals above the unconjugated cyclin B1 fragment. The asterisk indicates a non-specific background band observed in the presence of cyclin substrate alone (far left lane). Note that APC activity in wild type asynchronous cells is normally lower than that in G1-arrested cells (Charles et al., 1998); in this experiment, the high activity in asynchronous cells is due to the relatively high protein levels in these samples (see anti-Cdc28 western blot in panel B). **(B)** Lysates (approx. 35 μg) from the experiment in panel (A) were subjected to western blotting with polyclonal antibodies against Clb2 (upper) and Cdc28 (lower). (C) Histone H1 kinase activity was measured in anti-Clb2 immunoprecipitates from 100 μg cell lysate. (D) Cell lysates (100 μg) were subjected to immunoblotting with affinity-purified polyclonal antibodies against Sic1.

Figure 2-4

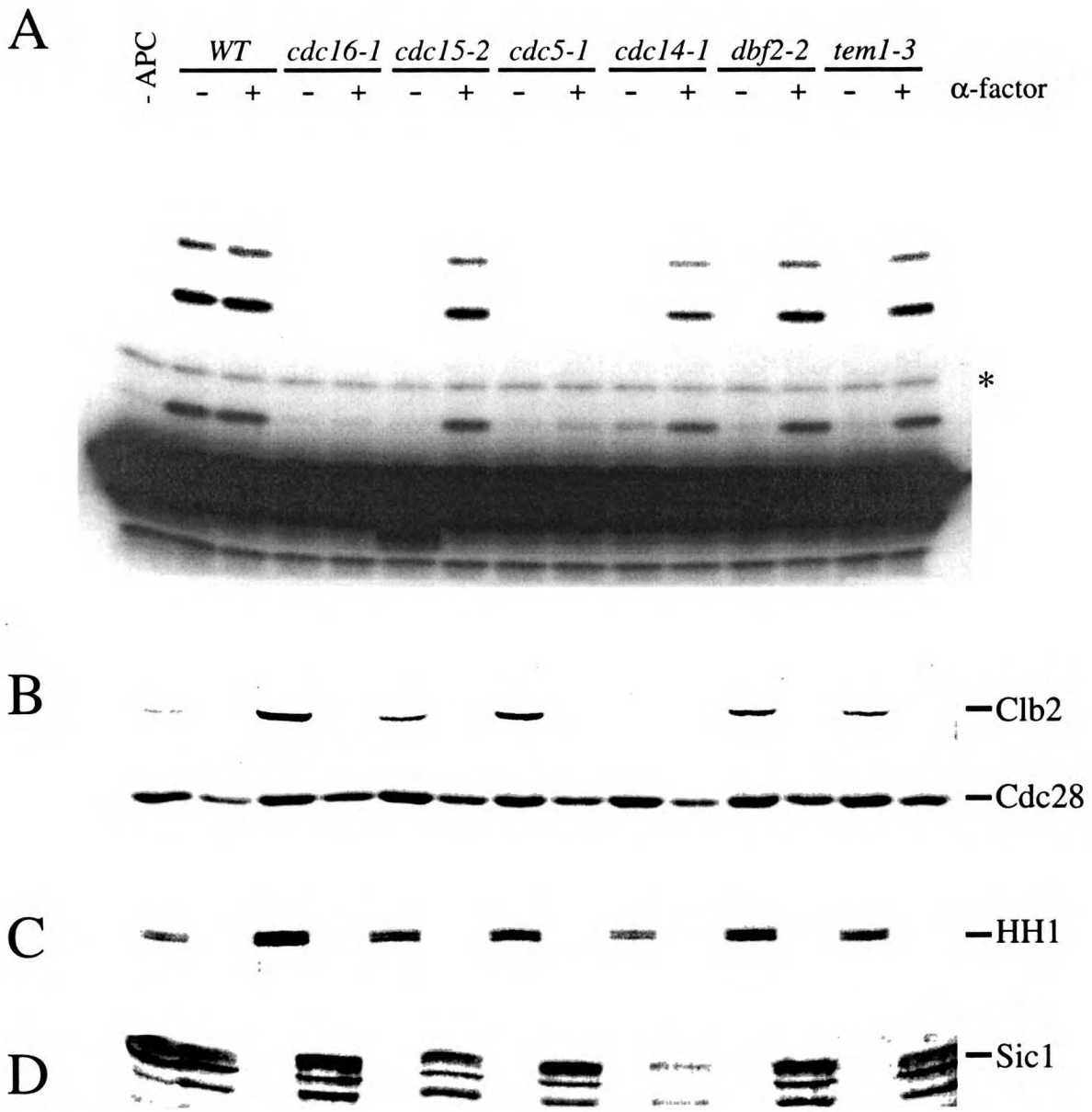

A
-APC
WT
cdc16-1
cdc15-2
cdc5-1
cdc14-1
dbf2-2
tem1-3
- + - + - + - + - + - + - +
α-factor
*
B
Clb2
Cdc28
C
HH1
D
Sic1

Figure 2-5. *CDC15* encodes a protein kinase.

(A) A version of *CDC15* carrying a C-terminal triple HA tag was used to replace the endogenous *CDC15* gene (SLJ23; lane 2) or was cloned onto a 2μ plasmid (pSJ103; lane 4). Lysates from the indicated asynchronous cultures (120 μg in lanes 1, 2; 35 μg in lanes 3-5) were subjected to western blotting with 12CA5 antibodies.

(B) 12CA5 immunoprecipitates from 1 mg (lanes 1, 2) or 250 μg (lanes 3-5) of cell extract were tested for their ability to phosphorylate myelin basic protein (MBP) in a standard kinase reaction. A protein the size of Cdc15HA3 was also labeled in these immunoprecipitates. In other experiments with singly tagged Cdc15HA, this band migrates slightly faster, indicating that it represents the Cdc15 protein itself (our unpublished data). In lane 5, the kinase reaction was performed with a version of Cdc15 (pSJ59) carrying a point mutation (K54L) that is predicted to abolish kinase activity.

Figure 2-5

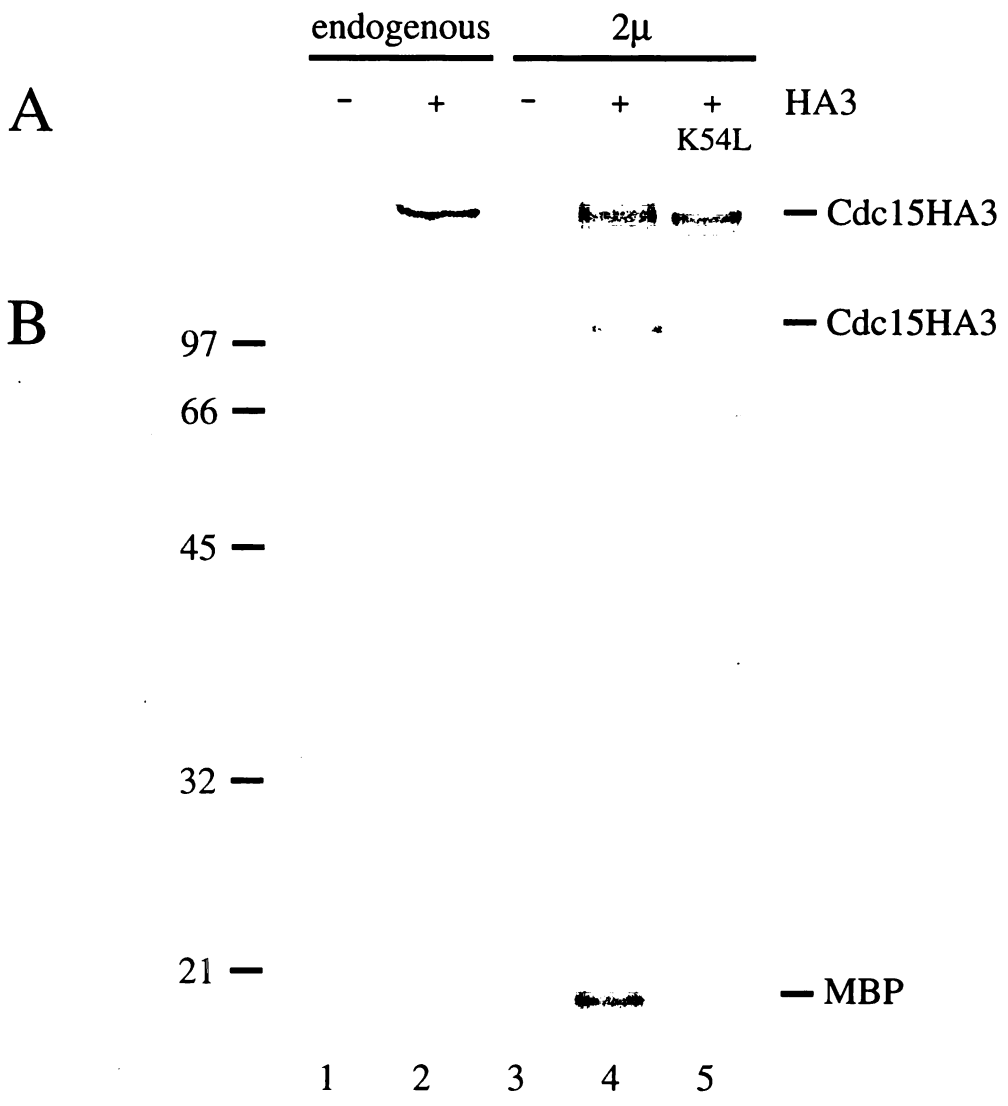
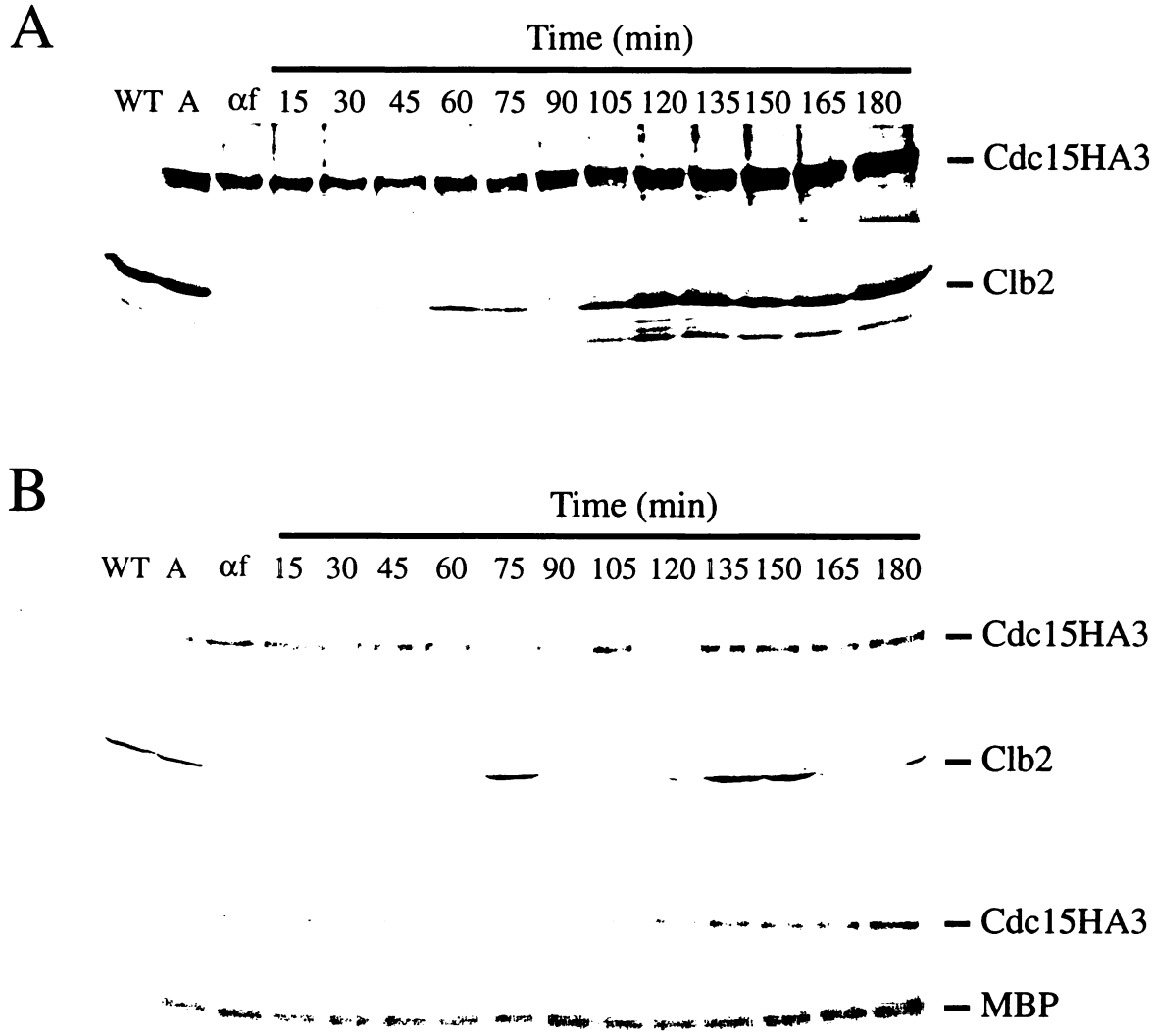

endogenous
2μ
A
- + - + +
K54L
HA3
— Cdc15HA3
B
— Cdc15HA3
97 —
66 —
45 —
32 —
21 —
— MBP
1 2 3 4 5

Figure 2-6. Cdc15 protein levels and kinase activity are constant during the cell cycle.

(A) A *cdc15::CDC15HA3* strain (SLJ23) was arrested for 3 hours at 30°C with 1 μg/ml α-factor, released from the arrest and allowed to grow at 30°C. Cells were harvested at the indicated times, and lysates (100 μg) were analyzed by western blotting with 12CA5 (top) or anti-Clb2 antibodies (bottom).

(B) Wild-type cells carrying *CDC15HA3* on a 2μ plasmid were arrested for 3 hours at 30°C with 1 μg/ml α-factor, released from the arrest and allowed to progress through the cell cycle at 30°C. Cells were harvested at the indicated times, and lysates (35 μg) were analyzed by western blotting with 12CA5 (upper) or anti-Clb2 antibodies (second panel). Cdc15HA3 was immunoprecipitated from 250 μg lysate and tested for its ability to phosphorylate itself (third panel) or MBP (bottom panel).

Figure 2-6


A
Time (min)
WT A αf 15 30 45 60 75 90 105 120 135 150 165 180
Cdc15HA3
Clb2
B
Time (min)
WT A αf 15 30 45 60 75 90 105 120 135 150 165 180
Cdc15HA3
Clb2
Cdc15HA3
MBP

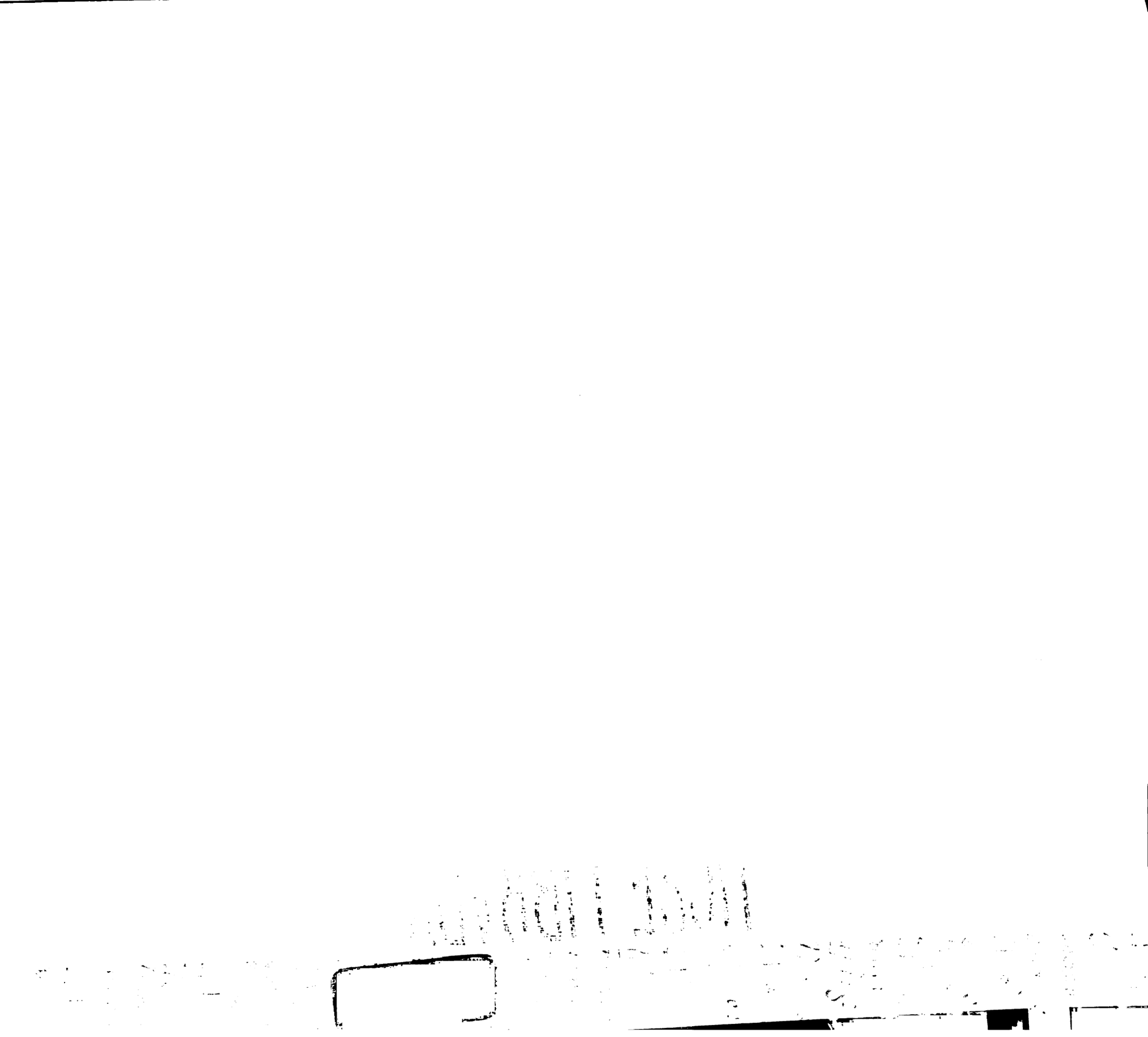
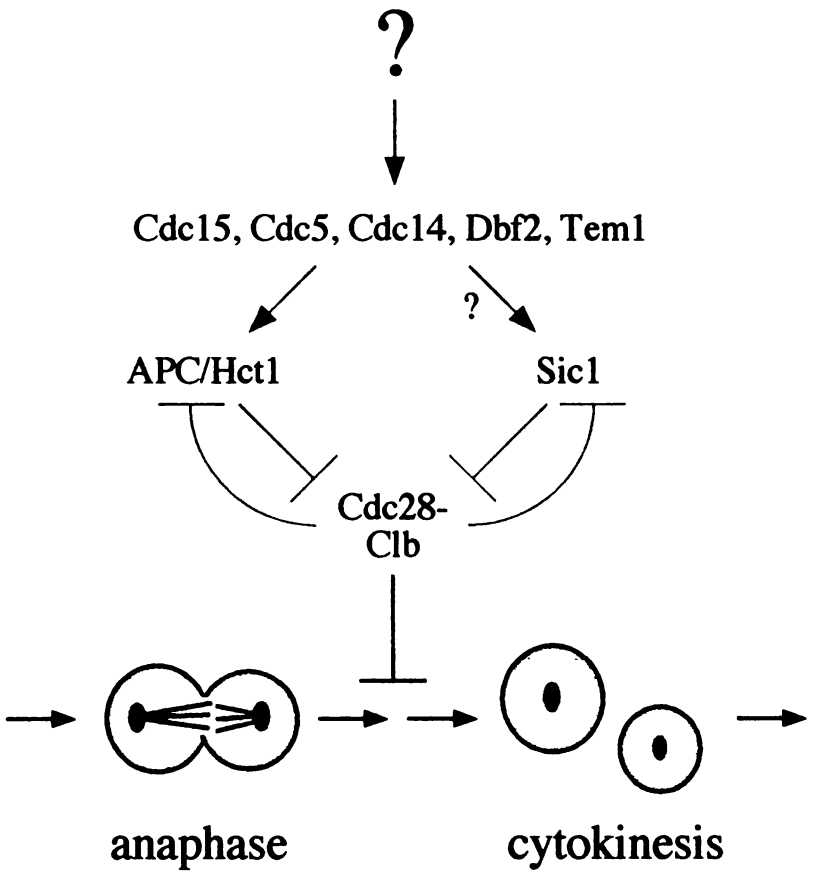


Figure 2-7. Model of regulatory pathways governing Cdc28 activity in late mitosis.

Late mitotic gene products stimulate mitotic cyclin destruction and may also induce increased levels of Sic1 (see Discussion). This model also accomodates evidence that Cdc28 inhibits APC activity (Amon, 1997) and also inhibits *SIC1* transcription and Sic1 stability (Moll et al., 1991; Toyn et al., 1996; Verma et al., 1997), resulting in a feedback system that triggers rapid and complete Cdc28 inactivation when Cdc28 activity is reduced to some threshold. For simplicity, this diagram does not include an additional feedback loop suggested by the observation that Cdc28-Clb complexes stimulate *CLB* transcription (Amon et al., 1993).

Figure 2-7


?
Cdc15, Cdc5, Cdc14, Dbf2, Tem1
?
APC/Hct1
Sic1
Cdc28-
Clb
anaphase
cytokinesis

Chapter Three

Inhibitory phosphorylation of the APC regulator Hct1 is controlled by the kinase Cdc28 and the phosphatase Cdc14

Inhibitory phosphorylation of the APC regulator Hct1 is controlled by the kinase Cdc28 and the phosphatase Cdc14

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Running title: Control of Hct1 by Cdc28 and Cdc14

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Abstract

Background: Exit from mitosis requires inactivation of mitotic cyclin-dependent kinases (CDKs). A key mechanism of CDK inactivation is ubiquitin-dependent cyclin proteolysis, which is triggered by the late mitotic activation of a ubiquitin ligase known as the anaphase-promoting complex (APC). APC activation requires association with substoichiometric activating subunits known as Cdc20 and Hct1/Cdh1.

Results: We explored the molecular function and regulation of the APC regulatory subunit Hct1 in the budding yeast *Saccharomyces cerevisiae*. Recombinant Hct1 activated the cyclin-ubiquitin ligase activity of APC isolated from multiple cell cycle stages. The APC from cells arrested in G1, or in late mitosis by a *cdc14-1* mutation, was more responsive to Hct1 than the APC from other stages. We found that Hct1 is phosphorylated *in vivo* at multiple CDK consensus sites during cell cycle stages when Cdc28/Cdk1 activity is high and APC activity is low. Purified Hct1 was phosphorylated *in vitro* at these sites by purified Cdc28-cyclin complexes, and phosphorylation abolished the ability of Hct1 to activate the APC *in vitro*. The phosphatase Cdc14, which is known to be required for APC activation *in vivo*, was able to reverse the effects of Cdc28 by catalyzing Hct1 dephosphorylation and activation.

Conclusion: We conclude that Hct1 phosphorylation is a key regulatory mechanism in the control of cyclin destruction. Phosphorylation of Hct1 provides a mechanism by which Cdc28 blocks its own inactivation during S phase and early mitosis. Following anaphase, dephosphorylation of Hct1 by Cdc14 may help initiate cyclin destruction.

Introduction

Exit from mitosis in *Saccharomyces cerevisiae* requires the ubiquitin-dependent proteolysis of multiple proteins, including the anaphase inhibitor Pds1 and the mitotic cyclins (Clbs) (Surana et al., 1993; Cohen-Fix et al., 1996). A key step in the ubiquitination of these proteins is catalyzed by a multi-subunit ubiquitin ligase known as the anaphase-promoting complex (APC) (King et al., 1996; Townsley and Ruderman, 1998b), whose activity increases in late mitosis and remains high throughout G1 (Amon et al., 1994; Zachariae and Nasmyth, 1996; Charles et al., 1998). Mechanisms governing APC activation in late mitosis are poorly understood. In higher eukaryotes, phosphorylation of APC subunits is thought to promote APC activity (Felix et al., 1990; Lahav-Baratz et al., 1995; Sudakin et al., 1995; Peters et al., 1996; Kotani et al., 1998; Patra and Dunphy, 1998). In budding yeast, phosphorylation of the APC has not been reported, but there is evidence that the protein kinase Cdc28/Cdk1 inhibits APC-dependent cyclin proteolysis *in vivo* (Amon, 1997).

The function of the APC is regulated by the WD40-repeat proteins Cdc20 and Hct1/Cdh1. Based on genetic evidence in budding yeast, it is thought that Pds1 degradation at the metaphase-to-anaphase transition is promoted by Cdc20, after which Hct1 stimulates proteolysis of the cyclin Clb2 (Schwab et al., 1997; Visintin et al., 1997; Lim et al., 1998; Shirayama et al., 1998). Recent biochemical evidence from vertebrates suggests that Cdc20 and Hct1 act as substoichiometric APC subunits that stimulate ubiquitin ligase activity and may also contribute to substrate recognition (Fang et al., 1998b; Fang et al., 1998a; Kallio et al., 1998; Kramer et al., 1998a).

As key regulators of APC function, Cdc20 and Hct1 are likely to be critical targets for many mitotic regulatory pathways. Cdc20 appears to be regulated at multiple levels. It is an unstable protein whose concentration peaks in mitosis, after which APC-dependent proteolysis leads to decreased levels in G1 (Fang et al., 1998a; Fang et al., 1998b; Kramer

et al., 1998a; Prinz et al., 1998; Shirayama et al., 1998). In cells arrested in metaphase by spindle damage, Cdc20 function is inhibited by association with checkpoint signaling proteins (Fang et al., 1998a; Hwang et al., 1998; Kallio et al., 1998; Kim et al., 1998). The regulation of Hct1 activity is poorly understood. Hct1 levels do not change during the cell cycle, although in human cells the amount of Hct1 associated with the APC increases in G1 (Fang et al., 1998a; Kramer et al., 1998a; Prinz et al., 1998). Thus, post-translational mechanisms probably regulate Hct1-dependent stimulation of APC activity.

Activation of the cyclin-ubiquitin ligase activity of the APC in late mitosis also requires the function of a large family of regulatory proteins, including the protein kinase Cdc15 (Schweitzer and Philippsen, 1991; Jaspersen et al., 1998), the Polo-like kinase Cdc5 (Kitada et al., 1993; Charles et al., 1998; Shirayama et al., 1998), and the protein phosphatase Cdc14 (Wan et al., 1992; Taylor et al., 1997). Cells lacking the function of these proteins arrest in late mitosis with high cyclin levels and profound defects in Hct1-dependent APC activity (Charles et al., 1998; Jaspersen et al., 1998). The mechanism by which the late mitotic regulatory proteins promote cyclin-specific APC activation is not clear.

We analyzed Hct1 phosphorylation and its effects on the ability of Hct1 to act as an activator of the cyclin-ubiquitin ligase activity of the APC. We found that Cdc28-dependent phosphorylation occurs at multiple sites on Hct1 *in vivo*. Phosphorylation of Hct1 by Cdc28 completely inhibited Hct1 activity *in vitro*, providing a mechanism by which Cdc28 blocks its own inactivation. Inhibitory phosphorylation of Hct1 was removed by the phosphatase Cdc14, suggesting that the requirement for Cdc14 in late mitosis is due, at least in part, to its ability to activate Hct1.

Results

Activation of the APC by Hct1 in vitro

We first set out to explore mechanisms of APC regulation by analyzing the ability of purified Hct1 to activate purified APC *in vitro*. Our initial step was to develop a conventional purification strategy to purify active APC from G1-arrested cells and inactive APC from cells arrested in late mitosis by the *cdc15-2* mutation (Figure 3-1). In both preparations, we observed approximately 12 co-purifying subunits that correspond to the 12 subunits observed in previous reports (Figure 3-1A) (Zachariae et al., 1996; Zachariae et al., 1998b). During the purification, the G1 APC maintained activity while the anaphase APC remained inactive (Figure 3-1B), suggesting that differences in activity were due to intrinsic subunits or modifications. However, in comparisons of several preparations from G1 and anaphase cells, we did not detect any differences in APC subunit composition or the electrophoretic mobility of any subunit on polyacrylamide gels (Figure 3-1A). Thus, activity in these experiments must be influenced by substoichiometric subunits or by modifications that do not affect gel mobility under these conditions.

Cyclin-ubiquitin ligase activity of the purified APC from *cdc15-2* cells was potently stimulated by hexahistidine-tagged Hct1 (Hct1-6His) purified from baculovirus-infected insect cells (Figure 3-2A). Thus, Hct1 alone is sufficient to activate the cyclin-ubiquitin ligase activity of the APC.

Hct1 was also able to activate the APC isolated by immunoprecipitation from several other late mitotic mutants (*cdc5-1*, *cdc14-1*, *dbf2-2*, and *tem1-3*) (Figure 3-2B). Hct1 also activated the APC from cells arrested at the DNA replication checkpoint (hydroxyurea, HU) and the spindle assembly checkpoint (nocodazole), indicating that the APC inhibitory mechanisms acting in these checkpoint arrests do not completely block APC responsiveness to Hct1 (Figure 3-2B). Similarly, Hct1 activated the APC from a *cdc20-1* mutant, indicating that no previous function of Cdc20 is required for Hct1

responsiveness *in vitro* (Figure 3-2B). Finally, we observed that Hct1 was able to hyperactivate the active APC isolated from cells arrested in G1 with the mating pheromone α-factor (Figure 3-2B). Thus, a major fraction of the APC from multiple cell cycle stages can be activated by Hct1.

Hct1 responsiveness of APC increases in late mitosis

Analysis of APC activation over a range of Hct1 concentrations *in vitro* revealed that differences in Hct1 responsiveness exist at different cell cycle stages (Figure 3-2C, D). We analyzed activation of the APC from cells arrested in metaphase (nocodazole), anaphase (*cdc15-2*), or G1 (α-factor), and in four independent experiments we found that the Hct1 concentration required for half-maximal activation of the G1 APC (5 nM) was consistently 4-fold lower than that required for half-maximal activation of the APC from cells arrested by nocodazole or the *cdc15-2* mutation (20 nM) (Figure 3-2C).

Interestingly, analysis of the APC from three late mitotic mutants (*cdc15-2*, *cdc5-1* and *cdc14-1*) revealed that all late mitotic mutants do not arrest with equally responsive APC (Figure 3-2D). Whereas the APC from *cdc5*-arrested cells behaved similarly to that of *cdc15*- and nocodazole-arrested cells (half-maximal activation at 20-25 nM Hct1), the APC from the *cdc14-1* mutant exhibited a higher Hct1 sensitivity similar to that seen in G1-arrested cells (half-maximal activation at 5 nM Hct1).

The simplest interpretation of these experiments is that increasing Hct1 concentration leads to increased occupancy of the APC, which is then reflected in stimulation of APC activity. As these experiments were performed at very low APC concentrations (roughly nanomolar), the Hct1 concentration at which half-maximal stimulation occurs probably provides an estimate of Hct1-APC affinity, although more direct binding analyses will be required to rigorously assess this possibility. Nevertheless, these results suggest that APCs from *cdc14*- and G1-arrested cells have a relatively high affinity for Hct1.

Hct1 is phosphorylated by Cdc28

Our results indicate that the APC from multiple cell cycle stages is responsive to Hct1 *in vitro*, raising the question of why Hct1 does not activate these APCs *in vivo*. Hct1 levels are constant during the normal cell cycle (Prinz et al., 1998), indicating that Hct1 activity *in vivo* may be regulated by post-translational modification. To explore potential Hct1 modifications, we constructed a strain containing an integrated copy of hemagglutinin (HA) epitope-tagged *HCT1* under the control of the *GAL* promoter. At least 3 electrophoretic mobility forms of Hct1HA were apparent in lysates from these cells: a closely spaced doublet at approximately 65 kDa and a heterogeneous series of bands migrating slightly slower than the doublet (Figure 3-3A, lane 2). The diffuse upper band, as well as the top band in the doublet, disappeared upon treatment of Hct1 immunoprecipitates with λ-phosphatase (Figure 3-3A), demonstrating that Hct1 is phosphorylated *in vivo*. The diffuse upper form of Hct1 was most prominent in cells arrested in S phase and mitosis, and was not detectable in cells arrested in G1 (Figure 3-3B). Thus, the presence of this form is inversely correlated with APC activity, suggesting that it may represent an inhibitory modification.

Hct1 phosphorylation increased during cell cycle stages (S and M) when Cdc28 activity is known to be high, and examination of the predicted amino acid sequence of Hct1 revealed the presence of six consensus Cdc28 phosphorylation sites (S/T*-P-X-K/R). We constructed a version of Hct1HA in which these sites (serines 16, 42, 227, 239, and 436 and threonine 176) were mutated to alanine (the Hct1-28A mutant). When this mutant was expressed in asynchronous yeast cells, only the lower two mobility forms of Hct1 were observed (Figure 3-3A). Similarly, in cells arrested in S or M phases, the diffuse upper Hct1 band was absent in the Hct1-28A mutant (Figure 3-3B). We conclude that Hct1 is phosphorylated *in vivo* at Cdc28 consensus sites when Cdc28 activity is high.

To further document a role for Cdc28 in Hct1 phosphorylation, we showed that purified, baculovirus-derived Hct1-6His was efficiently phosphorylated *in vitro* by purified

Cdc28-Clb2 complexes (Figure 3-4A), and that Cdc28 phosphorylation led to a heterogeneous electrophoretic mobility shift similar to the Hct1 mobility shifts observed *in vivo* (Figure 3-4A, B). Quantitation of phosphate incorporation in these experiments indicated that approximately 3-5 phosphates were transferred to the Hct1-6His by Cdc28-Clb2.

We also analyzed phosphorylation of the mutant Hct1-28A protein by Cdc28-Clb2 *in vitro* (Figure 3-4A). Because the Hct1-28A protein was poorly expressed in insect cells and therefore less homogeneous upon purification, phosphorylation of the protein by a contaminating kinase was more pronounced; nevertheless, mutation of the six Cdc28 sites abolished over 80% of the Cdc28-dependent Hct1 phosphorylation at high Cdc28-Clb2 concentrations. Based on this result, as well as our evidence from intact cells above, we conclude that Hct1 is phosphorylated by Cdc28 *in vivo* during S and M phases, and that phosphorylation occurs at several CDK consensus sites in the protein.

Five of the six CDK consensus sites in Hct1 are found in the amino-terminal half of the protein, while one site (Ser 436) is located within the WD40 repeats of the carboxy-terminal half. We therefore constructed another Hct1 mutant in which only the five amino-terminal sites were changed to alanine. Mutation of these sites also abolished the diffuse upper forms of Hct1, suggesting that these five sites are the major sites of Cdc28-dependent phosphorylation *in vivo* (data not shown).

Cdc28-dependent phosphorylation inactivates Hct1

To assess the effects of Cdc28-Clb2-dependent phosphorylation on Hct1 activity, we developed methods that allowed us to treat Hct1-6His with Cdc28-Clb2 and then re-purify it (Figure 3-4B). Phosphorylation of Hct1-6His dramatically inhibited its ability to stimulate the cyclin-ubiquitin ligase activity of the purified APC from *cdc15* cells (Figure 3-4C, center lanes). Treatment of the Hct1-28A mutant with Cdc28-Clb2 kinase had no effect on its ability to stimulate APC activity (Figure 3-4D). We conclude that

phosphorylation by Cdc28-Clb2 severely impairs stimulation of cyclin-ubiquitin ligase activity by Hct1.

To assess the role of Cdc28-dependent phosphorylation in Hct1 function *in vivo*, we analyzed the effects of moderate Hct1-28A expression on cell proliferation and cyclin levels. Expression of Hct1-28A, but not that of wild type Hct1, inhibited cell proliferation (Figure 3-5A) and caused a decrease in Clb2 levels in cells arrested in mitosis with nocodazole (Figure 3-5B). Expression of Hct1-28A was also able to partially suppress the growth defect of the late mitotic mutant *cdc14-1* (Figure 3-5C), which is defective in activation of cyclin-specific ubiquitin ligase activity (Jaspersen et al., 1998). These results are consistent with the possibility that the Cdc28 phosphorylation sites in Hct1 are involved in negative regulation of APC activity.

The phosphatase Cdc14 dephosphorylates and activates Hct1

Our results raise the possibility that activation of cyclin destruction in late mitosis is triggered by dephosphorylation of inhibitory sites on Hct1. The phosphatase Cdc14 is an excellent candidate for the Hct1 phosphatase, as it is required for late mitotic APC activation and cyclin destruction (Jaspersen et al., 1998), and is upregulated during mitosis (Wan et al., 1992). That the growth defect in *cdc14-1* cells is rescued by Hct1-28A expression (Figure 3-5C) also supports the possibility that Hct1 is a key target of Cdc14.

We tested the ability of Cdc14 to dephosphorylate Hct1 by adding purified GST-Cdc14 to preparations of Hct1 that had been radioactively phosphorylated by purified Cdc28-Clb2 (Figure 3-6A). In these experiments, Hct1 was labeled by Cdc28 at approximately one site per molecule, resulting in only a partial shift in mobility on the gel. Under these conditions, small amounts of GST-Cdc14 rapidly catalyzed the removal of 50% of the Cdc28-dependent phosphorylation on Hct1, resulting in the collapse of the labeled band into the high mobility form (Figure 3-6A). We also assessed the Hct1 phosphatase activity of Cdc14 by measuring the radioactive phosphate released from Hct1

into solution. Here again, Cdc14 catalyzed the release of phosphate from Hct1 in a dose-dependent fashion (Figure 3-6B).

Most importantly, treatment of Cdc28-phosphorylated Hct1 with Cdc14 partially restored the ability of Hct1 to activate the APC *in vitro* (Figure 3-6C). There was no effect when we added GST-Cdc14 proteins bearing point mutations in the active site (C283S or C283S/R289A), which are known to abolish phosphatase activity (Taylor et al., 1997). Thus, Cdc14 is able to dephosphorylate key Cdc28-dependent inhibitory sites on Hct1.

Addition of purified GST-Cdc14 alone did not stimulate the inactive APC from *cdc15-2* cells, and pre-incubation of inactive APC with GST-Cdc14 did not affect subsequent activation by purified Hct1 (data not shown). Thus, Cdc14 does not have a direct action on the APC itself in our experiments.

A fraction of phosphorylated Hct1 in these experiments appeared resistant to dephosphorylation by Cdc14 (Figure 3-6A). Similarly, the activity of phosphorylated Hct1 was not fully restored by Cdc14 treatment (Figure 3-6C). It therefore appears that all Cdc28-dependent phosphorylation sites on Hct1 are not equally effective substrates for Cdc14 under these conditions.

To further explore the function of Cdc14, we added GST-Cdc14 to crude lysates prepared from *cdc14*-arrested cells expressing Hct1HA from the *GAL* promoter (Figure 3-7A). Cdc14 treatment abolished the Cdc28-dependent low mobility forms of Hct1, resulting in a collapse of the diffuse Hct1 band to the doublet seen in G1 cells (Figure 3-7A, top). APC activity in the lysate increased (Figure 3-7A, bottom). Addition of large quantities of λ-phosphatase had only slight effects on Hct1 mobility and no effect on APC activity, suggesting that Cdc14 is a more specific Hct1 phosphatase under these conditions. APC activation was also seen when GST-Cdc14 was added to lysates of *cdc14*-arrested cells lacking overexpressed Hct1HA (Figure 3-7B), showing that APC re-activation by Cdc14 in these experiments is not dependent on high levels of exogenous Hct1.

We next confirmed the role of Cdc14 in Hct1 dephosphorylation *in vivo*. Overexpression of *CDC14* in mitotically-arrested cells abolished Hct1 phosphorylation and triggered APC activation and Clb2 destruction (Figure 3-7C). Similar results were obtained in cells lacking the CDK inhibitor Sic1 (Figure 3-7C), whose levels are known to increase in cells overexpressing *CDC14* (Visintin et al., 1998). Thus, Cdc14 does not trigger Hct1 dephosphorylation indirectly by decreasing Cdc28 activity.

Discussion

We conclude that inhibitory phosphorylation of Hct1 is an important mechanism of APC regulation. Several lines of evidence suggest that Cdc28-cyclin complexes are responsible for catalyzing this phosphorylation. First, Hct1 undergoes phosphate-dependent mobility shifts during cell cycle stages when Cdc28 is known to be active, and these shifts are abolished when Cdc28 consensus phosphorylation sites in Hct1 are changed to alanine. Second, purified Cdc28-Clb2 complexes catalyze phosphorylation at a large subset of these sites *in vitro*. Third, Cdc28-dependent phosphorylation inhibits Hct1 function *in vitro*, explaining previous observations that the activities of Cdc28 and APC are inversely correlated during the cell cycle (Amon et al., 1994), that inhibition of Cdc28 activity is sufficient to allow nocodazole-arrested cells to exit mitosis, and that artificial induction of Cdc28-Clb2 kinase activity in G1 cells leads to Clb2 stabilization (Amon, 1997).

While this manuscript was in preparation, Zachariae et al. published evidence also suggesting that Hct1 phosphorylation by Cdc28 inhibits APC activity *in vivo* (Zachariae et al., 1998a). They also observed phosphate-dependent Hct1 mobility shifts during cell cycle stages when Cdc28 activity is high, and found that Hct1 phosphorylation was inversely correlated with cyclin destruction *in vivo*. Overexpression of an Hct1 mutant lacking CDK consensus sites blocked cyclin accumulation *in vivo*. The mutant Hct1 protein associated

with the APC *in vivo* at cell cycle stages when Cdc28 activity is high, suggesting that Cdc28-dependent Hct1 phosphorylation blocks APC activation by inhibiting the Hct1-APC interaction.

We found that Cdc28-dependent Hct1 phosphorylation was prevented by mutation of five residues in Hct1, while Zachariae et al. observed a complete loss of phosphorylation only when nine sites were mutated (Zachariae et al., 1998a). This apparent discrepancy is readily explained. Whereas Zachariae et al. mutated all serines or threonines followed by a proline, we focused on the subset of sites that also have a basic residue two positions after the proline (as is often the case in CDK substrates). Zachariae et al. observed a reduction in Hct1 phosphorylation only when their mutations included one or more of the basic sites we mutated.

Inhibitory Hct1 phosphorylation thus provides a mechanism by which Cdc28 suppresses cyclin-specific APC activity during S phase and early mitosis. However, it seems unlikely that this is the only mechanism governing APC activity. Additional regulatory modifications may govern Hct1 function, since some Hct1 phosphorylation is observed in the Hct1-28A mutant and in G1 cells (Figure 3-3). In addition, cyclin destruction is probably controlled in part by modification of the APC core itself, as suggested by our observation (Figure 3-2C, D) that the APC from *cdc14*- and G1-arrested cells is more responsive to Hct1 than the APC from other late mitotic mutants. These results are reminiscent of recent evidence in vertebrates that the affinity of the APC for Cdc20 varies in the cell cycle (Fang et al., 1998b), and are also consistent with previous evidence that APC activity in vertebrates is regulated by phosphorylation of core APC subunits (Felix et al., 1990; Lahav-Baratz et al., 1995; Sudakin et al., 1995; Peters et al., 1996; Kotani et al., 1998; Patra and Dunphy, 1998).

Based on these considerations, we speculate that some modification of the APC in late mitosis results in enhanced affinity for Hct1 (Figure 3-8). Cdc14 is not required for this modification, since the APC from *cdc14* cells, unlike that from *cdc5* or *cdc15* cells,

displays the increased Hct1 sensitivity that is seen in G1 cells. In addition, the APC from *cdc14*-arrested cells has low but reproducibly higher activity than the completely inactive APC from *cdc5* and *cdc15* cells (Jaspersen et al., 1998). Finally, expression of the Hct1-28A mutant is able to partially suppress the late mitotic arrest of the *cdc14-1* mutant (Figure 3-5C), but has no effect on the growth defects in *cdc15-2* and *cdc5-1* cells (data not shown). These lines of evidence all point to the possibility that the major restraint on APC activity in *cdc14*-arrested cells is the presence of inhibitory Hct1 phosphorylation, whereas additional APC defects prevent cyclin destruction in the *cdc5* and *cdc15* mutants (Figure 3-8).

The ability of Cdc28 and the APC to antagonize each other's activity leads to the potential for a regulatory loop that could enhance the abrupt, all-or-none kinetics of Cdc28 inactivation in late mitosis. The switch-like features of Cdc28 inactivation may also be enhanced by a similar antagonistic relationship between Cdc28 and its inhibitor Sic1, whose synthesis and stability are inhibited by Cdc28 activity (Moll et al., 1991; Toyn et al., 1996; Verma et al., 1997) (Figure 3-8). A key issue remains unresolved, however: what initiating event is responsible for reducing Cdc28 activity (or increasing APC or Sic1 activity) to some threshold where these regulatory relationships bring on rapid and complete Cdc28 inactivation? Our studies suggest that Cdc14 could help initiate this process by catalyzing Hct1 dephosphorylation. In addition, Visintin et al. recently found that Cdc14 dephosphorylates Sic1 (leading to its stabilization) and the transcription factor Swi5, leading to an increase in *SIC1* expression (Visintin et al., 1998). We suspect that additional mechanisms, probably involving Cdc5 and Cdc15, also contribute to the late mitotic decline in Cdc28 activity.

Materials and Methods

Yeast strains and protein methods

All strains were derivatives of W303. To construct strains for APC purification, *CDC23HA* from pRS239 (gift from P. Hieter) was integrated at the *CDC23* locus of LH131 (Matα *cdc23-1*; a gift of L. Hwang) by one-step gene replacement. A 6His-tag was inserted immediately before the stop codon of *CDC16*, and this construct was used to replace endogenous *CDC16* of SLJ128 (*MAT***a** *bar1 pep4Δ::URA3*) with *CDC16-6His.* These strains were crossed and sporulated to produce SLJ290 (*MAT***a** *bar1 CDC23::CDC23HA CDC16::CDC16-6His-LEU2 pep4Δ::URA3*). Additional strains used for APC purification were derived from crosses to SLJ290. Wild-type and mutant *HCT1* genes were cloned into a pRS304-based plasmid containing the *GAL1/10* promoter and a single C-terminal HA tag (Jaspersen et al., 1998); these constructs were integrated at the *TRP1* locus of a wild type, an *hct1Δ::LEU2* (gift of W. Seufert), a *cdc14-1*, or a *sic1Δ::LEU2* (gift of A. Rudner) strain. For Cdc14 overproduction experiments, the *CDC14* gene was cloned into pDK20 (Tjandra et al., 1998) under control of the *GAL1/10* promoter; this construct was integrated at the *URA3* locus of wild type and *sic1Δ* strains containing *GAL-HCT1HA* integrated at the *TRP1* locus.

Yeast lysate preparation, immunoblotting, immunoprecipitation, and phosphatase treatment were as described (Espinoza et al., 1998; Jaspersen et al., 1998). Cyclin-ubiquitin ligase activity of the APC from yeast extracts was measured as described (Charles et al., 1998). Ubiquitination activity was quantitated on a PhosphorImager using the ImageQuant program (Molecular Dynamics).

APC Purification

A pellet from 5 x 10^{10} log phase cells was resuspended in 50 ml APC lysis buffer (50 mM Hepes-NaOH pH 7.4, 75 mM KCl, 50 mM NaF, 1 mM $MgCl_2$, 1 mM EGTA, 0.1% NP-40, 10% glycerol, 2 mM phenylmethylsulfonylfluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 4 μg/ml pepstatin) and cells were lysed by bead beating in a Mega Beater (Biospec) at 4°C. Lysates were clarified by centrifugation for 10 min at 9,000 x g at 4°C followed by ultracentrifugation (1 h, 80,000 x g, 4°C). Lysate was loaded onto a 5 ml HiTrap Chelating column (Pharmacia) charged with cobalt and equilibrated in APC-Buffer A (50 mM Hepes-NaOH pH 7.4, 200 mM KCl, 50 mM NaF, 0.1% NP-40, 10% glycerol). Following a wash with APC-Buffer A containing 10 mM imidazole, bound proteins were then eluted with a linear gradient (10-200 mM) of imidazole in APC-Buffer A. Fractions containing the APC were pooled, diluted to 50 mM KCl in APC-Buffer B (20 mM Tris-HCl, pH 7.6, 1 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol [DTT]), and loaded onto a 1 ml HiTrap SP column equilibrated in APC-Buffer B containing 50 mM KCl. Proteins were eluted with a 50-350 mM KCl gradient in APC-Buffer B. The APC fractions were pooled, diluted to 100 mM KCl with APC-Buffer C (APC-Buffer B with 0.1% Tween-20), and loaded onto a 1 ml HiTrap Q column. Following a wash in APC-Buffer C plus 250 mM KCl, the APC was eluted from the column with a 250-750 mM KCl gradient in APC-Buffer C. Fractions containing the APC were pooled, diluted with an equal volume of APC-Buffer A, and repurified on a 1 ml HiTrap Chelating column charged with cobalt. APC containing fractions were pooled and insulin (Sigma) was added to 0.1 mg/ml. The concentration of the purified APC was approximated based on the silver staining intensity of the Cdc16 band.

Hct1 purification

HCT1 was amplified from genomic DNA by PCR and cloned into pFB-6His at the NcoI site to create pFB-Hct1H6. The Cdc28 phosphorylation site mutant, pFB-Hct1H6-

28A, was generated by oligonucleotide-mediated mutagenesis of pFB-Hct1H6 (Kunkel, 1985) to change serines 16, 42, 227, 239, and 436 and threonine 176 to alanines. Baculoviruses encoding wild-type or mutant Hct1-6His were generated using the Bac-to-Bac expression system (Gibco BRL). Wild-type and mutant Hct1-6His were purified from baculovirus-infected Sf9 cells by metal affinity chromatography as described (Fisher et al., 1995). Tween-20 (0.1%) and insulin (0.1 mg/ml) were added prior to storage.

Cdc28-Clb2 purification

To produce an active Cdc28-Clb2 kinase, lysate was prepared from insect cells co-infected with baculoviruses encoding Cdc28HA and Cak1HA3 (a gift of A. Farrell), and combined with a lysate of bacteria expressing a Clb2MBP fusion protein (a gift of R. Deshaies). The Cdc28-Clb2MBP complex was then purified on an amylose column (NEB), followed by cation exchange chromatography (Pharmacia SP Sepharose Fast Flow). Approximately equimolar amounts of Cdc28 and Clb2 were present in the purified complex.

Cdc14 purification

CDC14 was amplified from genomic DNA by PCR and cloned into the BamHI site of pGEX-3X (Pharmacia). Bacteria transformed with this construct were grown to an OD_{600} of 0.6, and expression of GST-Cdc14 was induced with IPTG (0.1 mM) for 16 hours at 23°C. The recombinant protein was purified on a glutathione-sepharose 4B column (Pharmacia), followed by anion exchange chromatography (Pharmacia HiTrap Q). Point mutants in the Cdc14 active site were generated by subcloning *CDC14* into pBSSKII+ (Stratagene) for oligonucleotide-directed mutagenesis.

Phosphorylation of Hct1 by Cdc28-Clb2

Purified Cdc28-Clb2MBP was incubated for 20 min at 23°C in a 20 µl reaction mixture containing 100 µM ATP, 150 ng Hct1-6His, and 2.5 µCi [γ-^{32}P]ATP (3000 mCi/mmol) in kinase buffer (50 mM Hepes-NaOH pH 7.4, 10 mM $MgCl_2$, and 1 mM DTT). Reaction products were analyzed on 8% SDS-PAGE gels followed by autoradiography. To determine the effect of Cdc28-Clb2 phosphorylation on Hct-6His, the Cdc28-Clb2 complex was immobilized on protein A-sepharose beads (Sigma) by immunoprecipitation with α-Clb2 (Gerber et al., 1995) and α-MBP (NEB) polyclonal antibodies. Immobilized Cdc28-Clb2 complexes were used to phosphorylate Hct1-6His in reactions containing 1 mM ATP. Following a 30 min incubation at 25°C, Hct1-6His was separated from the Cdc28-Clb2 by removal of the beads.

Hct1 phosphate release assay

2.7 µg Hct1-6His was radiolabeled by 20 min incubation at 25°C with 1.1 µg Cdc28-Clb2 and 100 mCi ^{32}P-γ-ATP (3000 Ci/mmol) in a 560 µl reaction. Free ATP was removed by gel filtration on a 4 ml Sephadex G25 column equilibrated in HBS (25 mM Hepes-NaOH pH 7.4, 150 mM NaCl, 1 mM DTT), followed by addition of glycerol (10%) and insulin (0.1 mg/ml). Aliquots of labeled Hct1 (90 ng; 200,000 cpm) were incubated 5 min at 25°C in 20 µl reactions with GST or GST-Cdc14, followed by addition of 10 µl BSA (10 mg/ml) and 180 µl ice-cold 20% Trichloroacetic acid. Following incubation on ice for 30 min, the mixture was centrifuged (16,000xg, 10 min, 4°C), and 100 µl of the supernatant was removed for quantitation by scintillation counting.

Acknowledgements

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6His baculovirus, Sue Biggins for purified GST, and Andrew Murray, David Toczyski, Rachel Tinker-Kulberg, Sue Biggins, Jamison Nourse, Catherine Takizawa, Jeff Ubersax, Adam Rudner, Alex Szidon, and Hironori Funabiki for valuable discussions and comments on the manuscript. This work was supported by funding from the National Institute of General Medical Sciences (to D.O.M.), a University of California Dean's Fellowship (to J.F.C.), and a Howard Hughes Medical Institute Predoctoral Fellowship (to S.L.J.).

Figure 3-1. Purification of APC from G1 and anaphase cells.

The APC was purified from cells arrested in anaphase using a *cdc15-2* mutation or in G1 using a *cdc28-13* mutation.

(A) Peak fractions from the final purification step were pooled and analyzed by electrophoresis on a 8.5% polyacrylamide gel, followed by silver staining. In multiple preparations, we observed twelve subunits similar in mobility to those seen in previous reports (Zachariae et al., 1996; Zachariae et al., 1998b). Nine subunits are marked at left; the three smallest subunits are apparent only on higher percentage gels. Proteins migrating at about 50 kDa (asterisk) are contaminants that do not co-purify with the APC.

(B) Activities of purified anaphase and G1 APCs were measured by immunoprecipitating equivalent amounts of APC with 12CA5 (which recognizes the HA-tagged Cdc23 subunit in these preparations) and measuring ubiquitination of a ^{125}I-labeled cyclin fragment. The asterisk indicates a non-specific background band.

Figure 3-1

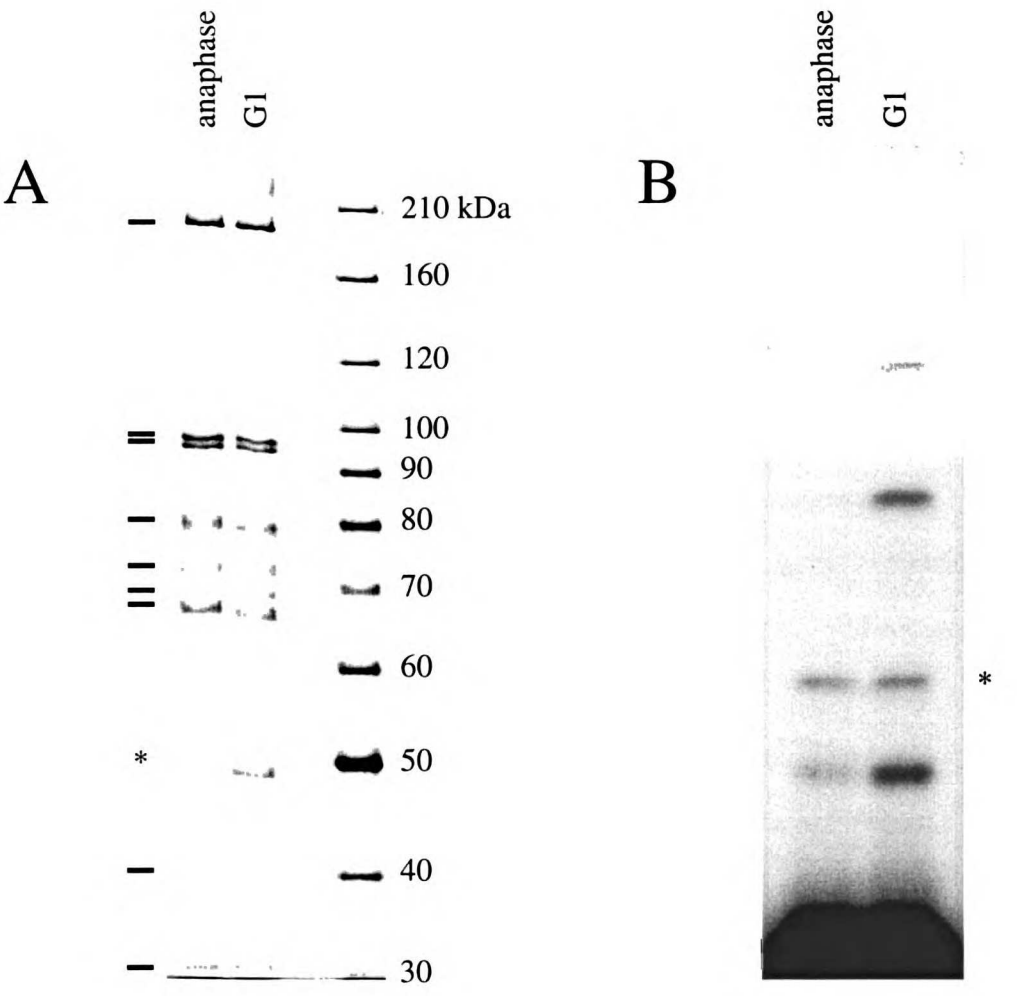

A
anaphase
G1
210 kDa
160
120
100
90
80
70
60
50
40
30
*
B
anaphase
G1
*

Figure 3-2. Activation of APC by recombinant Hct1.

(**A**) Increasing amounts of purified Hct1-6His were added to approximately 6 nM purified APC from *cdc15-2* cells, and cyclin-ubiquitin ligase activity was measured. The asterisk indicates a non-specific background band.

(B) Cells were arrested at various cell cycle stages with the indicated temperature-sensitive mutations or by treatment with α-factor (αf), hydroxyurea (HU), or nocodazole (Noc). The *hct1Δ* strain was grown asynchronously at 23°C. APC was immunoprecipitated from 250 μg yeast lysate with anti-Cdc26 antibodies (a gift of L. Hwang), and incubated either with 15 nM Hct1-6His (+) or buffer (-), followed by measurement of cyclin-ubiquitin ligase activity. Control experiments were performed without added yeast lysate (-APC).

(C) APC was immunoprecipitated from lysates (70 μg) of wild type cells treated with α-factor or nocodazole, or from *cdc15-2* cells arrested at 37°C. Recombinant Hct1-6His was added in increasing amounts, and APC activity was measured. In the top panel, the average APC activity in four independent experiments is expressed as a percentage of maximum activity for that APC. In the bottom panel, the data has been linearized with a double-reciprocal plot. Standard errors are indicated, although in most cases they were smaller than the diameters of the symbols. We estimate that APC concentrations in all four experiments were in the nanomolar range.

(D) APC was immunoprecipitated from lysates (70 μg) of *cdc5-1*, *cdc15-2*, and *cdc14-1* cells arrested at 37°C. Following incubation with increasing amounts of Hct1, APC activity in a representative experiment was analyzed as in (c). Similar results were obtained in three independent experiments.

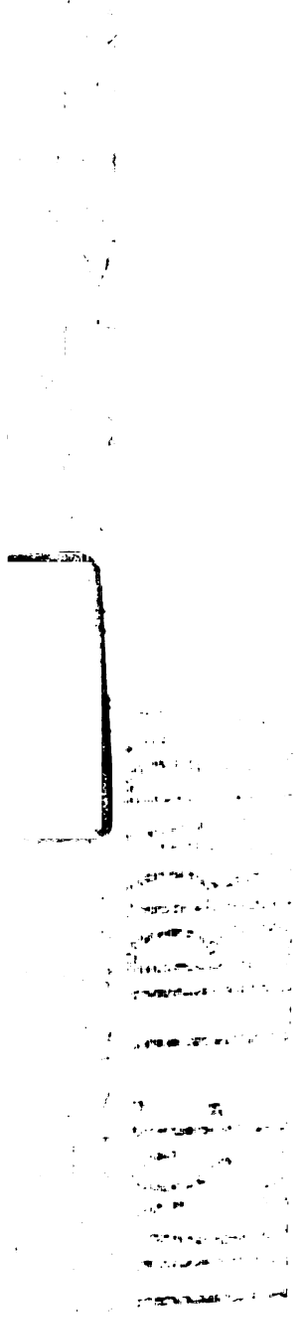
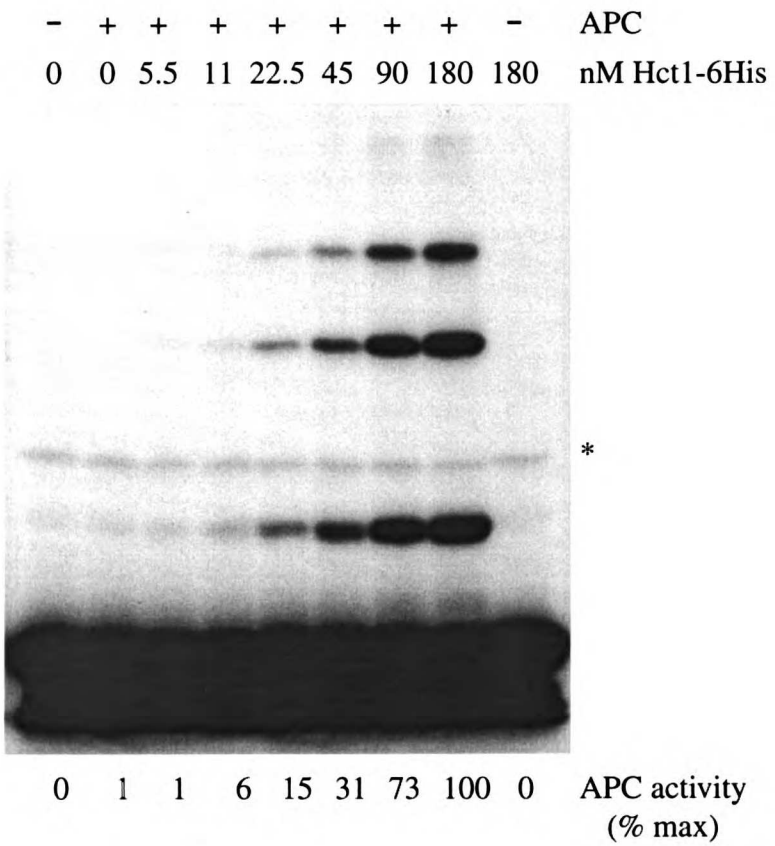
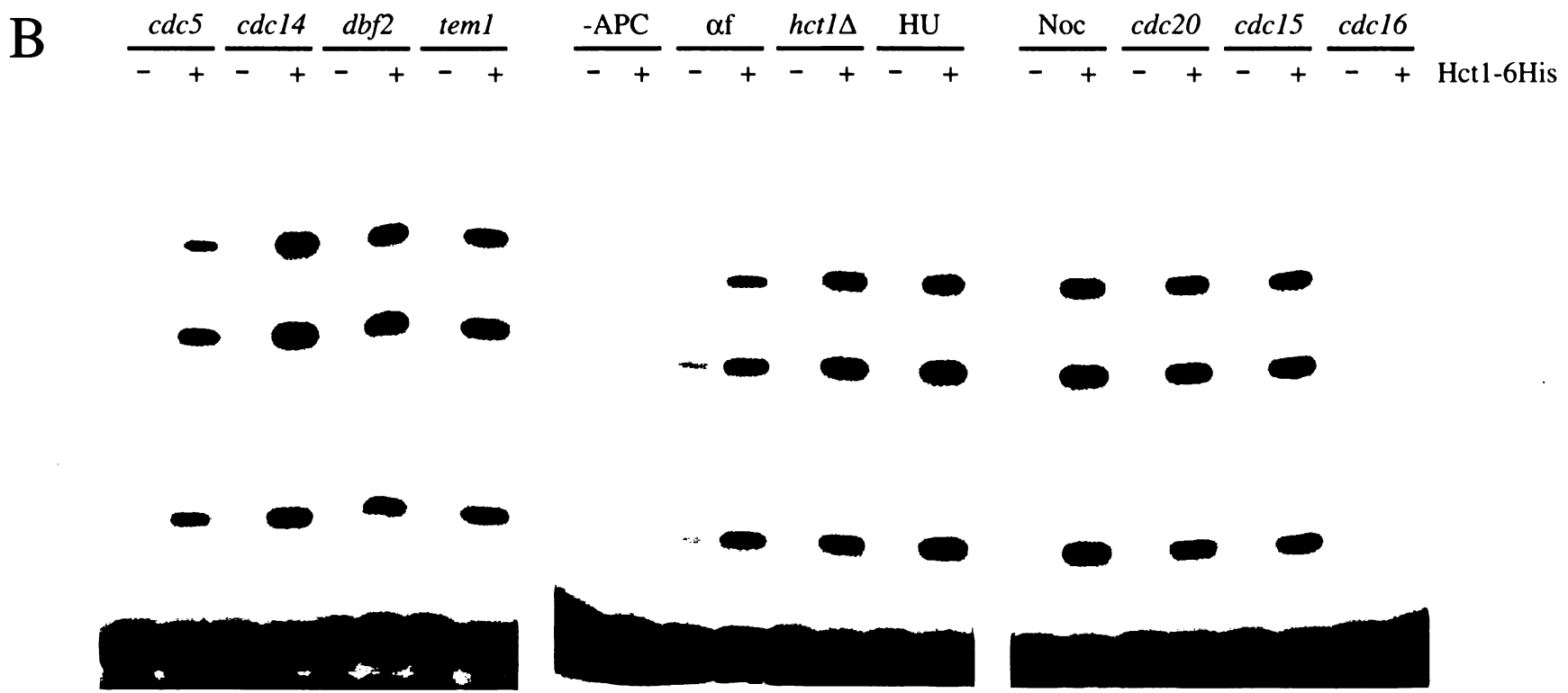


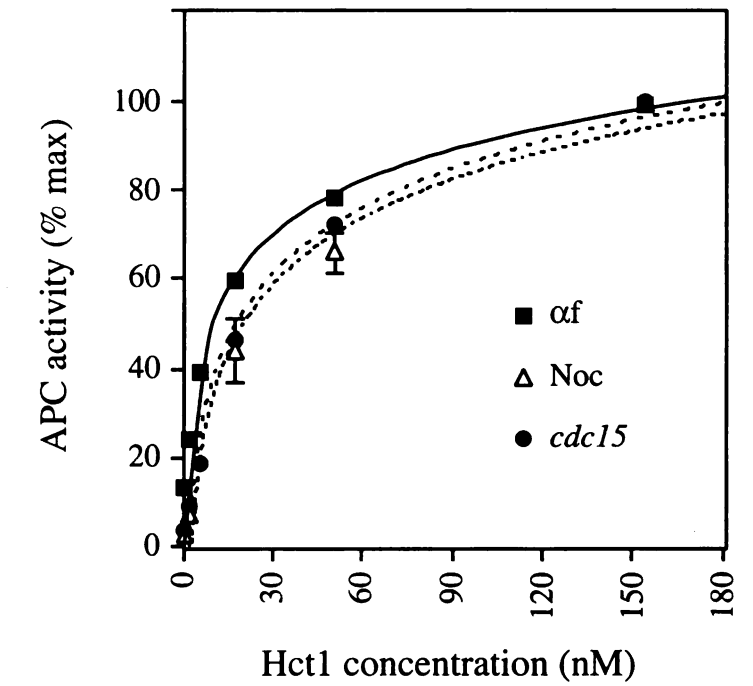
Figure 3-2

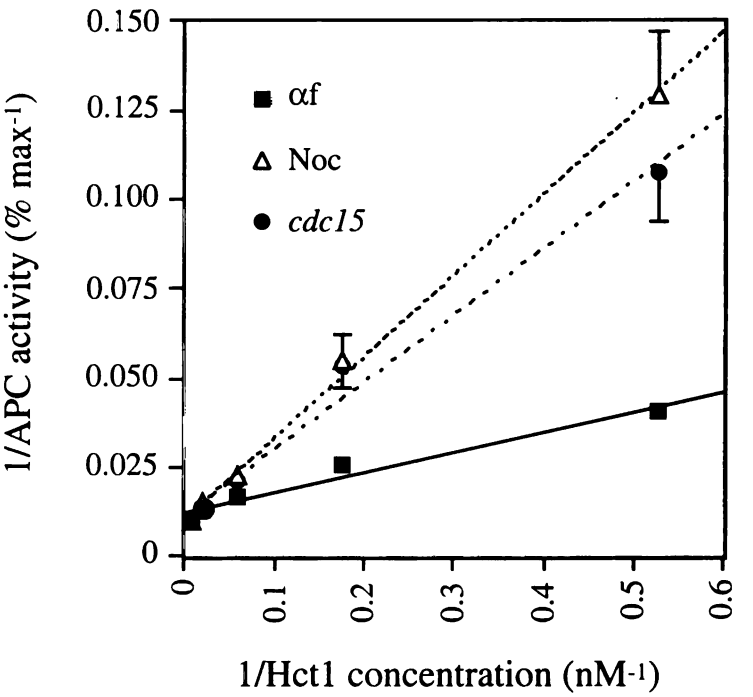
A


- + + + + + + + - APC
0 0 5.5 11 22.5 45 90 180 180 nM Hct1-6His
*
0 1 1 6 15 31 73 100 0 APC activity (% max)

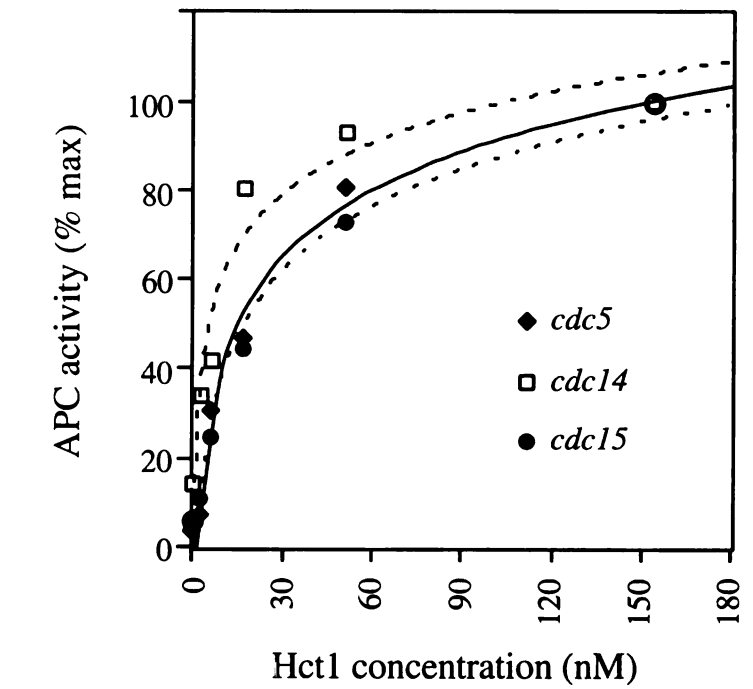

B
cdc5
cdc14
dbf2
tem1
-APC
αf
hct1Δ
HU
Noc
cdc20
cdc15
cdc16
- + - + - + - + - + - + - + - + - + - + - + - + - +
Hct1-6His

C


APC activity (% max)
100
80
60
40
20
0
0
30
60
90
120
150
180
Hct1 concentration (nM)
αf
Noc
cdc15


1/APC activity (% max-1)
0.150
0.125
0.100
0.075
0.050
0.025
0
0
0.1
0.2
0.3
0.4
0.5
0.6
1/Hct1 concentration (nM-1)
αf
Noc
cdc15

D


APC activity (% max)
100
80
60
40
20
0
0
30
60
90
120
150
180
Hct1 concentration (nM)
cdc5
cdc14
cdc15

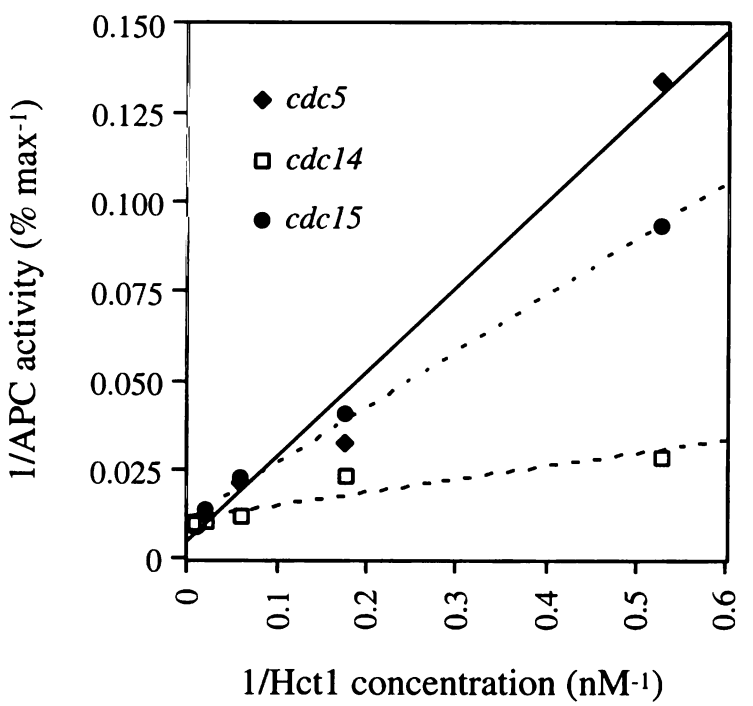

1/APC activity (% max-1)
0.150
0.125
0.100
0.075
0.050
0.025
0
0
0.1
0.2
0.3
0.4
0.5
0.6
1/Hct1 concentration (nM-1)
cdc5
cdc14
cdc15

Figure 3-3. Hct1 phosphorylation *in vivo* at Cdc28 consensus sites.

(A) Wild type strains containing *GAL-HCT1-HA* or *GAL-HCT1-28A-HA* (a mutant in which the six Cdc28 consensus sites are changed to alanine) were grown in galactose for 2.5 h. Wild-type or mutant Hct1HA proteins were immunoprecipitated from cell lysates (500 μg) and treated with phosphatase buffer plus phosphatase inhibitors (lanes 2,5), 100 U λ-phosphatase (lanes 3, 6), or both λ-phosphatase and phosphatase inhibitors (lanes 4,7). Lane 1 is an immunoprecipitate from a lysate of cells lacking the tagged Hct1. Immunoprecipitates were immunoblotted with the anti-HA antibody 16B12.
(B) The same strains as in (a) were arrested in α-factor (αf), hydroxyurea (HU), or nocodazole (Noc), or left untreated as asynchronous cultures (A). Galactose was then added to 2%, except in the uninduced control (U). Anti-HA immunoprecipitates of cell lysates were then probed by immunoblotting with anti-HA antibody 16B12. Partial Hct1 degradation in this experiment resulted in the appearance of anti-HA-reactive bands below the main Hct1 bands.

Figure 3-3

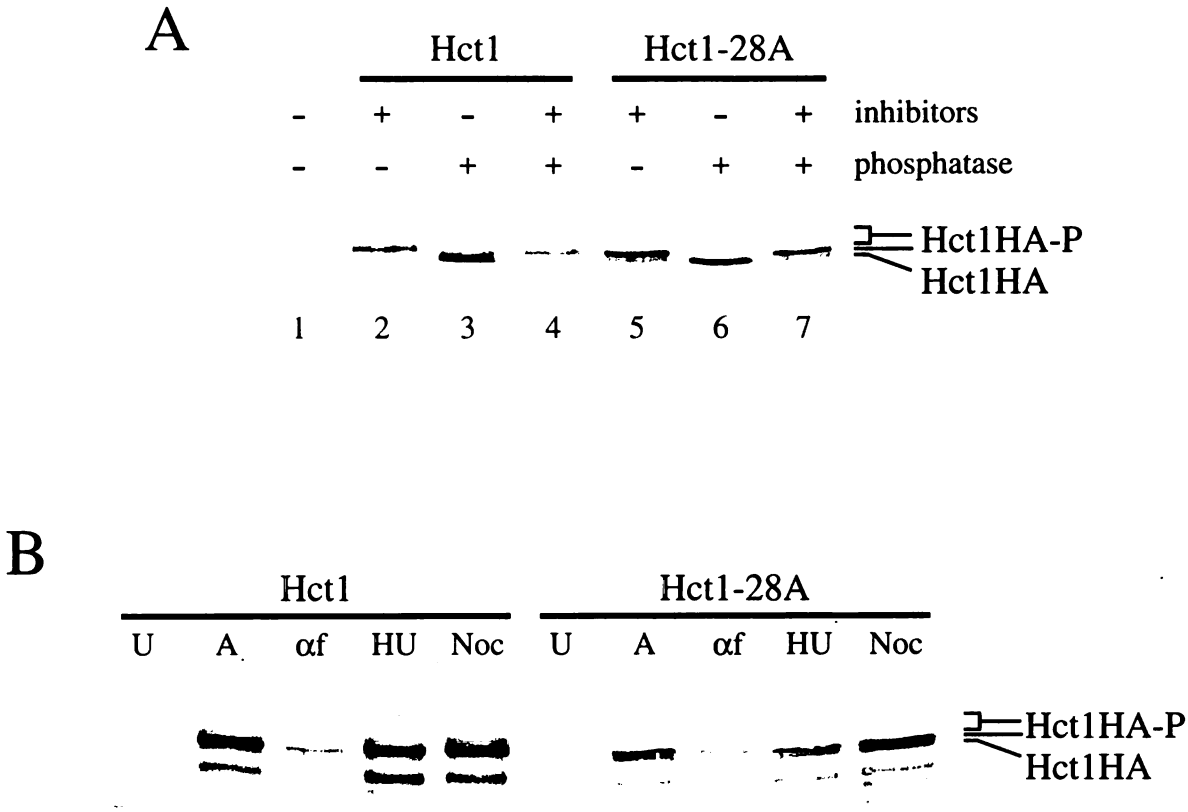

A
Hct1
Hct1-28A
- + - + + - +
inhibitors
- - + + - + +
phosphatase
Hct1HA-P
Hct1HA
1 2 3 4 5 6 7
B
Hct1
Hct1-28A
U A αf HU Noc U A αf HU Noc
Hct1HA-P
Hct1HA

Figure 3-4. Phosphorylation of Hct1 by Cdc28-Clb2 abolishes its ability to activate the APC.

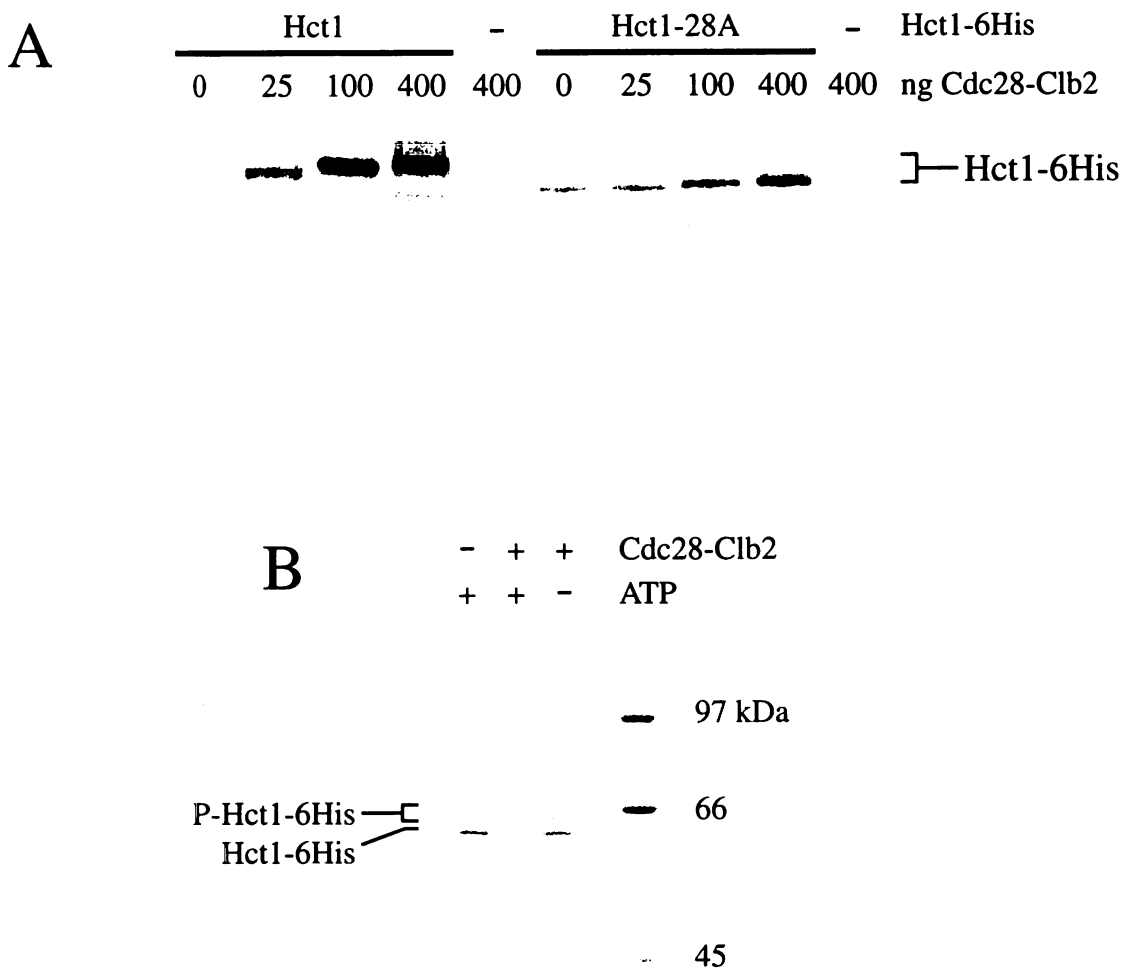
(A) Increasing amounts of Cdc28-Clb2 were tested for their ability to phosphorylate 150 ng purified Hct1-6His or the Hct1-28A-6His mutant. Quantitation of phosphate incorporation in these experiments indicated that approximately 4.4 mol phosphate was incorporated per mol wild type Hct1-6His at the highest Cdc28-Clb2 concentrations; in other experiments, phosphate incorporation varied from 3 to 5 mol/mol.

(B) 3 μg purified Hct1-6His was incubated with ATP and 10 μg Cdc28-Clb2 complex immobilized by immunoprecipitation on beads (center lane). To control for contaminating kinases, Hct-6His was incubated in a reaction with ATP and beads alone (left lane); to verify that the effects of Cdc28-Clb2 on Hct1-6His were dependent on Cdc28-Clb2 kinase activity, ATP was omitted from the kinase reaction (right lane). Following removal of the Cdc28-Clb2, 25% of the reaction was analyzed by gel electrophoresis and Coomassie Blue staining..

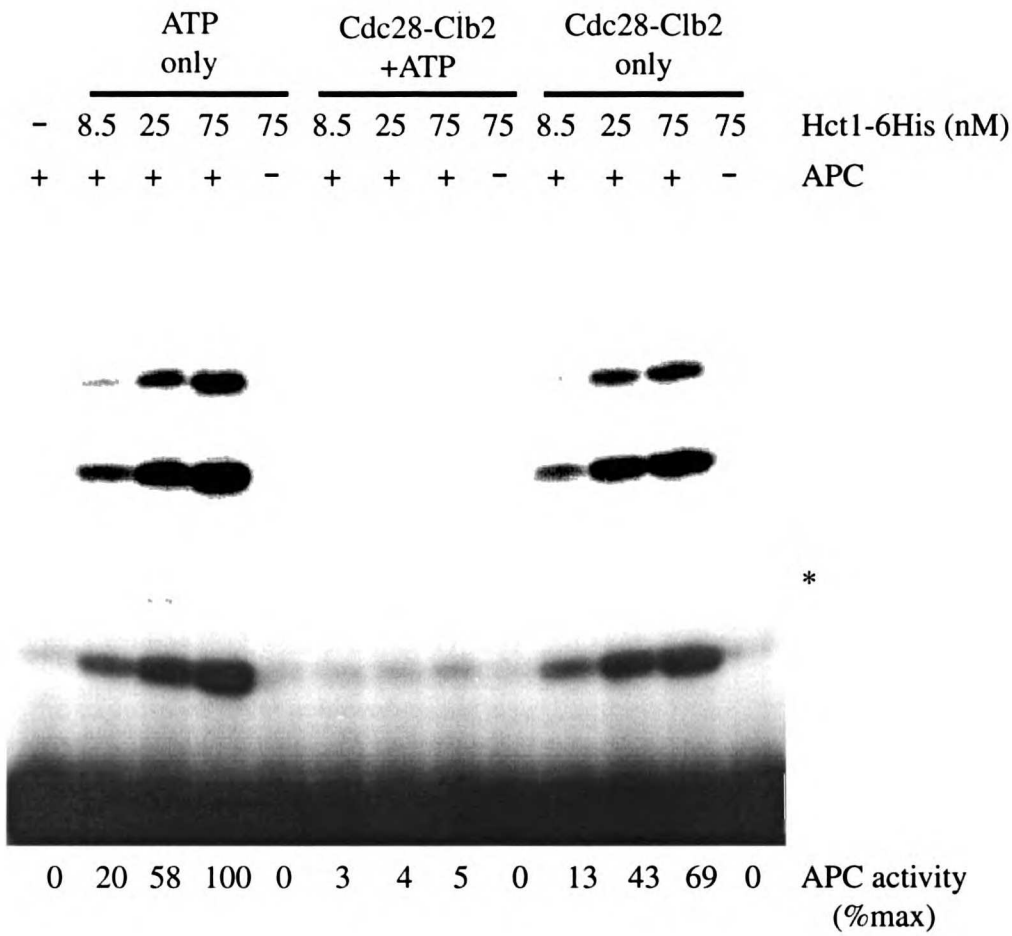
(C) The three Hct1-6His preparations shown in panel (b) were incubated in solution with 6 nM APC purified from *cdc15-2* arrested cells, and cyclin-ubiquitin ligase activity in the reaction was measured.

(D) Wild-type Hct1-6His and mutant Hct1-28A-6His (3 μg) were treated with Cdc28-Clb2 (10 μg), and activation of a purified *cdc15-2* APC was tested as in panel (c). Because the mutant Hct1-6His purified from insect cells appears unstable and is not as effective in APC activation as the wild type protein, wild-type Hct1-6His was diluted 1:30 after treatment with kinase and prior to incubation with the APC. Average APC activity in four independent experiments is shown as percent of maximal activity achieved with untreated Hct1-6His.

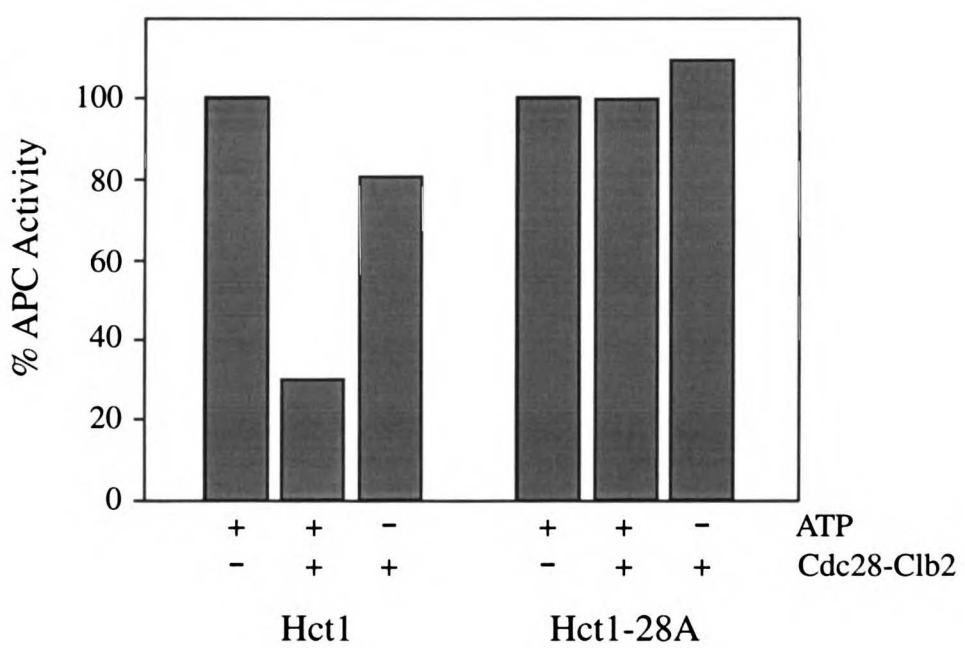
Figure 3-4


A
Hct1
-
Hct1-28A
-
Hct1-6His
0 25 100 400 400 0 25 100 400 400 ng Cdc28-Clb2
Hct1-6His
B
- + + Cdc28-Clb2
+ + - ATP
97 kDa
66
45
P-Hct1-6His
Hct1-6His

C


ATP only
Cdc28-Clb2 +ATP
Cdc28-Clb2 only
- 8.5 25 75 75 8.5 25 75 75 8.5 25 75 75 Hct1-6His (nM)
+ + + + - + + + - + + + - APC
*
0 20 58 100 0 3 4 5 0 13 43 69 0 APC activity (%max)

D


% APC Activity
100
80
60
40
20
0
+ + - + + - ATP
- + + - + + Cdc28-Clb2
Hct1
Hct1-28A

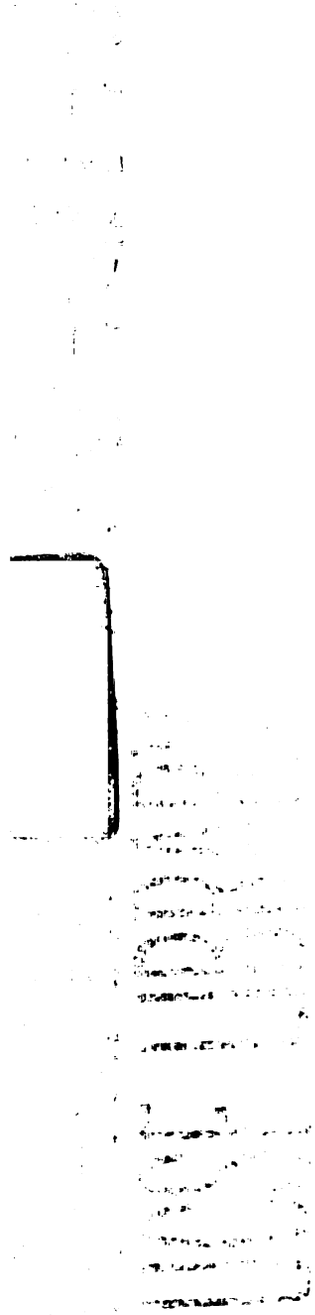


Figure 3-5. Effects of Hct1-28A *in vivo*.

(A) Log-phase *hct1Δ* strains containing vector, *GAL-HCT1HA* or the mutant *GAL-HCT1-28A-HA* were serially diluted 2-fold and spotted onto YPD and YP/galactose-raffinose plates. Plates were incubated for 2.5 days at 23°C.

(B) The same strains used in panel (a) were grown to mid-log phase, arrested with hydroxyurea, washed and treated 2 h with nocodazole. Galactose was added, and samples were harvested at the indicated times for immunoblotting with the indicated antibodies. In all samples, greater than 90% of cells remained large-budded throughout the experiment. Note that wild type *HCT1* expression had little effect in these experiments, in contrast to results in previous studies (Schwab et al., 1997; Visintin et al., 1997). This may be due to differences in the expression level achieved in our work, and may also indicate that the single carboxy-terminal HA tag on Hct1 reduces Hct1 function.

(C) Log-phase *cdc14-1* strains containing vector, *GAL-HCT1HA* or the mutant *GAL-HCT1-28A-HA* were serially diluted 2-fold and spotted onto YP/galactose-raffinose plates. Plates were incubated for 3 days at 23° or 4 days at 37°C. No growth was observed on control plates containing dextrose at 37°C (data not shown).

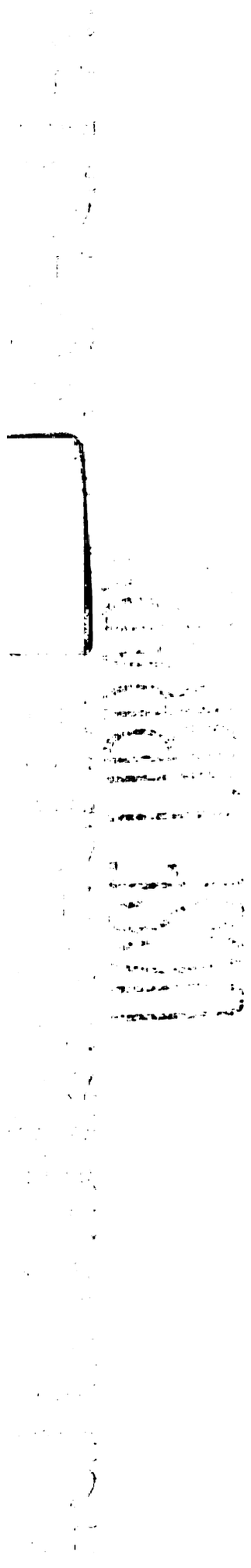


Figure 3-5

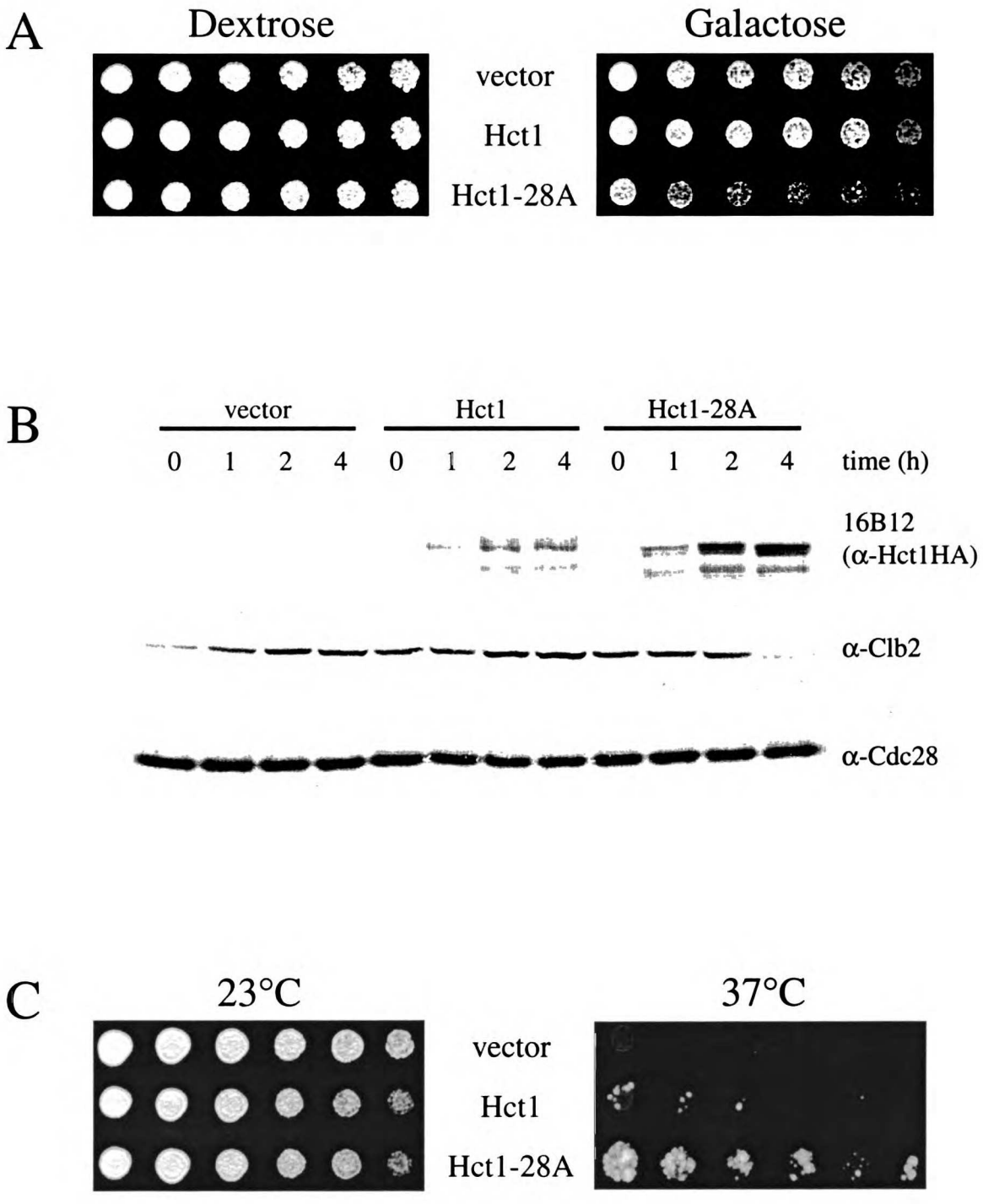

A
Dextrose
Galactose
vector
Hct1
Hct1-28A
B
vector
Hct1
Hct1-28A
0 1 2 4 0 1 2 4 0 1 2 4
time (h)
16B12
(α-Hct1HA)
α-Clb2
α-Cdc28
C
23°C
37°C
vector
Hct1
Hct1-28A

Figure 3-6. Cdc14 promotes Hct1 dephosphorylation and APC activation.

(A) Hct1-6His (50 ng) was incubated with ^{32}P-γ-ATP and 20 ng Cdc28-Clb2 complexes immobilized on beads, or incubated with ATP in the presence of beads alone. The soluble Hct1 was incubated 30 min at 30°C with purified GST or with the indicated amounts of purified GST-Cdc14. Cdc28-dependent phosphate remaining on Hct1 is indicated below the figure.

(B) ^{32}P-phospholabeled Hct1-6His (final concentration 3 nM) was incubated for 5 min at 25°C with the indicated amounts of GST or GST-Cdc14. Proteins in the reaction were precipitated with acid, and radioactivity in the resulting supernatant was measured. Background phosphate release (in presence of GST alone) has been subtracted. Because phosphorylated Hct1 is not readily prepared in large quantities, we could not use saturating substrate concentrations, and the rate of phosphate release in these experiments (0.1-0.3 nmol mg^{-1} min^{-1}) is probably far below maximum.

(C) Hct1-6His (100 ng) was incubated with 20 ng Cdc28-Clb2, either in the presence (+) or absence (-) of 1 mM ATP. We then added the indicated amounts of GST (lane G), GST-Cdc14 (lanes marked 14), or phosphatase-deficient mutant forms of GST-Cdc14 (C283S, lane C; C283S/R289A, lane C/R). After 30 min incubation at 30°C, treated Hct1 was tested for its ability to activate the cyclin-ubiquitin ligase activity of the APC in anti-Cdc26 immunoprecipitates from 400 μg lysates of *cdc15-2* cells arrested at 37°C.

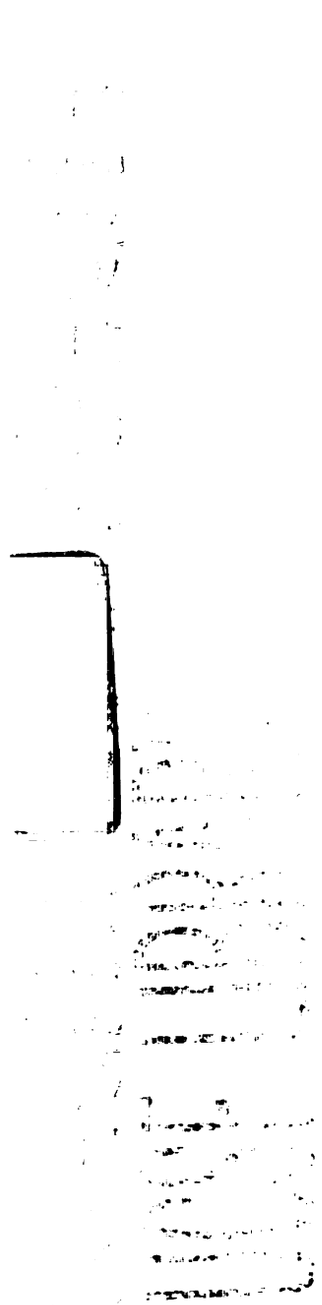
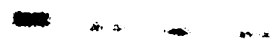


Figure 3-6

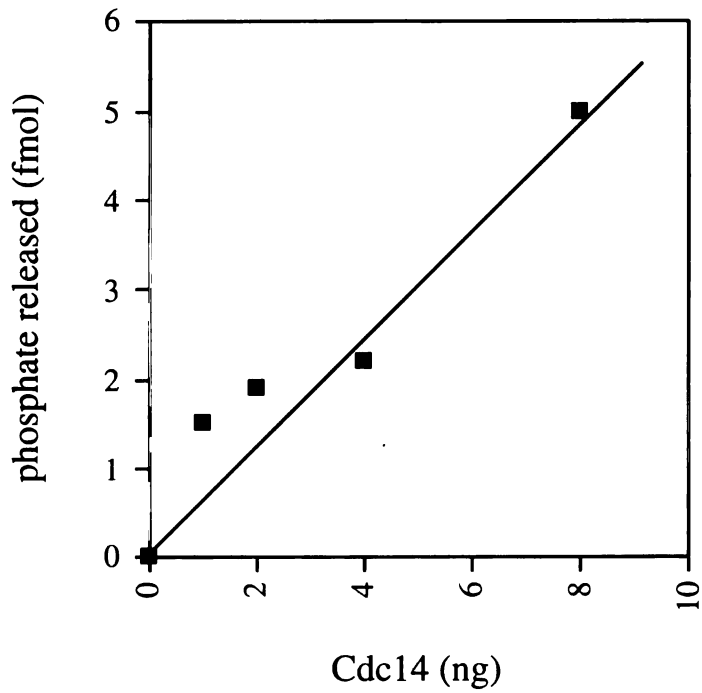
A

-	-	+	+	+	+	Cdc28-Clb2
-	80	-	10	20	40	ng GST-Cdc14
80	-	80	-	-	-	ng GST



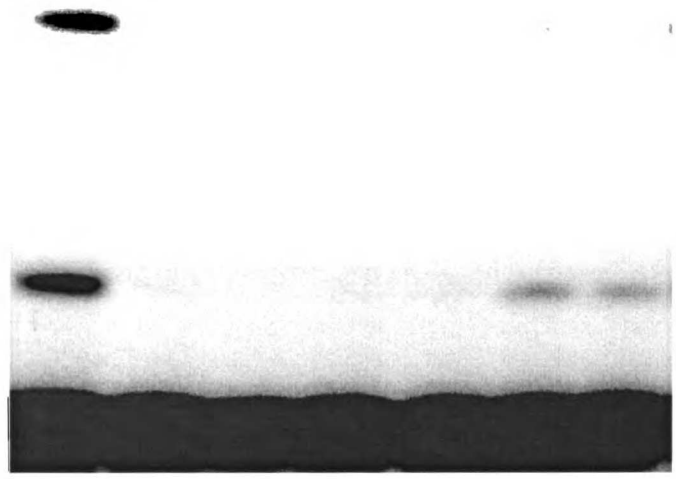
100	72	57	50	% Hct1-P

B



C

-	+	+	+	+	+	+	ATP
-	-	C	C/R	G	14	14	
		80	80	80	40	80	ng protein



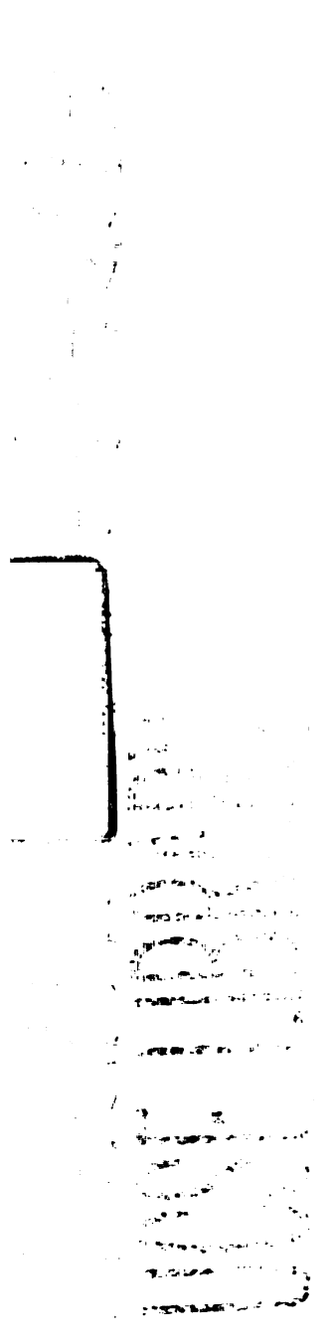


Figure 3-7. Cdc14 promotes APC activation in crude cell extracts and *in vivo*.

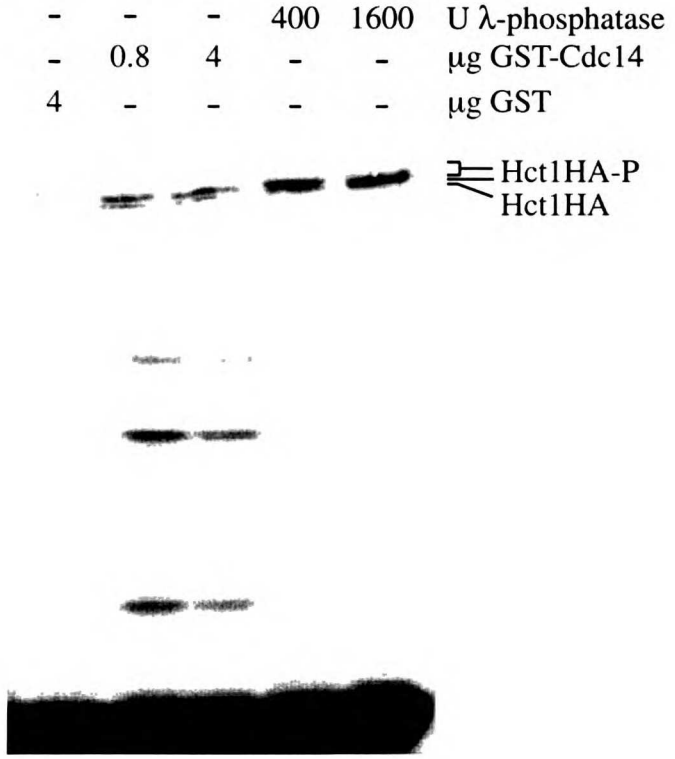
(A) The indicated amounts of GST, GST-Cdc14, or λ-phosphatase were added to cell lysates (1 mg) prepared from *cdc14-1* cells arrested at 37°C and expressing Hct1HA from the *GAL* promoter. Anti-HA immunoprecipitates were immunoblotted with anti-HA antibody 16B12 (top), or cyclin-ubiquitin ligase activity was measured in anti-Cdc26 immunoprecipitates (bottom).

(B) The indicated amounts of GST (lane G), GST-Cdc14 (lanes marked 14), phosphatase-deficient GST-Cdc14 point mutants (lanes C and C/R), or λ-phosphatase were added to cell lysates (1 mg) prepared from *cdc14-1* cells arrested at 37°C. Cyclin-ubiquitin ligase activity was then measured in anti-Cdc26 immunoprecipitates.

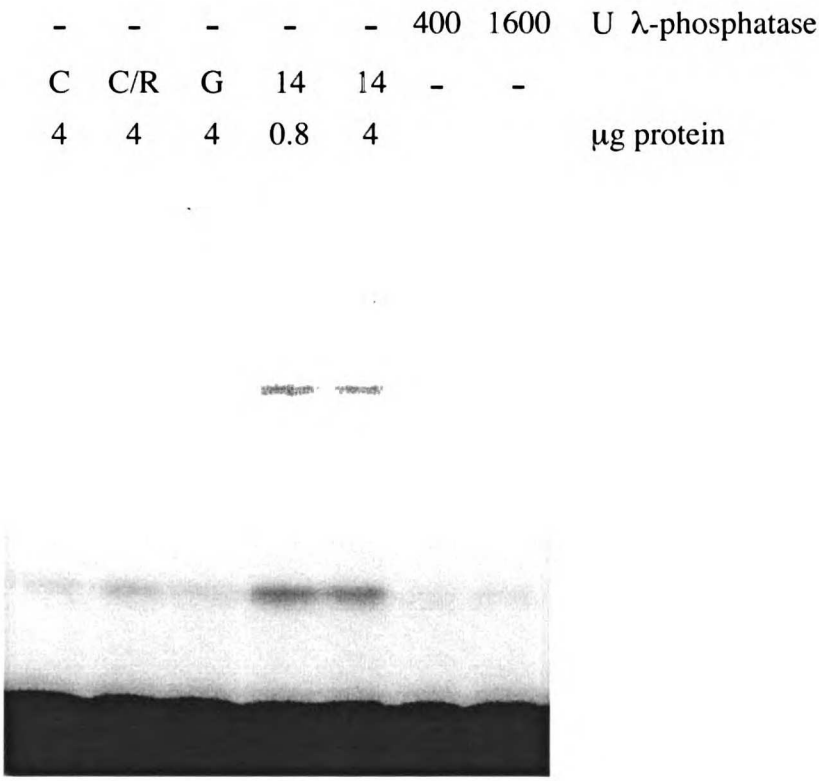
(C) Wild type or *sic1Δ* cells containing *GAL-HCT1HA* alone or in combination with *GAL-CDC14* were arrested in mitosis by treatment with 15 μg/ml nocodazole, followed by addition of galactose (4%) for 3 h. APC activity was measured in anti-Cdc26 immunoprecipitates from 600 μg of cell extract (top panel). Clb2 protein was detected by western blotting with anti-Clb2 antibody (middle). Hct1HA protein was immunoprecipitated from 1 mg of cell extract and analyzed by western blotting (bottom).

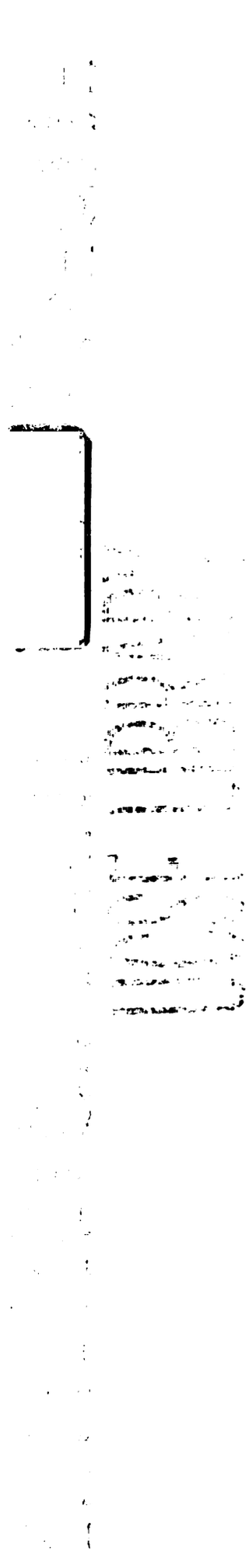
Figure 3-7

A


- - - 400 1600 U λ-phosphatase
- 0.8 4 - - μg GST-Cdc14
4 - - - - μg GST
Hct1HA-P
Hct1HA

B


- - - - - 400 1600 U λ-phosphatase
C C/R G 14 14 - -
4 4 4 0.8 4 μg protein



C

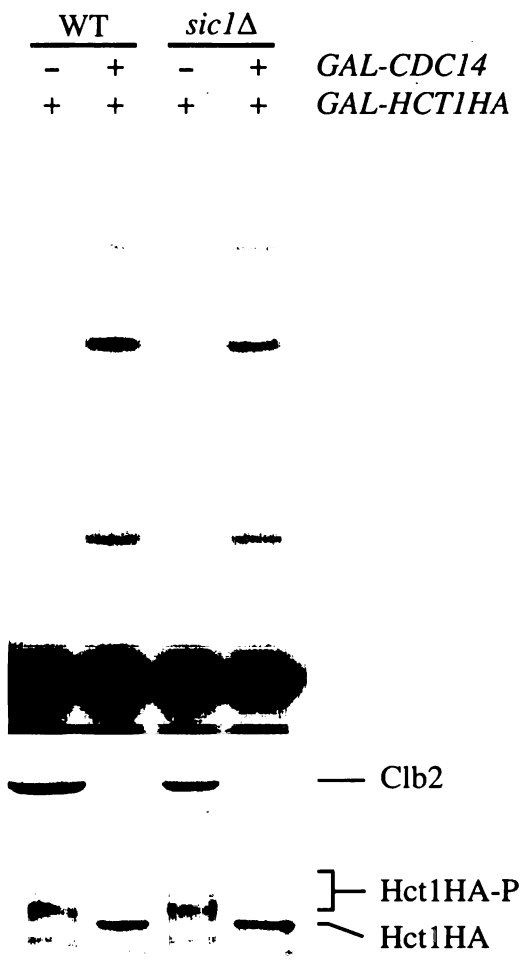


Figure 3-8. Model of the regulatory system governing Cdc28 inactivation in late mitosis.

This scheme accounts for our evidence that the APC core undergoes a Cdc5- and Cdc15-dependent increase in Hct1 sensitivity in late mitosis (indicated by an asterisk). Cdc14 is not required for this process but is required for the activation of APC by Hct1 dephosphorylation. It remains possible that Cdc5 and Cdc15 are also required for Hct1 dephosphorylation and for upregulation of Sic1 (Charles et al., 1998; Jaspersen et al., 1998).

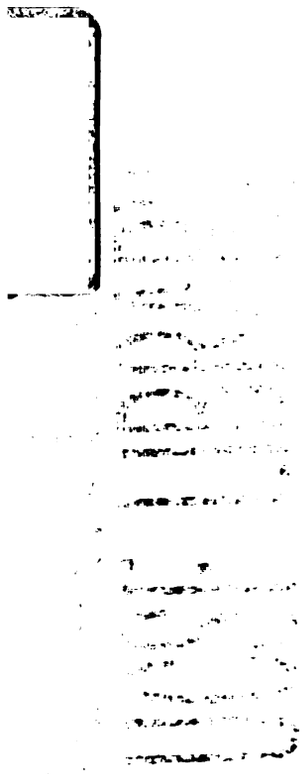
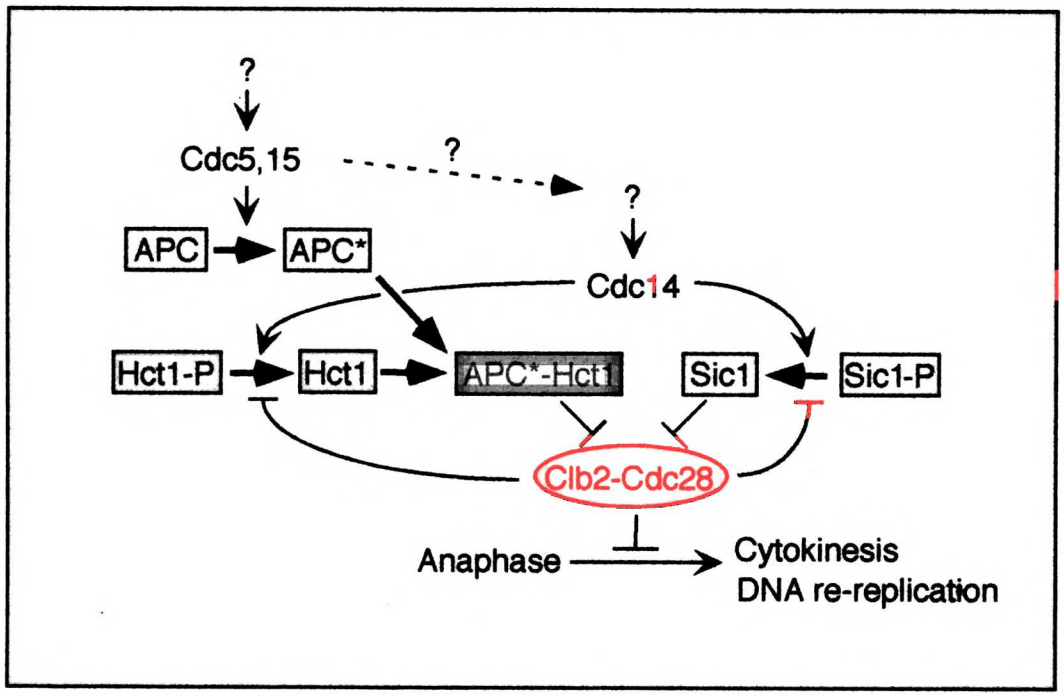
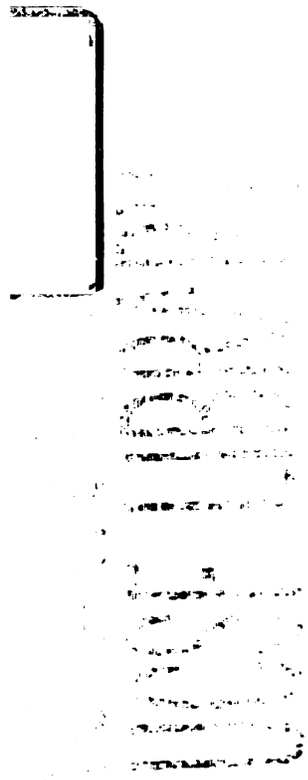


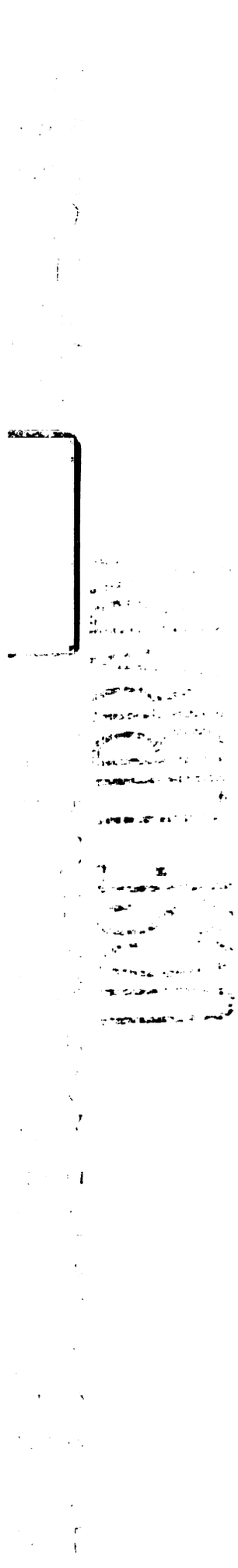
Figure 3-8


?
Cdc5,15
?
?
APC
APC*
Cdc14
Hct1-P
Hct1
APC*-Hct1
Sic1
Sic1-P
Clb2-Cdc28
Anaphase
Cytokinesis
DNA re-replication



Chapter Four

Cdc14 activates Cdc15 to promote mitotic exit in budding yeast



Cdc14 activates Cdc15 to promote mitotic exit in budding yeast

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Running title: Control of mitotic exit by Cdc14 and Cdc15

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Abstract

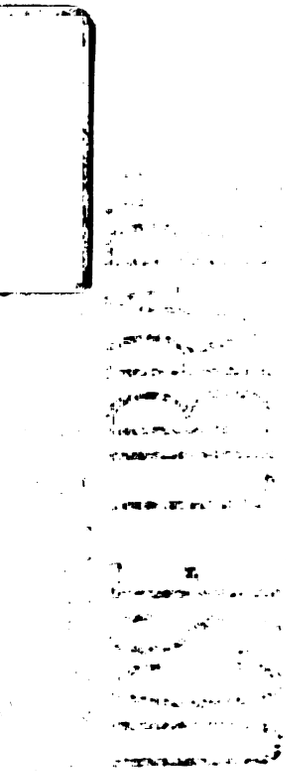
Inactivation of mitotic cyclin-dependent kinases (Cdks) is required for cells to exit from mitosis and enter the next cell cycle. In the budding yeast *Saccharomyces cerevisiae*, Cdk inactivation is triggered by the phosphatase Cdc14, which is activated by a complex network of regulatory proteins that includes the protein kinase Cdc15. Here we show that the ability of Cdc15 to promote mitotic exit is negatively regulated by phosphorylation. Cdc15 is phosphorylated *in vivo* at multiple Cdk-consensus sites during most of the cell cycle. However, transient dephosphorylation of Cdc15 occurs in late mitosis, at the time when Cdc15 is required to promote Cdk inactivation and cell division. Although phosphorylation appears to have no effect on Cdc15 kinase activity *in vitro*, a non-phosphorylatable mutant of Cdc15 is a more potent stimulator of mitotic exit than wild-type Cdc15, indicating that phosphorylation inhibits Cdc15 function *in vivo*. Interestingly, inhibitory phosphorylation of Cdc15 is removed by the phosphatase Cdc14 *in vitro*; in addition, overproduction of Cdc14 leads to Cdc15 dephosphorylation *in vivo*. Thus, Cdc15 serves both as an activator and substrate of Cdc14. Although this scheme raises the possibility that positive feedback promotes Cdc14 activation, we present evidence that such feedback is not essential for Cdc14 activation *in vivo*. Instead, Cdc15 dephosphorylation may promote some additional function of Cdc15 that is independent of its effects on Cdc14 activation.

Introduction

Exit from mitosis and entry into the next cell cycle requires the inactivation of mitotic cyclin-dependent kinases (Cdks). In the budding yeast *Saccharomyces cerevisiae*, mitotic Cdk-cyclin complexes (Cdc28-Clbs) are inactivated at the end of anaphase by two mechanisms: binding of the Cdk-inhibitor Sic1 and ubiquitin-dependent proteolysis of the cyclin subunit (Morgan, 1999; Zachariae and Nasmyth, 1999). Both Sic1 accumulation and cyclin proteolysis are tightly controlled during the cell cycle by multiple regulatory mechanisms, ensuring that Cdk inactivation and cell division occur only when anaphase events are completed.

A large multi-subunit ubiquitin ligase, known as the anaphase-promoting complex (APC), mediates the key regulatory step in cyclin proteolysis (King et al., 1996; Morgan, 1999; Zachariae and Nasmyth, 1999). APC activity is low during most of the cell cycle, increases during mitosis, and remains high throughout G1 (Amon et al., 1994; King et al., 1995; Lahav-Baratz et al., 1995; Sudakin et al., 1995; Zachariae and Nasmyth, 1996; Charles et al., 1998). The major mechanism of APC regulation is the transient association of the core APC with the substoichiometric regulatory subunits Cdc20 and Hct1/Cdh1 (Fang et al., 1998b; Kramer et al., 1998a; Zachariae et al., 1998a; Jaspersen et al., 1999). As well as being required for APC activity, Cdc20 and Hct1 may also confer substrate specificity to the APC *in vivo* (Schwab et al., 1997; Visintin et al., 1997). At the metaphase-to-anaphase transition, the Cdc20-dependent APC is required for destruction of the anaphase inhibitor Pds1 and the S-phase cyclin Clb5, whereas Hct1 directs the destruction of the major mitotic cyclin Clb2 in late mitosis and G1 (Yamamoto et al., 1996; Schwab et al., 1997; Lim et al., 1998; Shirayama et al., 1999).

In budding yeast, late mitotic Cdk inactivation is also promoted by the inhibitor Sic1. The levels of Sic1 protein increase in late mitosis as a result of two mechanisms (Donovan et al., 1994). First, increased levels and nuclear accumulation of the transcription



factor Swi5 increase *SIC1* transcription (Lydall et al., 1991; Moll et al., 1991; Knapp et al., 1996; Toyn et al., 1996). Second, the ubiquitin-dependent destruction of Sic1 (mediated by the ubiquitin-ligase SCF^{Cdc4}) (Deshaies, 1997; Peters, 1998) decreases in late mitosis, resulting in increased Sic1 stability (Schwob et al., 1994; Verma et al., 1997).

The functions of both Hct1 and Sic1 are inhibited by Cdc28 activity (Amon, 1997). Cdc28-dependent phosphorylation of Hct1 blocks the ability of Hct1 to bind and activate the APC (Zachariae et al., 1998a; Jaspersen et al., 1999). Cdc28 controls Sic1 levels by multiple mechanisms: Cdc28-dependent phosphorylation of Sic1 promotes its ubiquitination by SCF^{Cdc4} (Schwob et al., 1994; Verma et al., 1997), and phosphorylation of Swi5 prevents its nuclear accumulation and reduces *SIC1* transcription (Moll et al., 1991; Toyn et al., 1996). The ability of Cdc28 to inhibit its own inactivation by these mechansims allows the accumulation of Cdc28-Clb activity from late G1 to mitosis, and also provides the foundation for a biochemical switch that promotes rapid and complete Cdk inactivation following anaphase.

The Cdk inactivation switch is triggered in late mitosis by multiple regulatory events. Cdc20-dependent destruction of certain cyclins, such as Clb5 and Clb3, promotes the activation of Hct1 and Sic1, presumably by reducing their phosphorylation (Schwab et al., 1997; Alexandru et al., 1999; Shirayama et al., 1999). In addition, Cdk inactivation and mitotic exit require activation of the protein phosphatase Cdc14, which reverses the effects of Cdc28 by catalyzing dephosphorylation of Hct1, Sic1, and Swi5 (Visintin et al., 1998; Jaspersen et al., 1999).

Recent work has suggested that Cdc14 is sequestered in the nucleolus during most of the cell cycle, due to an association with the nucleolar protein Net1/Cfi1 (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999). Shortly after anaphase, Cdc14 is transiently released into the nucleus and the cytoplasm, where it is thought to dephosphorylate its targets and trigger mitotic exit. Although mechanisms of Cdc14 regulation remain obscure, Cdc14 redistribution requires the activities of a family of

regulatory proteins, including the protein kinases Cdc15 and Dbf2, the Polo-like kinase Cdc5, and the GTPase Tem1 (Shou et al., 1999; Visintin et al., 1999). Cells lacking any component of this mitotic exit network arrest after anaphase with high levels of Clb2, low levels of Sic1, and nucleolar Cdc14 (Surana et al., 1993; Shirayama et al., 1994b; Toyn and Johnston, 1994; Charles et al., 1998; Jaspersen et al., 1998; Shou et al., 1999; Visintin et al., 1999).

It is not clear if the sole function of the mitotic exit network is to trigger Cdc14 release from the nucleolus, or whether it has additional functions required for mitotic exit. Characterization of *Schizosaccharomyces pombe* homologs of the late mitotic genes, including the Cdc15 homolog Cdc7, has revealed no clear role for the fission yeast proteins in regulating mitotic cyclin-Cdk inactivation. Instead, these proteins are thought to control septum formation and cytokinesis in *S. pombe* (Gould and Simanis, 1997; LeGoff et al., 1999), raising the possiblity that Cdc15 and the other late mitotic proteins have similar functions in *S. cerevisiae*. Indeed, recent studies of the genetic requirements for cell division in budding yeast, as well as careful analysis of Dbf2 regulation and the phenotype of *tem1Δ net1-1* cells, support the possibility that the late mitotic proteins, including Cdc15, directly promote cytokinesis (Jiménez et al., 1998; Lippincott and Li, 1998; Fesquet et al., 1999; Shannon and Li, 1999; Shou et al., 1999). In addition, components of the late mitotic network have been proposed to play roles in licensing duplication of the spindle pole body and in regulating transcription of a variety of genes, including those involved in cell wall integrity (Liu et al., 1997; Komarnitsky et al., 1998; Luca and Winey, 1998). Whether the mitotic exit network directly controls these processes, or is indirectly involved as a result of a role in the regulation of mitotic-Cdk activity, is currently unclear.

To gain insight into the regulation of the mitotic exit network and its role during the cell cycle, we explored the mechanisms controlling the function of the kinase Cdc15. We found that Cdc15 is phosphorylated on multiple sites during most of the cell cycle, but is dephosphorylated for a brief period following anaphase. Genetic analysis of a non-

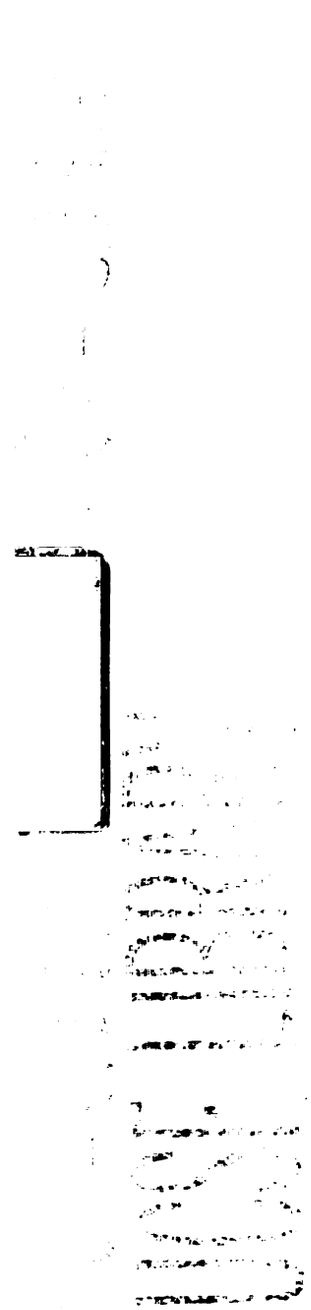
phosphorylatable *CDC15* mutant suggests that phosphorylation inhibits the ability of Cdc15 to stimulate Cdc28 inactivation and mitotic exit. Inhibitory phosphorylation of Cdc15 is removed by Cdc14, suggesting that feedback mechanisms may enhance Cdc14 activation by the mitotic exit network. However, dephosphorylation of Cdc15 does not appear to be required for Cdc14 release from the nucleolus, raising the possibility that Cdc15 dephosphorylation by Cdc14 might promote a second Cdc15 function in the control of mitotic exit.

Results

Cdc15 is phosphorylated in vivo on Cdk consensus sites

Our previous studies indicated that Cdc15 protein levels do not fluctuate during the cell cycle, even though Cdc15 function is not required until the end of mitosis (Jaspersen et al., 1998). We therefore pursued the possibility that Cdc15 activity is regulated by post-translational modifications. We constructed a strain containing an integrated copy of a gene encoding a hemagglutinin (HA) epitope-tagged form of Cdc15 under the control of the *GAL1/10* promoter. At least 5 electrophoretic mobility forms of Cdc15HA were apparent in lysates from asynchronously growing yeast cells (Figure 4-1A, lane 2). Treatment of Cdc15HA immunoprecipitates with λ-phosphatase resulted in the disappearance of the slower mobility forms (Figure 4-1A, lane 3), demonstrating that Cdc15 is phosphorylated *in vivo*. In addition, Cdc15 was labeled with ^{32}P when yeast cells were grown in media containing radioactive inorganic phosphate (Figure 4-1B).

Many mitotic regulatory proteins, such as Hct1 and Sic1, are substrates of Cdc28 (Verma et al., 1997; Zachariae et al., 1998a; Jaspersen et al., 1999). Examination of the predicted amino acid sequence of Cdc15 revealed the presence of seven consensus Cdk phosphorylation sites (S/T*-P-X-K/R or K/R-S/T*-P, in single letter amino acid code where X represents any amino acid). To determine if these are sites of phosphorylation *in*

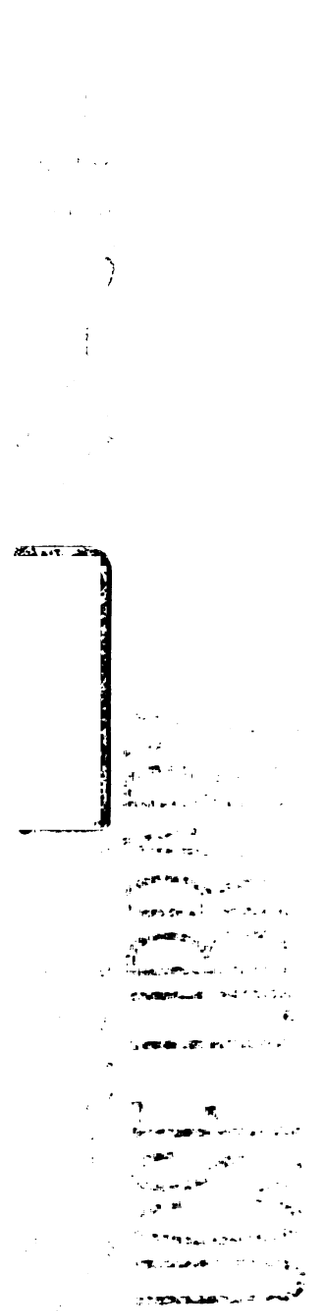


vivo, we constructed a version of Cdc15 in which these sites (serines 309, 562, 574, 578, 590, 946, and threonine 448) were mutated to alanine (termed the Cdc15-7A mutant). When this mutant was expressed in yeast cells, only two electrophoretic mobility forms were observed (Figure 4-1A, lane 5). In addition, ^{32}P-labeling *in vivo* indicated that Cdc15 phosphorylation was undetectable in the Cdc15-7A mutant (Figure 4-1B). Based on this result and on the heterogeneous gel mobility of Cdc15, we conclude that Cdc15 is phosphorylated *in vivo* at a large subset of the protein's seven Cdk consensus sites.

Cdc15 is briefly dephosphorylated during late mitosis

We found that Cdc15 was highly phosphorylated in cells arrested in G1, S-phase, or mitosis (Figure 4-2A). In addition, Cdc15 was highly phosphorylated in cells arrested in anaphase due to loss of function in any of the late mitotic genes (Figure 4-2B). Finally, we found that Cdc15 phosphorylation appeared constant throughout the cell cycle in cells released from a G1 arrest (data not shown).

However, careful analysis of cells passing synchronously through anaphase revealed that Cdc15 is transiently dephosphorylated in late mitosis. For these experiments, we employed a *cdc20Δ* strain in which the sole *CDC20* gene is under the control of the *GAL1/10* promoter (Lim et al., 1998). These cells arrest in metaphase in the absence of galactose. Addition of galactose then leads to a highly synchronous progression through anaphase and cytokinesis. Analysis of Cdc15HA3 during this release revealed a highly reproducible, transient decrease in Cdc15 phosphorylation at approximately the time that cells were exiting anaphase (30 min; Figure 4-2C). Cdc15 dephosphorylation coincided with cyclin destruction and Sic1 accumulation, suggesting that Cdc15 dephosphorylation might help promote the exit from mitosis.



What is the kinase that phosphorylates Cdc15?

Since phosphorylation of Cdc15 occurs on Cdk consensus sites, we next tested the possibility that Cdc28 is the kinase that phosphorylates Cdc15 *in vivo*. Consistent with this possibility, we found that purified, baculovirus-derived Cdc15-His_6 was efficiently phosphorylated *in vitro* by purified Cdc28-Clb2 complexes (data not shown). However, several lines of evidence argue against a central role for Cdc28 in the phosphorylation of Cdc15 *in vivo*. First, Cdc15 phosphorylation is not significantly reduced in cells arrested in G1 by α-factor (Figure 4-2A, 4-3A), where Cdc28 activity is absent. Second, Cdc15 is rapidly rephosphorylated in cells progressing out of mitosis into the following G1, despite the absence of Cdc28 activity (Figure 4-2C). Finally, we found that Cdc15 phosphorylation *in vivo* is only slightly inhibited in *cdc28* mutant cells arrested at the restrictive temperature (Figure 4-3A). While we cannot exclude the possibilty that Cdc28 contributes to Cdc15 regulation, it appears that Cdc28 cannot be the sole kinase responsible for Cdc15 phosphorylation *in vivo*.

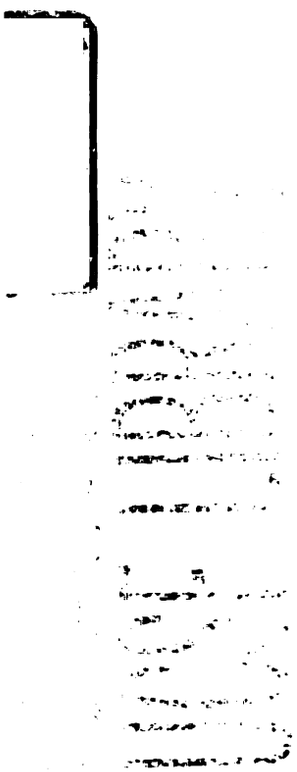
Cdc15 is known to autophosphorylate *in vitro* (Jaspersen et al., 1998), and so we next tested the possibility that Cdc15 itself is responsible for its phosphorylation. We expressed from the *GAL1/10* promoter a catalytically inactive Cdc15 mutant (K54L) and two fragments of Cdc15 (the N-terminal kinase domain, KD, and the C-terminal domain of unknown function, CTD, which contains the 7 Cdk sites) in *cdc15-2* arrested cells. In the absence of any detectable Cdc15 kinase activity, we still observed phosphorylation of Cdc15 and the CTD (Figure 4-3B), suggesting that Cdc15 is not solely responsible for its own phosphorylation. In addition, we found that autophosphorylation *in vitro* was not abolished in the Cdc15-7A mutant (Figure 4-3B, middle panel), indicating that autophosphorylation *in vitro* probably occurs at sites that are not phosphorylated *in vivo*.

Because Cdc15 phosphorylation occurs primarily on Cdk consensus sites, the kinase or kinases that phosphorylate Cdc15 may be proline-directed. In addition to Cdc28, yeast contains another Cdk, Pho85, that has been reported to phosphorylate targets at Cdk

consensus sites (Huang et al., 1996; O'Neill et al., 1996). However, we found that Cdc15 was still phosphorylated in G1-arrested cells lacking both Cdc28 and Pho85 activities (Figure 4-3C). Next, we tested the role of mitogen-activated protein kinases (MAPKs), which are also thought to be proline-directed (Davis, 1993). Budding yeast contain six non-essential MAPKs, five of which are expressed in mitotic cells (Herskowitz, 1995; Hunter and Plowman, 1997). We found that Cdc15 was still phosphorylated in asynchronous cells from which the five mitotic MAPKs were deleted (Figure 4-3D). We also found that Cdc15 phosphorylation was normal in cells with mutations in the PAK-kinases (*ste20Δ cla4-75* (Cvrckova et al., 1995), data not shown) or mutations in the late mitotic kinases Cdc5 and Dbf2 (Figure 4-2B). We have thus been unable to conclusively identify a single protein kinase responsible for Cdc15 phosphorylation *in vivo*.

The phosphatase Cdc14 dephosphorylates Cdc15

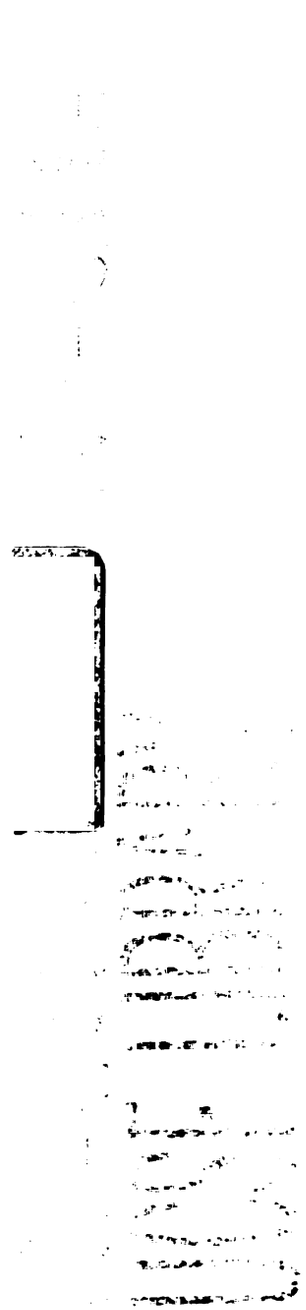
The fact that Cdc15 is phosphorylated during most of the cell cycle and only briefly dephosphorylated during mitosis might suggest that its phosphorylation is constitutive. A phosphatase, rather than a kinase, might therefore be the limiting regulator of Cdc15 phosphorylation and function. The phosphatase Cdc14 is an excellent candidate for the Cdc15 phosphatase, as it is required for Cdc28 inactivation at the end of mitosis (Jaspersen et al., 1998; Visintin et al., 1998), it is transiently released from the nucleolus following anaphase and then rapidly sequestered again in G1 (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999), and it is known to dephosphorylate other mitotic regulatory proteins at Cdk consensus sites (Visintin et al., 1998; Jaspersen et al., 1999). Further evidence that Cdc14 might positively regulate Cdc15 comes from the observations that overexpression of *CDC14* rescues the growth defect of *cdc15* mutants (Shirayama et al., 1996; Jaspersen et al., 1998; Visintin et al., 1998) and that Cdc15 is phosphorylated in cells lacking Cdc14 function (Figure 4-2B).



We tested the ability of Cdc14 to dephosphorylate Cdc15 by adding purified glutathione-S-transferase (GST)-Cdc14 to Cdc15 immunoprecipitates. Treatment with Cdc14 resulted in Cdc15 dephosphorylation, similar to that seen when λ-phosphatase was added (Figure 4-4A). There was no effect when we added GST or a catalytically inactive version of Cdc14 (GST-Cdc14 C283S/R289A (Taylor et al., 1997)), indicating that the Cdc15 dephosphorylation we observed depends on the phosphatase activity of Cdc14. When we added Cdc14 and λ-phosphatase to crude yeast lysates prepared from cells expressing Cdc15HA from the *GAL1/10* promoter, only Cdc14 was able to abolish the upper mobility forms of Cdc15 (Figure 4-4B), suggesting that Cdc14 is a more specific Cdc15 phosphatase under these conditions. To confirm that Cdc14 is involved in regulation of Cdc15 phosphorylation *in vivo*, we demonstrated that overexpression of *CDC14* in mitotically-arrested cells abolished Cdc15 phosphorylation (Figure 4-4C, top panel). The effects of *CDC14* overexpression were not simply an indirect consequence of mitotic exit, since similar results were obtained in a *cdc23-1* mutant at the restrictive temperature (Figure 4-4C). Based on these results, we propose that Cdc14 is responsible for Cdc15 dephosphorylation at the end of anaphase.

Phosphorylation negatively regulates Cdc15 in vivo

Our observation that Cdc15 is dephosphorylated at the end of mitosis by Cdc14 might suggest that phosphorylation negatively regulates Cdc15 activity. However, immunoprecipitates of phosphorylated wild-type Cdc15 and un-phosphorylated Cdc15-7A from asynchronously growing yeast cells contained the same amounts of kinase activity when Myelin Basic Protein (MBP) was used as a general substrate (Figure 4-3B, bottom panels). In addition, dephosphorylation of Cdc15 *in vivo* by overexpression of Cdc14 resulted in little change in Cdc15-associated kinase activity (Figure 4-4C, bottom panels). Finally, Cdc15-associated kinase activity does not appear to fluctuate during the cell cycle, either in cells released from a G1 arrest (Jaspersen et al., 1998) or from a metaphase arrest



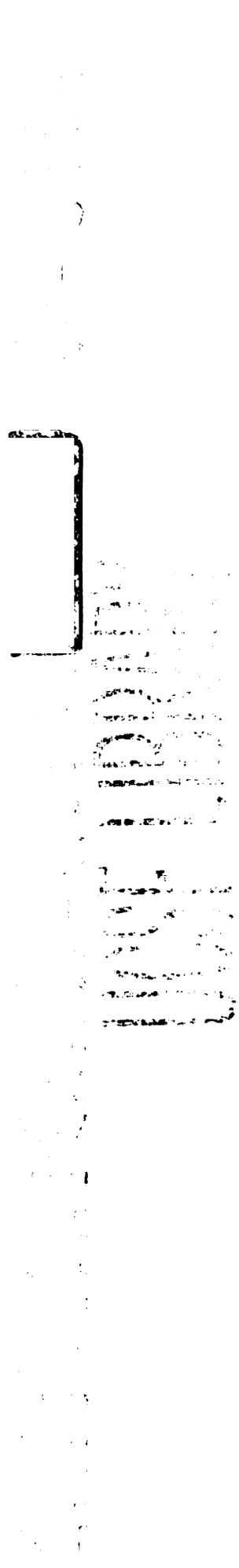
(Figure 4-2C). It therefore seems likely that phosphorylation does not affect the catalytic activity of Cdc15. However, because our assays were performed with MBP, we cannot exclude the possibility that phosphorylation affects activity toward physiological substrates.

To assess the role of phosphorylation in Cdc15 function *in vivo*, we analyzed the ability of Cdc15-7A overexpression to promote Cdk inactivation during mitosis. In cells arrested in mitosis with nocodazole, expression of Cdc15-7A, but not that of wild-type Cdc15, caused a decrease in the levels of the mitotic cyclins, Clb2 and Clb3, and an increase in the level of Sic1 (Figure 4-5A). Cdc15-7A expression resulted in a 50% decrease in the amount of Clb2-associated Cdc28 kinase activity (Figure 4-5A, HH1). Although cells overexpressing Cdc15-7A rebudded (Figure 4-5B), they did not complete all mitotic events: Pds1 levels remained high and sister chromatids did not separate (Figure 4-5A and data not shown). The ability of Cdc15-7A to induce Cdk inactivation was dependent on the functions of both Hct1 and Sic1 (Figure 4-5C), underscoring the importance of both pathways in exit from mitosis.

The ability of Cdc15-7A to promote Cdk inactivation supports the possibility that phosphorylation has a negative effect on Cdc15 function. Consistent with this possibility, we also found that overexpression of Cdc15-7A, but not that of wild type Cdc15, suppressed the growth defect of the late mitotic mutants *cdc5-1*, *dbf2-2*, and *tem1-3* (data not shown). However, like wild-type Cdc15, Cdc15-7A was unable to restore growth to *cdc14-1* arrested cells.

Effect of Cdc15-7A on cell cycle progression

To examine further the effect of Cdc15 phosphorylation on cell cycle progression, we constructed a yeast strain in which *CDC15-7A* was the only copy of *CDC15* in the cell. These cells were viable, morphologically identical to wild-type cells (data not shown), and progessed through the cell cycle with wild-type kinetics (Figure 4-6A). However, the *CDC15-7A* strain was unique in its genetic requirements for mitotic exit. Removal of the

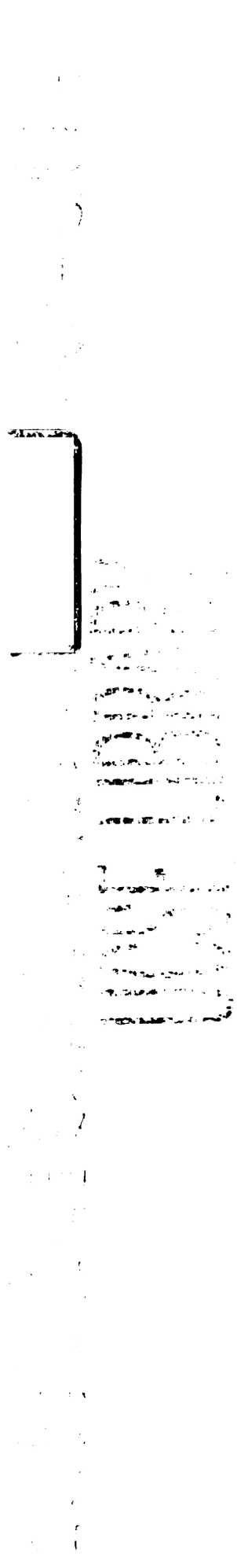


Cdc15 phosphorylation sites allowed proliferation of *cdc5-1*, *dbf2-2*, and *tem1-3* mutants at 37°C (Figure 4-6B). In particular, the growth of *tem1-3* strains expressing *CDC15-7A*, in the presence or absence of wild-type *CDC15*, was nearly as robust as the growth of wild-type cells (Figure 4-6B and data not shown). *CDC15-7A* did not suppress the growth defect of cells from which *TEM1* was deleted (data not shown). In addition, mitotic exit in *CDC15-7A* still required the function of Cdc14. The ability of *cdc5-1 CDC15-7A*, *dbf2-2 CDC15-7A*, and *tem1-3 CDC15-7A* strains to grow at the non-permissive temperature provides compelling evidence that Cdc15 dephosphorylation promotes mitotic exit.

Cdc15 dephosphorylation is not required for Cdc14 release from the nucleolus

There is considerable evidence that components of the mitotic exit network, including Cdc15, are upstream promoters of Cdc14 activation. Our present results, however, suggest that Cdc15 is also a downstream substrate of Cdc14. One interpretation of these results is that the ability of Cdc14 to activate Cdc15 leads to a positive feedback loop that enhances Cdc14 activation in late mitosis. To test this possibility, we analyzed the release of Cdc14 from the nucleolus in cells arrested in late mitosis by a *cdc14-1* mutation. These cells arrest with highly phosphorylated Cdc15 (Figure 4-2B); thus, if Cdc15 dephosphorylation is required for Cdc14 activation, then *cdc14-1* cells should arrest with Cdc14 still sequestered in the nucleolus.

We fused the endogenous *CDC14* gene in wild-type cells and in *cdc14-1* mutants with the gene encoding green-fluorescent protein (GFP) using a PCR-based tagging method (Longtine et al., 1998). *CDC14-GFP* and *cdc14-1-GFP* strains were synchronized in G1 by treatment with α-factor, released from the arrest, and shifted to a non-permissive temperature to inactivate the Cdc14-1 protein and arrest the mutant cells in anaphase (Figure 4-7N). Examination of Cdc14 localization by indirect immunofluoresence revealed that wild-type Cdc14 and the mutant Cdc14-1 protein were initially sequestered in the

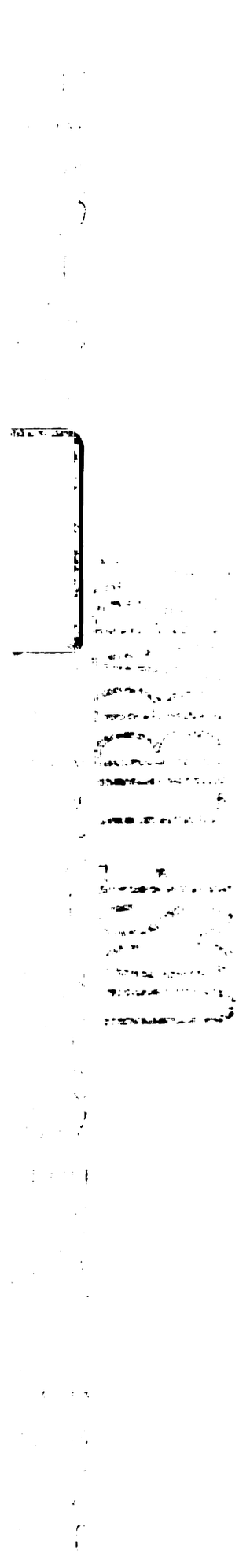


nucleolus. As wild-type cells progressed through anaphase and elongated the mitotic spindle, Cdc14 was efficiently redistributed into the nucleus and cytoplasm, and then returned to the nucleolus upon entry into the following G1 (Figure 4-7A-D, M). Although *cdc14-1* mutant cells entered anaphase slightly later than wild-type cells, the mutant Cdc14-1 protein also dispersed throughout the cell in late mitosis, and remained dispersed at the *cdc14-1* arrest point (Figure 4-7E-H, M). Release of mutant Cdc14-1 from the nucleolus was not simply a consequence of altered nucleolar structure, since a nucleolar marker protein, Nop1, remained sequestered in the nucleolus when Cdc14-1 protein was released (Figure 4-7I-L) (Aris and Blobel, 1988; Granot and Snyder, 1991). Thus, Cdc14 function does not seem to be required for its release from the nucleolus, suggesting that Cdc15 dephosphorylation and positive feedback are not essential for Cdc14 activation under these conditions.

Discussion

Our results argue that the ability of Cdc15 to promote the exit from mitosis is negatively regulated by phosphorylation. First, we observed that Cdc15 is transiently dephosphorylated at the time in mitosis when cyclin levels decrease, Sic1 levels increase, and Cdc15 function is known to be required. Second, ectopic expression of a non-phosphorylatable form of Cdc15, but not wild-type Cdc15, causes partial Cdk-inactivation and rebudding in metaphase-arrested cells. Finally, removal of Cdc15 phosphorylation sites allows proliferation of mutants with otherwise lethal defects in cyclin destruction and mitotic exit.

Our observation that Cdc15 is phosphorylated during most of the cell cycle suggests that the kinase(s) that phosphorylates Cdc15 is constitutively active. Alternatively, Cdc15 may be phosphorylated by multiple protein kinases, each acting during a different cell cycle stage. Despite the fact that Cdc15 was efficiently

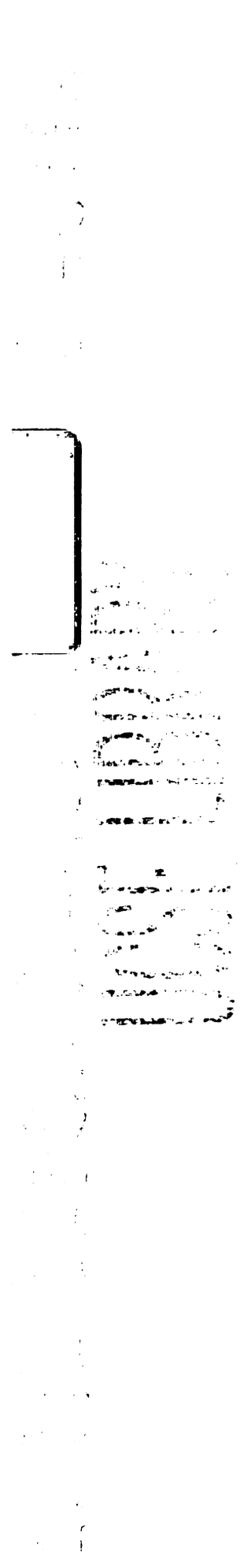


phosphorylated by Cdc28 *in vitro* and was phosphorylated *in vivo* on Cdk consensus sites, several lines of evidence argue that Cdc28 is not the sole kinase responsible for Cdc15 phosphorylation in cells. Presumably, other kinases are required for modification of Cdc15 *in vivo*. The sequence targeted by these kinases must be remarkably similar to that targeted by Cdc28, underscoring the dangers of assigning kinases based solely on the sequence surrounding phosphorylation sites.

The ability of non-phosphorylatable Cdc15-7A to promote the destruction of cyclin, but not that of Pds1, is consistent with previous evidence that the late mitotic mutants are defective primarily in activation the Hct1-dependent APC pathway (Jaspersen et al., 1998). The effects of Cdc15-7A on Cdk inactivation are dependent on Cdc14 function (data not shown), and expression of Cdc15-7A does not suppress the growth defect in the *cdc14-1* mutant. These results are consistent with abundant previous evidence that Cdc14 activation is dependent on Cdc15; for example, Cdc14 release from the nucleolus requires Cdc15 function (Shou et al., 1999; Visintin et al., 1999), and the growth defect in *cdc15* mutants is rescued by mutation of Net1, which anchors Cdc14 in the nucleolus (Shou et al., 1999; Visintin et al., 1999). In addition, moderate overexpression of *CDC14* can induce cyclin destruction in the absence of *CDC15* function (Visintin et al., 1998). Thus, it seems likely that Cdc15 induces cyclin destruction (and the accumulation of Sic1) by promoting activation of Cdc14. The mechanism by which this occurs remains unclear.

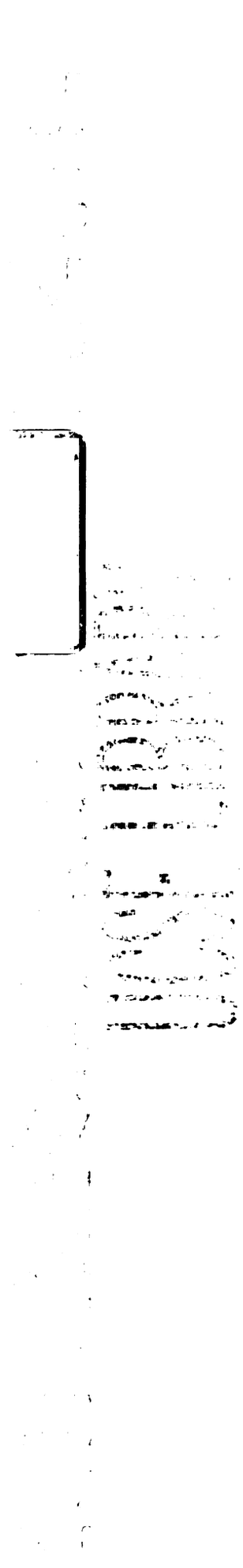
Our evidence suggests that Cdc15 is a substrate of the phosphatase Cdc14. Cdc15 is dephosphorylated during anaphase at the time of Cdc14 relocalization and activation, and Cdc15 phosphorylation state in the cell is dependent on Cdc14 activity. The rapid and transient dephosphorylation of Cdc15 in anaphase, in addition to the fact that Cdc15 is a substrate of Cdc14 *in vitro*, strongly suggest that Cdc14 acts directly on Cdc15.

The ability of Cdc14 to dephosphorylate and activate Cdc15 seems to contradict previous evidence that Cdc14 functions downstream of Cdc15 to promote Cdk inactivation. An obvious possibility is that this arrangement sets up a positive feedback loop, whereby



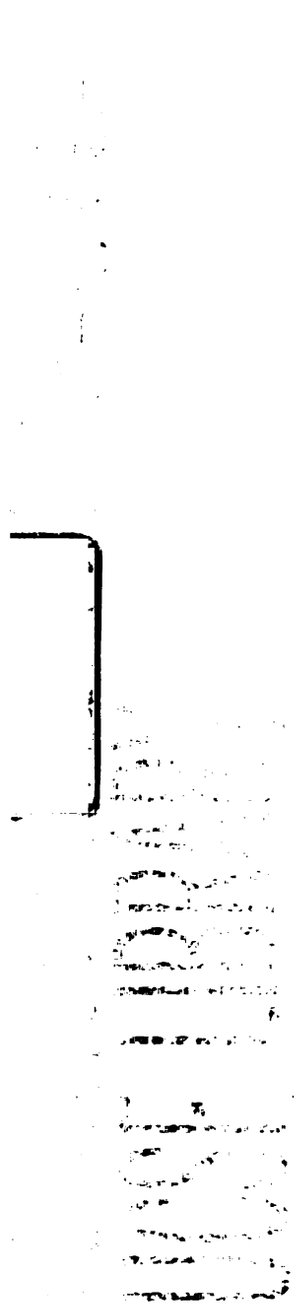
Cdc14 enhances its own activation by dephosphorylating its activator, Cdc15. However, we found that Cdc14 release from the nucleolus, which presumably reflects its activation (Shou et al., 1999; Visintin et al., 1999), can occur even when Cdc15 dephosphorylation does not occur (in a *cdc14* mutant). This result indicates that phosphorylated Cdc15 is fully functional in Cdc14 activation, and therefore suggests that positive feedback is not essential for Cdc14 activation. Nevertheless, considering that the Cdc15-7A mutant is a potent stimulator of Cdc14-dependent Cdk inactivation, it still seems plausible that feedback through Cdc15 increases the rapidity or completeness of Cdc14 activation to an extent that is not apparent under the conditions of our experiments (Figure 4-8). It also remains possible that Cdc15 has additional functions downstream of Cdc14, which may not be essential for cyclin destruction and Sic1 accumulation, but might help ensure rapid and complete Cdk inactivation once Cdc14 is activated.

An intriguing alternative explanation for our results is that Cdc15 dephosphorylation promotes a second Cdc15 function required for completion of mitosis (Figure 4-8). Recent studies of several *cdc15* mutant alleles suggest that Cdc15 might play multiple roles during mitosis (Shirayama et al., 1996; Jiménez et al., 1998). In particular, careful characterization of the *cdc15-lyt1* mutant phenotype suggests that Cdc15 function is critical for formation of an active division septum, which is required for cytokinesis. Based on this and other work in budding yeast (Jiménez et al., 1998; Lippincott and Li, 1998), as well as studies of the Cdc15 homolog in fission yeast (Fankhauser and Simanis, 1994; Sohrmann et al., 1998), it seems likely that Cdc15 is directly involved in controlling cytokinesis. We speculate that Cdc15 dephosphorylation promotes this or some related aspect of Cdc15 function (Figure 4-8). According to this model, the release of Cdc14 from the nucleolus not only activates cyclin destruction and Sic1 accumulation to induce Cdk inactivation, but also triggers Cdc15 dephosphorylation, allowing Cdc15 to perform an additional function in cytokinesis. Such a mechanism might be important for cells to link completion of anaphase with both Cdk inactivation and cytokinesis.



Dephosphorylation of Cdc15 alone is not sufficient to trigger mitotic exit, since *CDC15-7A* cells display normal mitotic progression. Presumably, exit from mitosis requires that Cdc15 dephosphorylation be accompanied by the activation of other components of the mitotic exit network, such as Cdc5 and Dbf2, which display increased activity during mitosis (Toyn and Johnston, 1994; Hardy and Pautz, 1996; Charles et al., 1998; Shirayama et al., 1998; Fesquet et al., 1999). Like Cdc15, recent studies in budding and fission yeasts support a role for Cdc5, Dbf2, Tem1, and even Cdc14 in the regulation of cytokinesis (Shirayama et al., 1996; Gould and Simanis, 1997; Jiménez et al., 1998; Fesquet et al., 1999; LeGoff et al., 1999; Shannon and Li, 1999; Shou et al., 1999). Activation of each of these components may be necessary but not sufficient for mitotic exit.

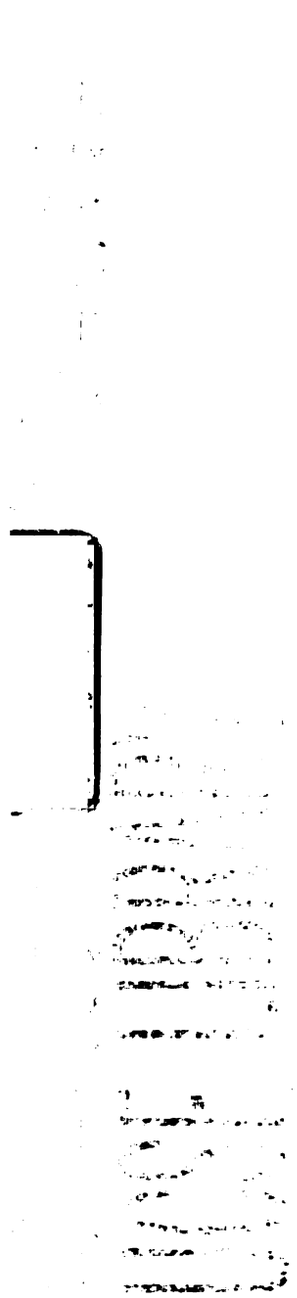
Cdc15 dephosphorylation has no detectable effect on Cdc15-associated kinase activity. While we cannot exclude the possibility that Cdc15 phosphorylation regulates kinase activity *in vivo*, our results suggest that phosphorylation regulates Cdc15 by some other mechanism. In *S. pombe*, the activity of the Cdc15 homolog, Cdc7, also remains constant during the cell cycle (Fankhauser and Simanis, 1994; Sohrmann et al., 1998). Cdc7 is instead regulated by changes in its subcellular localization: during interphase, Cdc7 is localized throughout the cell, but during mitosis it is localized first to both, then to one of the spindle pole bodies (Sohrmann et al., 1998). Recruitment of Cdc7 to the spindle pole is regulated by the nucleotide state of the GTPase Spg1, which bears homology to budding yeast Tem1, a component of the mitotic exit network (Schmidt et al., 1997; Sohrmann et al., 1998; Cerutti and Simanis, 1999). Perhaps Tem1-dependent recruitment of Cdc15 to the spindle pole is inhibited during most of the cell cycle by phosphorylation; dephosphorylation of Cdc15 would then allow binding to Tem1 and activation of some function, such as cytokinesis, in the mitotic exit pathway.



Materials and Methods

Yeast strains and plasmids

All strains were derivatives of W303 (*MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-1 can1-100*), except the *mapkΔ* strain whose genotype is described (Madhani et al., 1997). Oligonucleotide-directed mutagenesis of *CDC15* (Kunkel, 1985) was used to mutate lysine 54 to leucine (K54L), serines 309, 562, 574, 578, 590, 946 and threonine to alanine (7A), or to introduce a methionine (and a restriction site) immediately between amino acids 277 and 279 (truncation constructs). Wild-type and mutant *CDC15* genes were cloned into a pRS304-based plasmid containing the *GAL1/10* promoter and a single carboxy-terminal HA tag (Jaspersen et al., 1998). These were introduced at the *TRP1* locus of the following strains: AFS92 (WT *bar1-*); *cdc15-2*; *sicΔ::URA3*; *hct1Δ::LEU2*; *pds1::PDS1myc18-LEU2* (Jaspersen et al., 1998; Shirayama et al., 1998; Jaspersen et al., 1999). *CDC15* and *CDC15-7A* were also tagged at the carboxyl terminus with an HA3 tag and cloned with the endogenous promoter into pRS305 (Sikorski and Hieter, 1989). pRS305-*CDC15HA3* and pRS305-*CDC15HA3-7A* were introduced at the *LEU2* locus of AFS92, the late mitotic mutant strains, *cdc28-4*, *cdc28-13*, *cdc20Δ::LEU2 trp1::GAL-CDC20-TRP1* (a gift of U. Surana, (Lim et al., 1998)), *pho85Δ::LEU2* (a gift of E. O'Shea), and *cdc23-1 ura3::GAL-CDC14-URA3* (Jaspersen et al., 1999). A diploid in which one copy of *CDC15* was deleted by diruption with *HIS3* was also transformed with pRS305-*CDC15HA3* and pRS305-*CDC15HA3-7A.* Upon sporulation we obtained *MAT***a** *cdc15Δ::HIS3 leu2::CDC15HA3* (SLJ511) and *MAT***a** *cdc15Δ::HIS3 leu2::CDC15HA3-7A* (SLJ512), which were crossed to the late mitotic mutants to generate the strains used in Figure 4-6B and to a wild-type strain containing *ura3::GAL-CDC14-URA3* (Jaspersen et al., 1999). For *in vivo* labeling, *CDC15* and *CDC15-7A* were cloned into pDK20 (Tjandra et al., 1998), a myc12 tag was inserted immediately before the stop codon (Biggins et al., 1999), and these constructs were integrated at the *URA3* locus of AFS92, *cdc28-4*, and



cdc28-13. Using a similar cloning strategy, the full length *CDC15* (with the endogenous promoter) was tagged with myc12, subcloned into pRS306 (Sikorski and Hieter, 1989), and introduced into *fus3Δ kss1Δ hog1Δ mpk1Δ mlp1Δ* and an isogenic wild-type strain (a gift of H. Madhani (Madhani et al., 1997)). GFP(S65T)-*HIS3* was amplified from pFA6a-GFP(S65T)-HIS3MX6 by PCR as described (Longtine et al., 1998). This PCR product was transformed into AFS92 and *cdc14-1* to epitope tag the endogenous *CDC14* or *cdc14-1* gene with GFP immediately after the last codon, creating SLJ650 (*MAT***a** *bar1 CDC14::CDC14-GFP-HIS3*) and SLJ651 (*MAT***a** *bar1 cdc14-1::cdc14-1-GFP-HIS3*).

Lysate preparation and immunoblotting

Yeast lysates were prepared by bead beating into lysis buffer (50 mM Hepes-NaOH, pH 7.4, 75 mM KCl, 50 mM NaF, 50 mM ß-glycerophosphate, 1 mM EGTA, 0.1% NP40, 1 mM DTT, 1 mM phenylmethylsulfonylfluoride, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) as described (Jaspersen et al., 1998). For immunoblots, equal amounts of total protein were loaded on 8% SDS-PAGE gels. Clb2, Cdc28, Clb3, and Sic1 proteins were detected with affinity purified polyclonal antibodies as previously described (Gerber et al., 1995; Charles et al., 1998; Jaspersen et al., 1998; Alexandru et al., 1999). For detection of HA-tagged proteins, the mouse monoclonal antibody 16B12 (Babco) was used at a 1:1500 dilution; for detection of myc-tagged proteins, the anti-myc polyclonal antibody A-14 (Santa Cruz) was used at a 1:1000 dilution; for detection of GFP-tagged protein, the anti-GFP monoclonal antibody C163 (a gift of P. O'Farrell lab) was used at a 1:20 dilution. Overexpressed Cdc14 was detected using a 1:1000 dilution of anti-Cdc14 polyclonal antibodies (a gift of J. Charles).

Cell cycle synchronization

Standard protocols were used for cell propagation and induction from the *GAL1/10* promoter (Guthrie and Fink, 1991). Methods used to arrest cells with α-factor,

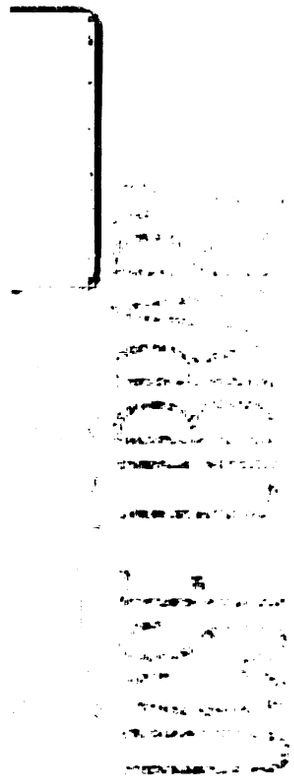
hydroxyurea, or nocodazole have been described (Jaspersen et al., 1998). To monitor progression through mitosis, *cdc20Δ GAL-CDC20* cells were grown to mid-log phase in YEP plus 2% galactose and raffinose. Cells were harvested, washed once in YEP, then resuspended in YEP plus 2% raffinose for 3 hours at 23°C. To release into G1, 2% galactose and 5 μg/ml α-factor were added. In these experiments, budding index in >200 cells was determined, and the morphology of the spindle was analyzed by indirect immunofluorescence (see below).

Immunofluorescence

To detect proteins by indirect immunofluorescence, 5 ml of cells were harvested, resuspended in 0.5 ml 0.1 M potassium phosphate, pH 7.5 containing 3.7% formaldehyde, and fixed for 30 minutes at room temperature. Following fixation, cells were processed for immunofluorescence as described (Rose et al., 1990; Biggins et al., 1999). Cdc14-GFP was detected using a 1:1000 dilution of affinity-purified rabbit polyclonal anti-GFP antibodies (a gift of the Murray lab), Nop1 was detected using a 1:10000 dilution of anti-Nop1 mouse monoclonal antibodies (a gift of J. Aris), and microtubules were detected using a 1:1000 dilution of the rat anti-tubulin antibody YOL 1/34 (Accurate Chemical & Scientific Corp.). Secondary antibodies included 1:10000 CY3-conjugated goat anti-rabbit IgG (Chemicon), 1:500 fluorescein-conjugated goat anti-mouse IgG (Cappel), and 1:500 fluorescein-conjugated goat anti-rat IgG (Cappel). DNA was visualized by staining with 1 μg/ml DAPI (4'6-diamidino-2-phenylindole) for 5 minutes immediately prior to mounting. Cells were examined with a Nikon fluorescence microscope using a 60X Nikon lens.

In vivo labeling

Cultures (50 ml) were grown overnight at 23°C in YEP plus 2% raffinose to OD_{600} of 0.5. Expression of Cdc15myc12 was induced by the addition of 2% galactose for 2



hours. Cells were harvested, washed twice with sterile ddH_2O, then resuspended in 1 ml synthetic complete minimal media lacking phosphate (Rothblatt and Schekman, 1989) that contained 2% galactose, 2% raffinose, and 0.5 mCi ^{32}P-orthophosphate (Amersham). After 1 hour of labeling, cells were harvested, washed once in ddH_2O, resuspended in 0.3 ml lysis buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 50 mM ß-glycerophosphate, 5 mM EDTA, 0.2% Triton X-100, 1 mM DTT, 1 mM phenylmethylsulfonylfluoride, 2 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin), and lysed by mechanical diruption as described (Jaspersen et al., 1998). Cdc15myc12 was immunoprecipitated from 3 mg yeast lysate with 50 µl protein G-sepharose (Pharmacia) and 2 µl 9E10 ascites fluid (Babco). Immunoprecipitates were washed 4 times with lysis buffer supplemented with an additional 150 mM NaCl then 2 times with 50 mM Tris-HCl pH 8.0. Samples were treated with 0.2 mg/ml RNaseA for 30 minutes at 4°C, then washed an additional 2 times with lysis buffer, and run on an 8% SDS-PAGE gel. Labeled proteins were detected by autoradiography, and ^{32}P-incorporation was quantitated on a Phosphorimager using the ImageQuant program (Molecular Dynamics). To quantitate protein levels, serial dilutions of each sample were analyzed by western blotting using the ECL Plus detection system (Amersham) and the Phosphorimager.

Kinase, phosphatase, and cyclin ubiquitination assays

Cdc15-associated kinase activity was measured in 16B12 immunoprecipitates and Cdc28-associated kinase activity was measured in Clb2 immunoprecipitates as described (Gerber et al., 1995; Jaspersen et al., 1998). For immunoprecipitation phosphatase assays, HA-tagged Cdc15 was immunoprecipitated from 0.5-1 mg cell lysate using 0.5 µl 16B12 (Babco) and protein A-sepharose (Sigma). Following three washes with lysis buffer and one wash with phosphatase buffer (25 mM Hepes-NaOH, pH 7.4, 150 mM NaCl, 0.1 mg/ml bovine serum albumin), immunoprecipitates were resuspended in 50 µl

phosphatase buffer to which either 2 mM $MnCl_2$, 2 mM $MnCl_2$ plus phosphatase, or 2 mM $MnCl_2$, phosphatase plus 2 mM $ZnCl_2$, 50 mM NaF, and 1 mM Na_3VO_4 were added. After incubation for 30 minutes at 30°C, immune complexes were washed two times in lysis buffer and analyzed by immunoblotting. Phosphatase activity in crude yeast extracts was measured by addition of phosphatase and 2 mM $MnCl_2$ for 30 minutes at 30°C. Cdc15HA3 was then immunoprecipitated and the phosphorylation state analyzed by western blotting. λ-phosphatase was purchased from NEBL, and recombinant GST-Cdc14 and GST-Cdc14-C/R were purified from bacterial lysates on a glutathione-sepharose 4B column (Pharmacia), followed by anion exchange chromatography (Pharmacia HiTrap Q) (Jaspersen et al., 1999). Cyclin ubiquitin ligase activity of the APC was measured in anti-Cdc26 immunoprecipitates from 500 μg yeast lysate as described (Charles et al., 1998).

Acknowledgements

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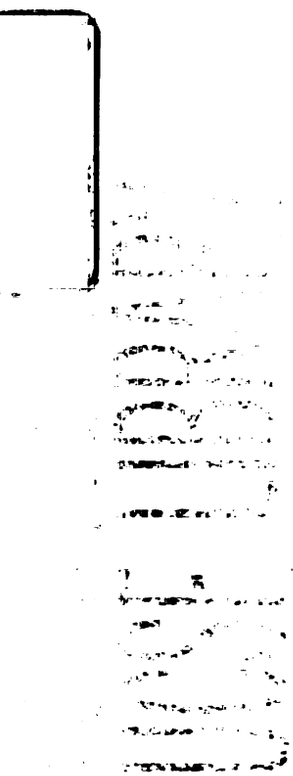


Figure 4-1. Cdc15 is phosphorylated *in vivo* at Cdk consensus sites.

(A) Wild-type strains containing *GAL-CDC15HA* or *GAL-CDC15HA-7A* (a mutant in which the seven Cdk consensus sites are changed to alanine) were grown in 4% galactose for 2.5 hours. Wild-type and mutant Cdc15HA proteins were immunoprecipitated with the anti-HA antibody 16B12 from cell lysates (500 μg) and treated with phosphatase buffer (lanes 2, 5), 100 U λ-phosphatase (lanes 3, 6), or both λ-phosphatase and phosphatase inhibitors (lanes 4, 7). Lane 1 is an immunoprecipitate from cells lacking the tagged Cdc15. Immunoprecipitates were immunoblotted with 16B12.

(B) Wild-type strains containing vector alone, *GAL-CDC15myc12*, or *GAL-CDC15myc12-7A* were grown in 2% galactose for 2 hours and then labeled for 1 hour with ^{32}P-orthophosphate. Cdc15myc12 proteins were immunoprecipitated from 3 mg yeast extract with the anti-myc antibody 9E10. One-tenth of the immunoprecipitate was immunoblotted with the anti-myc antibody A-14 (bottom panel); the remainder was analyzed by gel electrophoresis and autoradiography (top panel). The asterisk indicates a non-specific phosphoprotein present in the control lane; positions of molecular weight markers are indicated on the left.

The positions of unphosphorylated Cdc15 (Cdc15) and phosphorylated Cdc15 (P-Cdc15) forms are indicated.

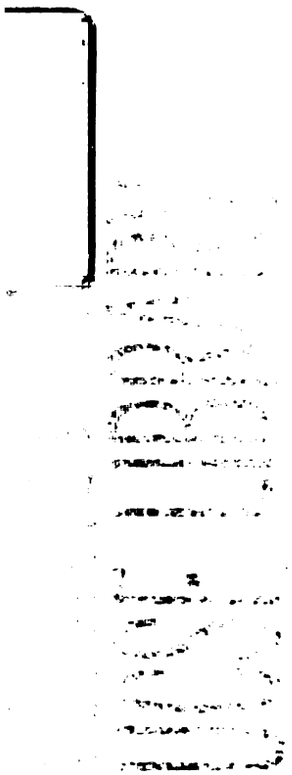
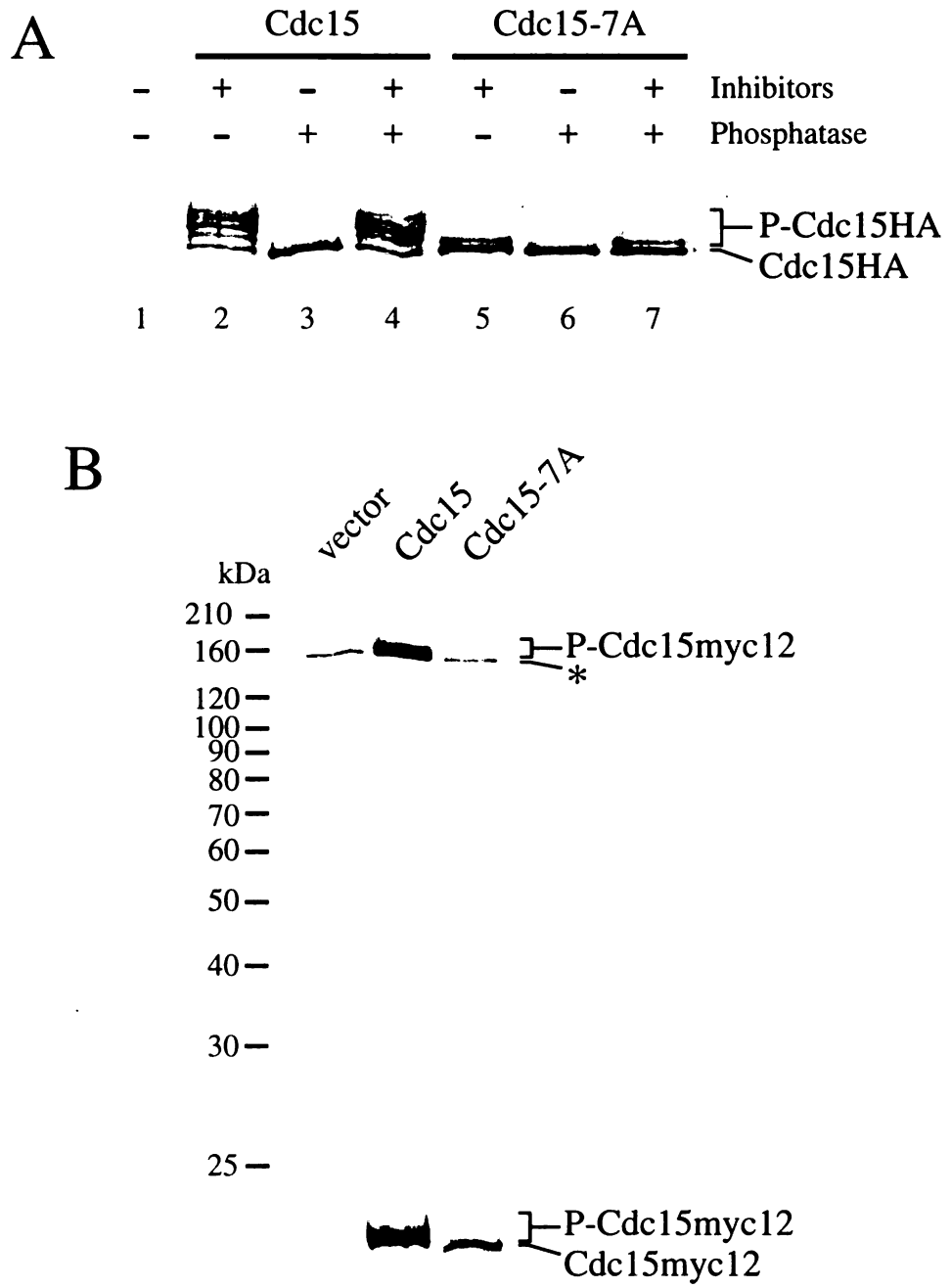


Figure 4-1


A
Cdc15
Cdc15-7A
- + - + + - + Inhibitors
- - + + - + + Phosphatase
P-Cdc15HA
Cdc15HA
1 2 3 4 5 6 7
B
vector
Cdc15
Cdc15-7A
kDa
210
160
120
100
90
80
70
60
50
40
30
25
P-Cdc15myc12
*
P-Cdc15myc12
Cdc15myc12

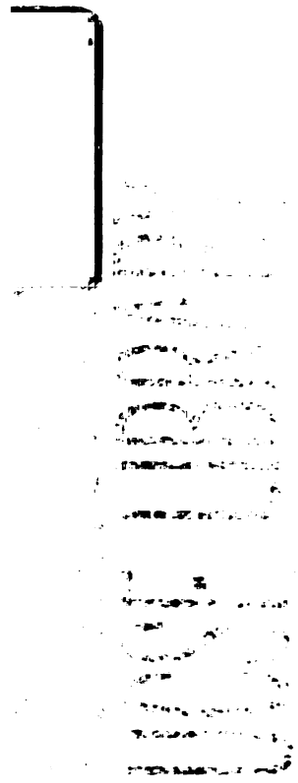
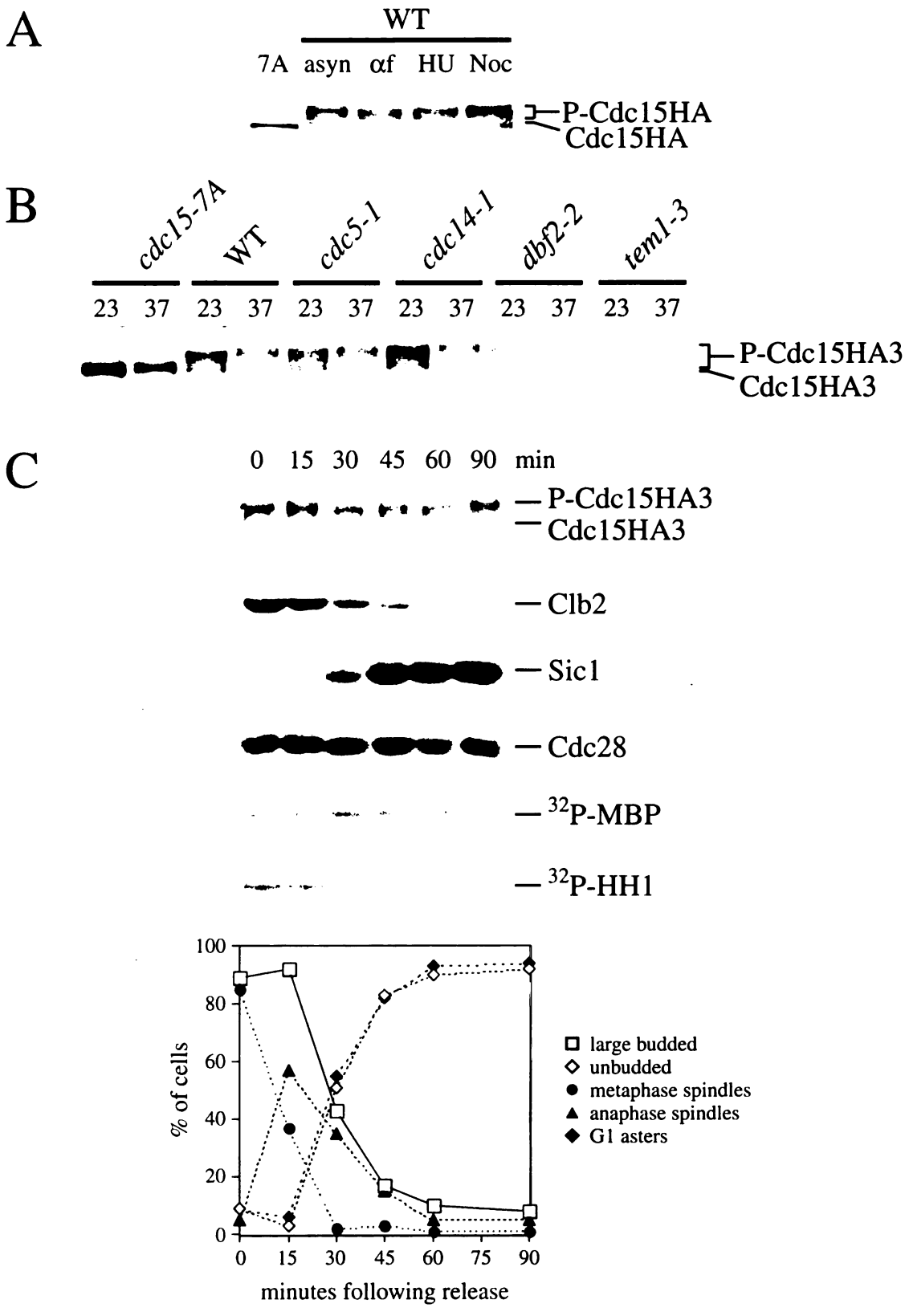


Figure 4-2. Cdc15 is dephosphorylated transiently at the end of anaphase.

(A) Wild-type strains containing *GAL-CDC15HA* (WT) or *GAL-CDC15HA-7A* (7A) were arrested in α-factor (αf), hydroxyurea (HU), or nocodazole (Noc), or left as asynchronous cultures (7A, asyn) for 3 hours. Galactose was then added to 4% for an additonal 1.5 hours. Lysates (50 μg) were analyzed by western blotting with 16B12.
(B) The indicated temperature-sensitive mutants containing *CDC15HA3* expressed from the endogenous promoter were grown at 23°C or were arrested in anaphase by shifting to 37°C for 4 hours. Cdc15HA3 was detected by western blotting with 16B12. The changes in Cdc15HA3 levels are due to differences in the amount of total protein loaded (data not shown).
(C) A *cdc20Δ GAL-CDC20* strain containing *CDC15HA3* expressed from the endogenous promoter was transferred to raffinose-containing media to arrest cells in metaphase. Cells were released from the arrest into G1 by the addition of 2% galactose and 5 μg/ml α-factor. At the indicated times, samples were withdrawn to analyze Clb2, Sic1, and Cdc28 levels, Cdc15 phosphorylation, Cdc15-associated kinase activity towards Myelin Basic Protein (MBP), and Clb2-associated Cdc28 kinase activity towards Histone H1 (HH1). The percentage of cells at each time point exhibiting the indicated budding and spindle morphologies is shown in the bottom panel.

In (A, B, C), the positions of unphosphorylated (Cdc15) and phosphorylated (P-Cdc15) forms are indicated. The dephosphorylation of Cdc15 in late mitosis (panel C) was observed reproducibly in four separate experiments.

Figure 4-2


A
WT
7A asyn αf HU Noc
P-Cdc15HA
Cdc15HA
B
cdc15-7A
WT
cdc5-1
cdc14-1
dbf2-2
tem1-3
23 37 23 37 23 37 23 37 23 37 23 37
P-Cdc15HA3
Cdc15HA3
C
0 15 30 45 60 90 min
P-Cdc15HA3
Cdc15HA3
Clb2
Sic1
Cdc28
32P-MBP
32P-HH1
100
80
60
40
20
0
% of cells
0 15 30 45 60 75 90
minutes following release
large budded
unbudded
metaphase spindles
anaphase spindles
G1 asters

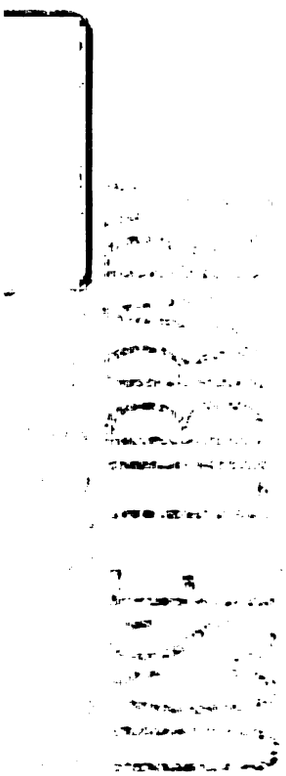


Figure 4-3. Analysis of Cdc15 phosphorylation by different kinases.

(A) Strains containing vector, *GAL-CDC15myc12* (WT), or *GAL-CDC15myc12-7A* (7A) were arrested in G1 by treatment with 5 μg/ml α–factor for 3 hours at 23°C (αf, *cdc28-4*, *cdc28-13*) or were left as asynchronous cultures (vector, 7A, asyn). One hour after α-factor addition, galactose was added to 2%. Cultures were shifted to 37°C for 30 minutes before labeling with ^{32}P-orthophosphate at 37°C. Samples were processed as in Figure 4-1B and the labeled proteins are shown in the top panel. The asterisk indicates a non-specific phosphoprotein present in the control lane. Because Cdc15 expression was not uniform (western blot; bottom panel), the amount of total Cdc15 protein was determined by quantitative immunoblotting and relative ^{32}P incorporation in each sample is shown below the gel. Incorporation in the vector and asynchronous samples were assigned values of 0 and 1, respectively.

(B) *cdc15-2* strains containing vector, *GAL-CDC15HA* (WT), *GAL-CDC15HA-7A* (7A), *GAL-CDC15HA-K54L* (K54L, a catalytically inactive point mutant in the Cdc15 kinase domain), *GAL-CDC15HA-CTD* (CTD, a version of Cdc15 in which the C-terminal, non-catalytic domain of the protein is expressed starting at amino acid 279), or *GAL-CDC15HA-KD* (KD, a truncation of Cdc15 at amino-acid 277, so that only the N-terminal kinase domain is produced) were grown in 4% galactose for 3 hours at 37°C. Cdc15HA was detected in 50 μg lysate by immunoblotting with 16B12 (top panel). Lower expression levels of K54L were consistently observed. The heterogenous mobility of the CTD protein is due to phosphorylation, since phosphatase treatment collapses these forms into a single band (data not shown); no change in the gel mobility of the kinase domain was observed upon treatment with phosphatase (data not shown). Positions of molecular weight markers are indicated on the left. To measure Cdc15-associated kinase activity,

Cdc15HA was immunoprecipitated from 500 μg of lysate and tested for its ability to phosphorylate MBP (bottom) or itself (center). No autophosphorylation of CTD or KD was observed (data not shown).

(C) The indicated strains expressing Cdc15HA3 (WT) or Cdc15HA3-7A (7A) from the *CDC15* promoter were arrested in G1 by treatment with 5 μg/ml α-factor (7A, WT, *pho85Δ*) or by shifting cells to 37°C for 4 hours (*cdc28-4*). Cdc15HA3 was detected in 75 μg yeast lysate by western blotting with 16B12. A strain lacking the tagged Cdc15 was also analyzed (V).

(D) A *CDC15myc12* gene under the control of the endogenous promoter was integrated into a *fus3Δ kss1Δ hog1Δ mpk1Δ mlp1Δ* (*mapkΔ*) and an isogenic wild-type (WT) stain at the *URA3* locus. Cdc15myc12 was detected in asynchronously growing cells by western blotting 75 μg yeast extract with the anti-myc antibody A-14.

In (A, C, D), the positions of unphosphorylated (Cdc15) and phosphorylated (P-Cdc15) forms are indicated.

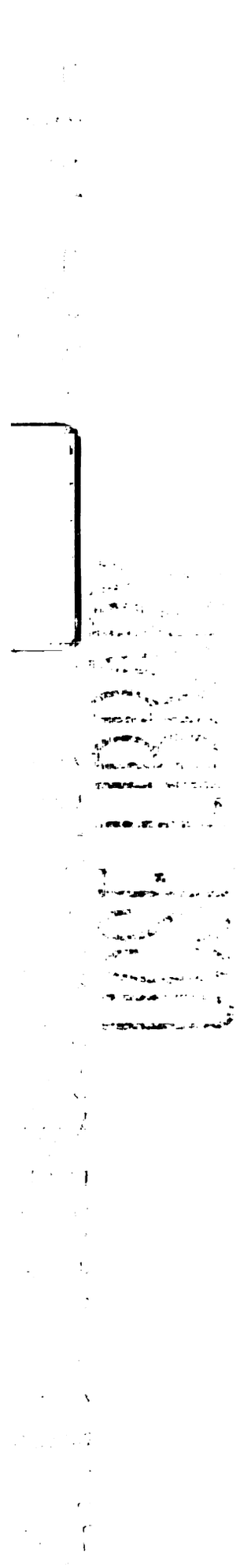
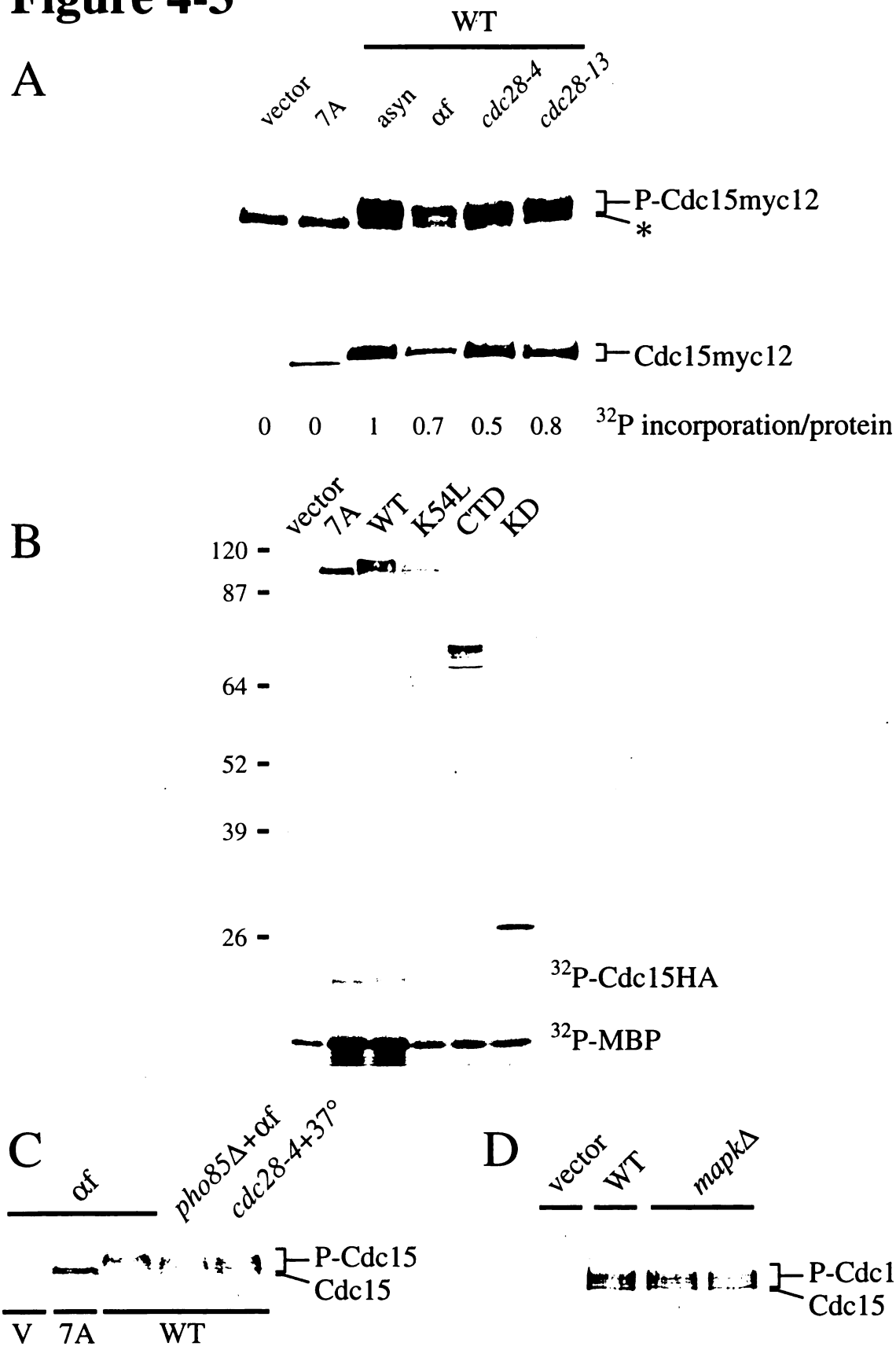


Figure 4-3


A
WT
vector
7A
asyn
αf
cdc28-4
cdc28-13
P-Cdc15myc12
*
Cdc15myc12
0 0 1 0.7 0.5 0.8
32P incorporation/protein
B
vector
7A
WT
K54L
CTD
KD
120
87
64
52
39
26
32P-Cdc15HA
32P-MBP
C
αf
pho85Δ+αf
cdc28-4+37°
P-Cdc15
Cdc15
V
7A
WT
D
vector
WT
mapkΔ
P-Cdc15
Cdc15

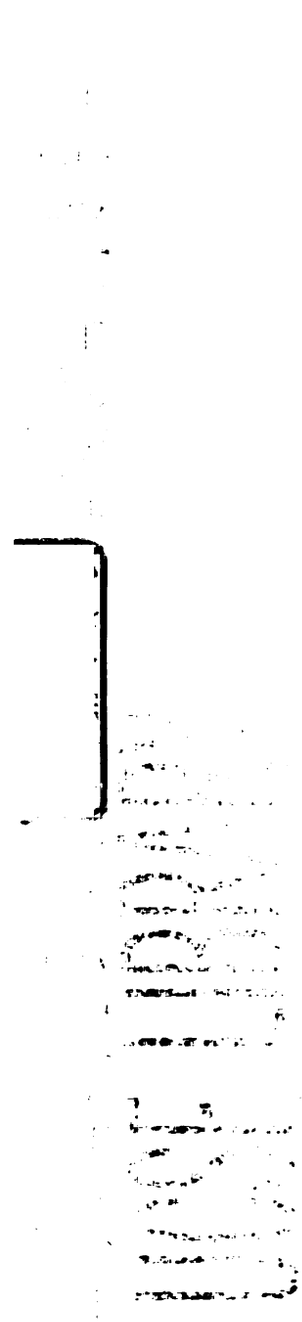


Figure 4-4. Cdc14 promotes Cdc15 dephosphorylation *in vitro* and *in vivo*.

(A) Wild-type strains containing *GAL-CDC15HA* (WT) or *GAL-CDC15HA-7A* (7A) were grown in 4% galactose for 2.5 hours and yeast extracts were prepared. Cdc15HA proteins were immunoprecipitated with 16B12 from 1 mg cell lysate and incubated with buffer alone (-), 1 μg GST, GST-Cdc14, GST-Cdc14-C/R (an inactive double mutant), or 200 U λ-phosphatase.

(B) Crude cell lysates (1 mg, prepared as in panel A) were incubated for 30 min at 30°C with buffer alone (-), 5 μg GST, GST-Cdc14, GST-Cdc14-C/R or 1600 U λ-phosphatase. Cdc15HA was immunoprecipitated from the reactions and detected by immunoblotting with 16B12.

(C) *cdc15Δ CDC15HA3*, *cdc15Δ CDC15HA3-7A*, *cdc23-1 CDC15HA3*, *cdc23-1 CDC15HA3-7A*, and *cdc23-1* strains, each containing *GAL-CDC14*, were arrested in mitosis by treatment with 15 μg/ml nocodazole for 2 hours. Cultures where shifted to 35°C for 30 minutes to inactivate *cdc23*, followed by addition of 2% dextrose (-) or 4% galactose (+) for 2 hours at 35°C. Cdc15HA3, Clb2, and Cdc14 were detected by western blotting 50 μg lysate with 16B12 (top panel), anti-Clb2 (second panel), and anti-Cdc14 (third panel) antibodies, respectively. Cdc15HA or Cdc15HA-7A were immunoprecipitated from 2 mg of lysate and tested for their ability to autophosphorylate (fourth panel) or phosphorylate MBP (bottom panel).

In (A, B, C), the positions of unphosphorylated (Cdc15) and phosphorylated (P-Cdc15) forms are indicated.

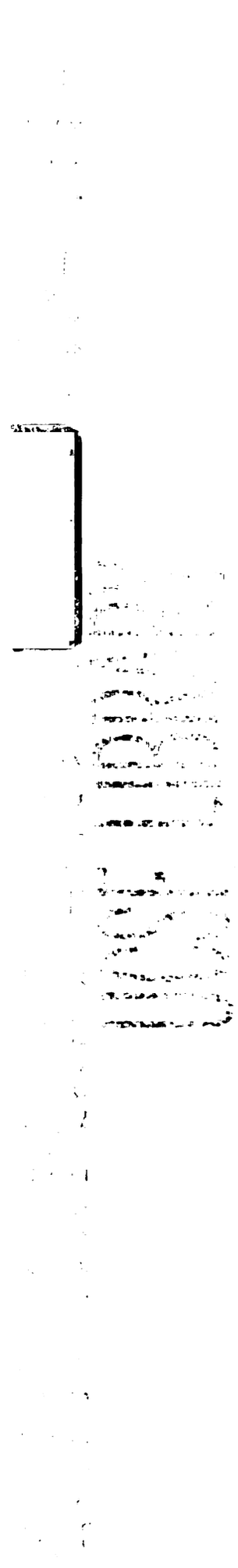


Figure 4-4

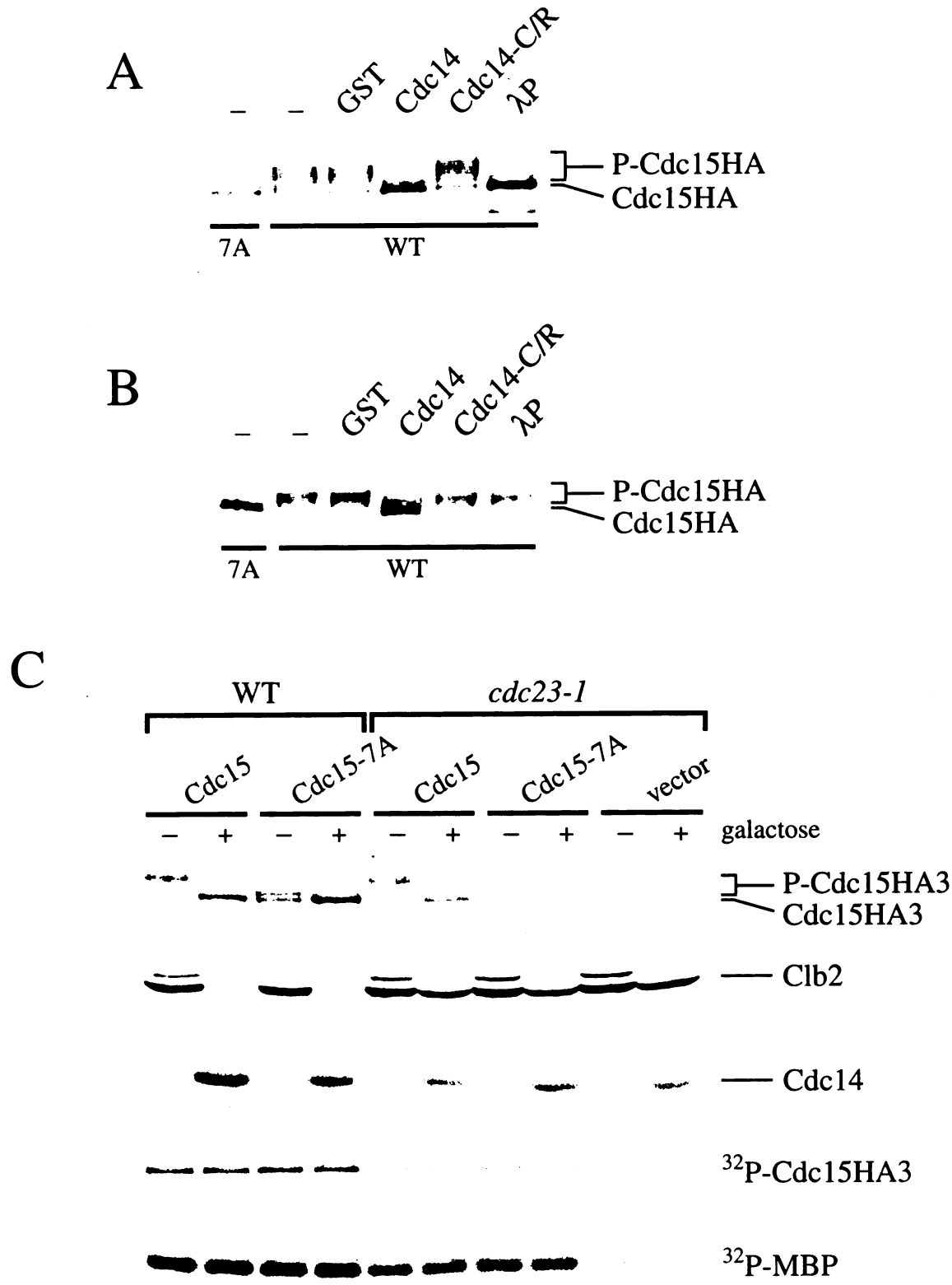

A
GST
Cdc14
Cdc14-C/R
λP
P-Cdc15HA
Cdc15HA
7A
WT
B
GST
Cdc14
Cdc14-C/R
λP
P-Cdc15HA
Cdc15HA
7A
WT
C
WT
cdc23-1
Cdc15
Cdc15-7A
Cdc15
Cdc15-7A
vector
galactose
P-Cdc15HA3
Cdc15HA3
Clb2
Cdc14
32P-Cdc15HA3
32P-MBP

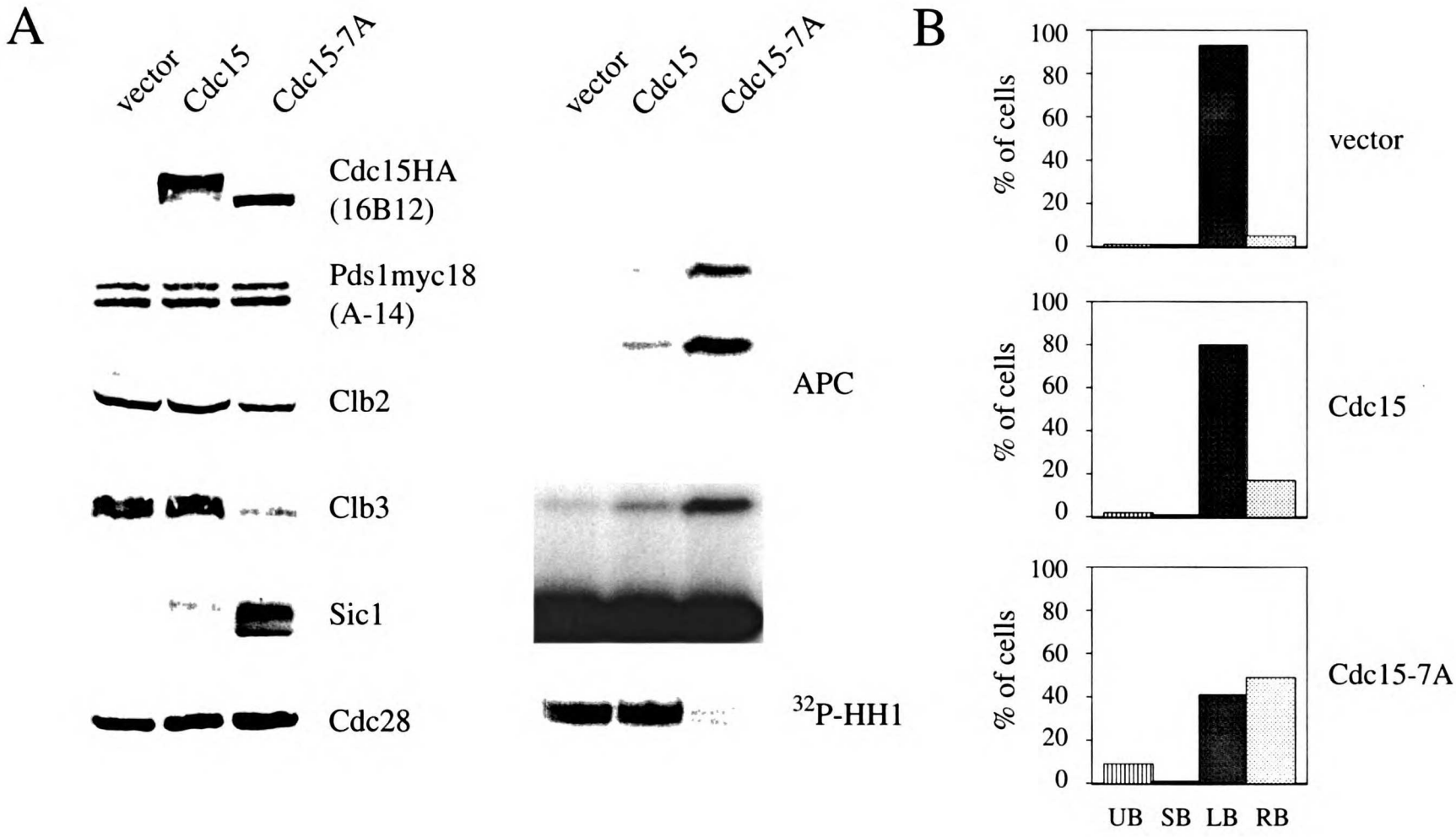
Figure 4-5. Cdc15-7A promotes Cdk inactivation *in vivo*.

(A) A *pds1Δ::PDS1myc18* strain containing vector, *GAL-CDC15HA*, or *GAL-CDC15HA-7A* was arrested in mitosis by treatment with 15 μg/ml nocodazole, followed by addition of 4% galactose for 3 hours. The levels of Cdc15, Pds1, Clb2, Clb3, Sic1, and Cdc28 in each of the extracts (50 μg) were measured by western blotting with the corresponding antibody. Cyclin-ubiquitin ligase activity of the APC was measured in anti-Cdc26 immunoprecipitates from 500 μg extract, and Cdc28-associated kinase activity toward HH1 was measured in anti-Clb2 immunoprecipitates from 100 μg lysate.

(B) Bud morphology of cells from panel A was analyzed, and bars indicate the percentage of unbudded cells (UB), small-budded cells (SB), large-budded cells (LB), or large-budded cells that have rebudded (RB).

(C) Wild-type, *hct1Δ*, and *sic1Δ* strains containing vector (V), *GAL-CDC15HA* (WT), or *GAL-CDC15HA-7A* (7A) were arrested in mitosis by treatment with 15 μg/ml nocodazole and 30 μg/ml benomyl. After 2 hours, galactose was added to 5% and cultures were grown at 30°C for an additional 3 hours. Samples were analyzed as in (A). Although approximately 50% of wild-type cells expressing Cdc15-7A rebudded, less than 10% of the *sic1Δ* and *hct1Δ* cells rebudded (data not shown).

Figure 4-5


A
vector
Cdc15
Cdc15-7A
Cdc15HA
(16B12)
Pds1myc18
(A-14)
Clb2
Clb3
Sic1
Cdc28
vector
Cdc15
Cdc15-7A
APC
32P-HH1
B
% of cells
100
80
60
40
20
0
vector
Cdc15
Cdc15-7A
UB
SB
LB
RB

C

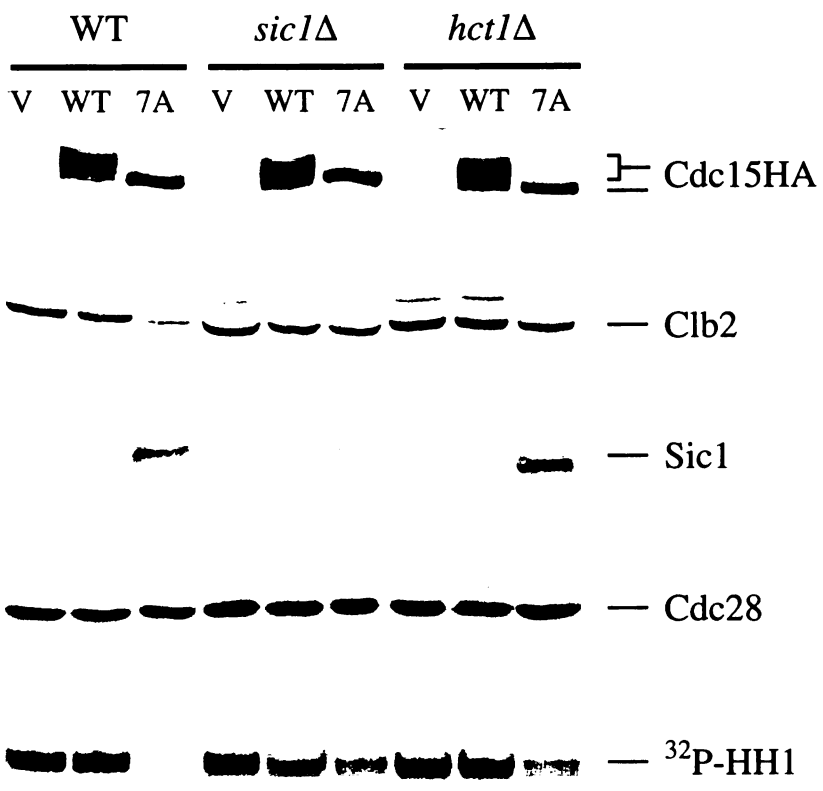

WT
sic1Δ
hct1Δ
V WT 7A V WT 7A V WT 7A
Cdc15HA
Clb2
Sic1
Cdc28
32P-HH1

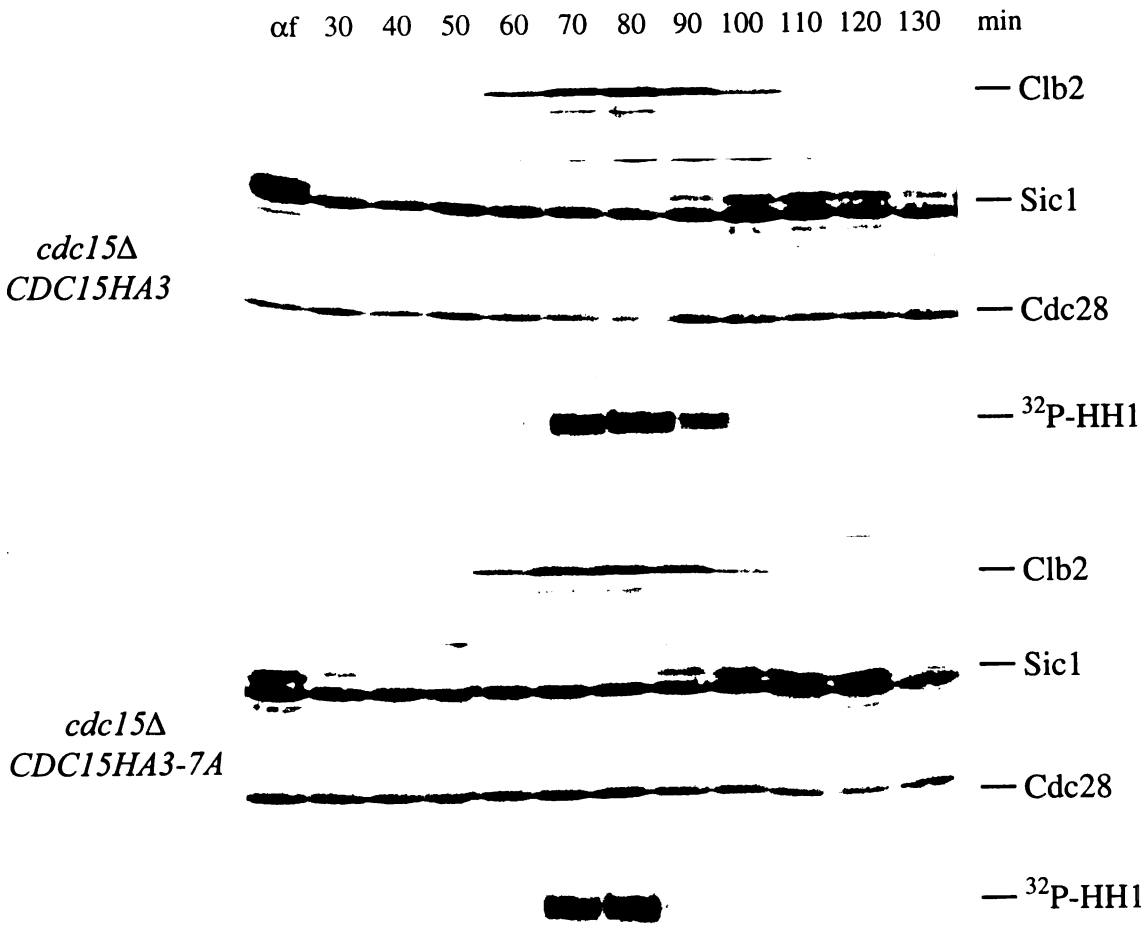
Figure 4-6. Phenotype of *cdc15Δ CDC15HA3-7A*.

(A) *cdc15Δ CDC15HA3* or *cdc15Δ CDC15HA3-7A* cells were synchronized in G1 by treatment with 5 μg/ml α-factor for 3 hours at 25°C. Cells were released into fresh media at 25°C and cell lysates were prepared from samples taken every 10 minutes. 5 μg/ml α-factor was added back to the culture at 60 minutes. Clb2, Sic1, and Cdc28 were detected by immunoblotting (25 μg). Kinase activity was measured in anti-Clb2 immunoprecipitates (100 μg).

(B) Log phase cells with the indicated genotypes (generated through genetic crosses, see Materials and Methods for details) were serially diluted three-fold and spotted onto YPD plates. Plates were incubated for 2 days at 23°C or 1.5 days at 37°C.

Figure 4-6

A


αf 30 40 50 60 70 80 90 100 110 120 130 min
— Clb2
— Sic1
cdc15Δ
CDC15HA3
— Cdc28
— 32P-HH1
— Clb2
— Sic1
cdc15Δ
CDC15HA3-7A
— Cdc28
— 32P-HH1

B

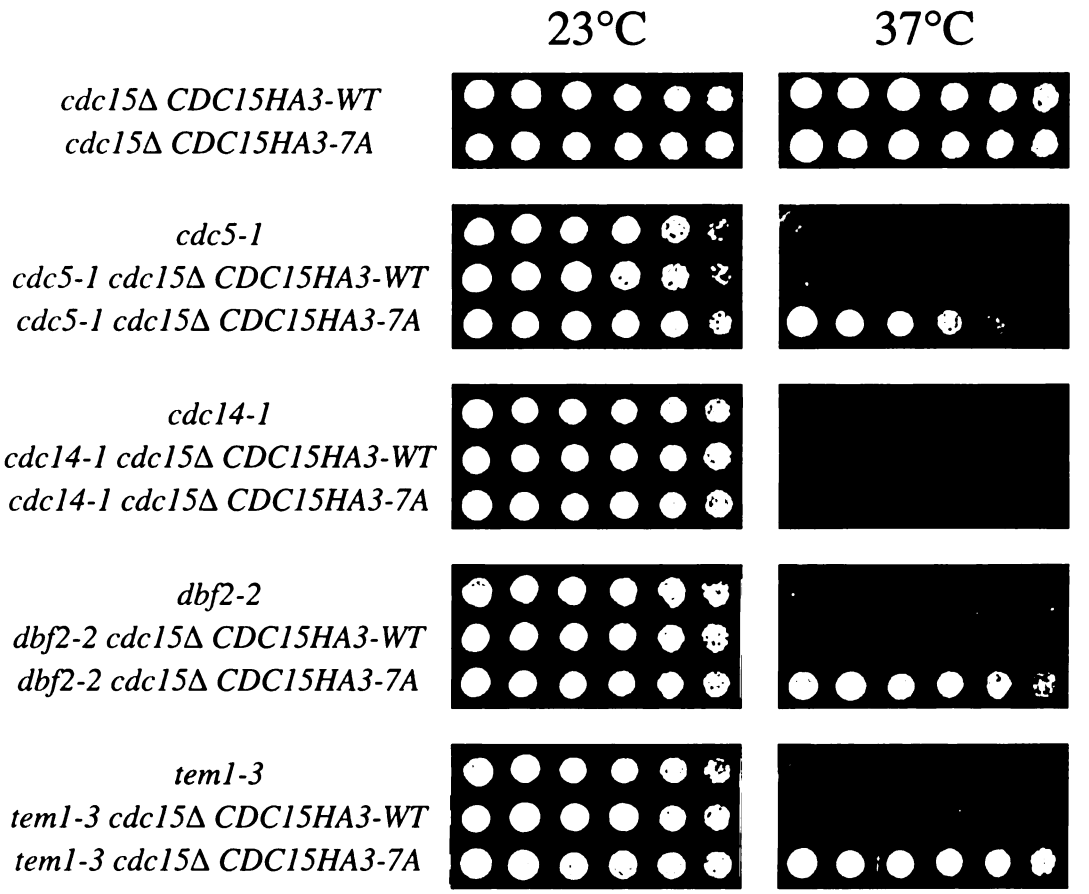
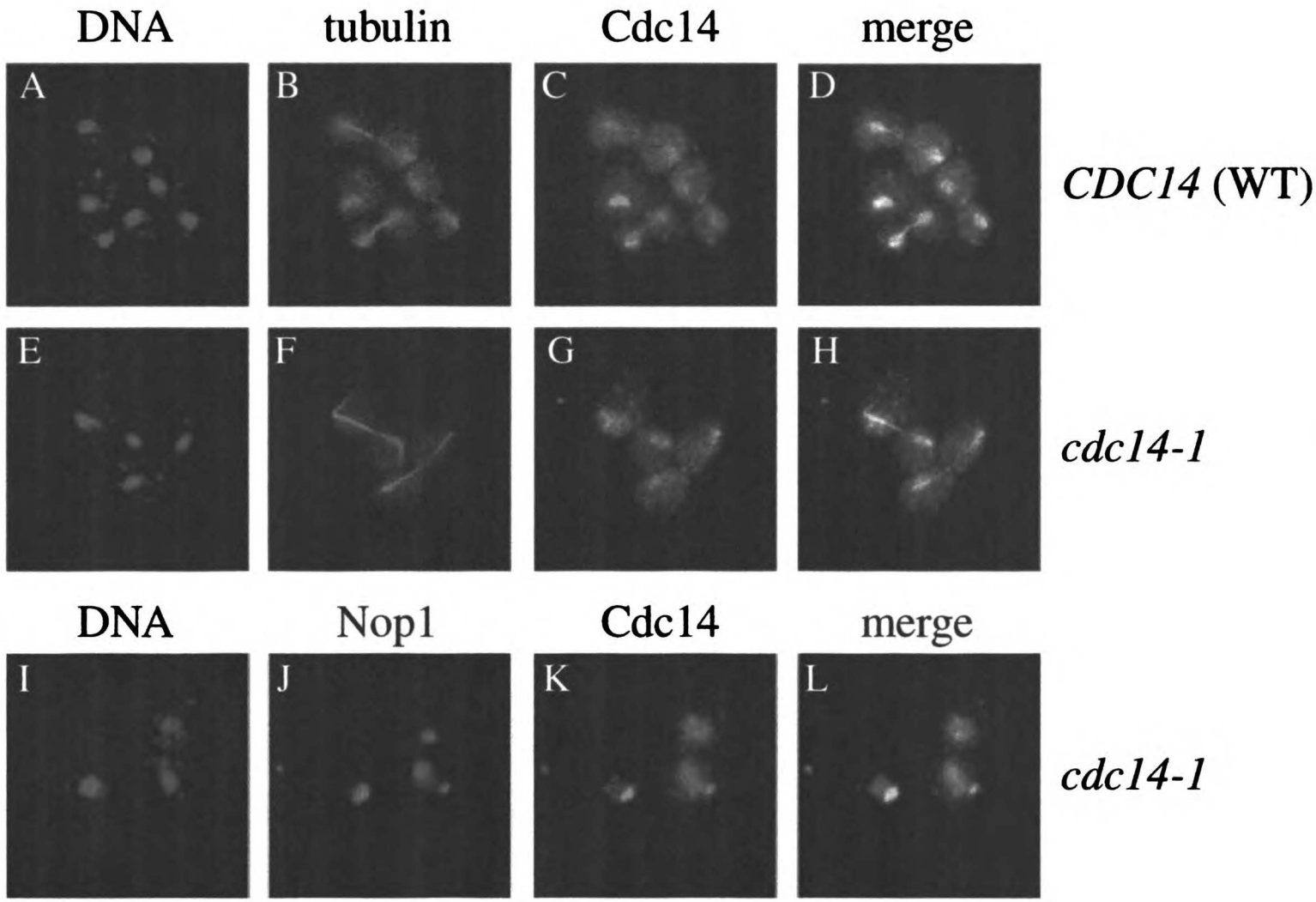

23°C
37°C
cdc15Δ CDC15HA3-WT
cdc15Δ CDC15HA3-7A
cdc5-1
cdc5-1 cdc15Δ CDC15HA3-WT
cdc5-1 cdc15Δ CDC15HA3-7A
cdc14-1
cdc14-1 cdc15Δ CDC15HA3-WT
cdc14-1 cdc15Δ CDC15HA3-7A
dbf2-2
dbf2-2 cdc15Δ CDC15HA3-WT
dbf2-2 cdc15Δ CDC15HA3-7A
tem1-3
tem1-3 cdc15Δ CDC15HA3-WT
tem1-3 cdc15Δ CDC15HA3-7A

Figure 4-7. Dephosphorylation of Cdc15 is not required for Cdc14 release from the nucleolus.

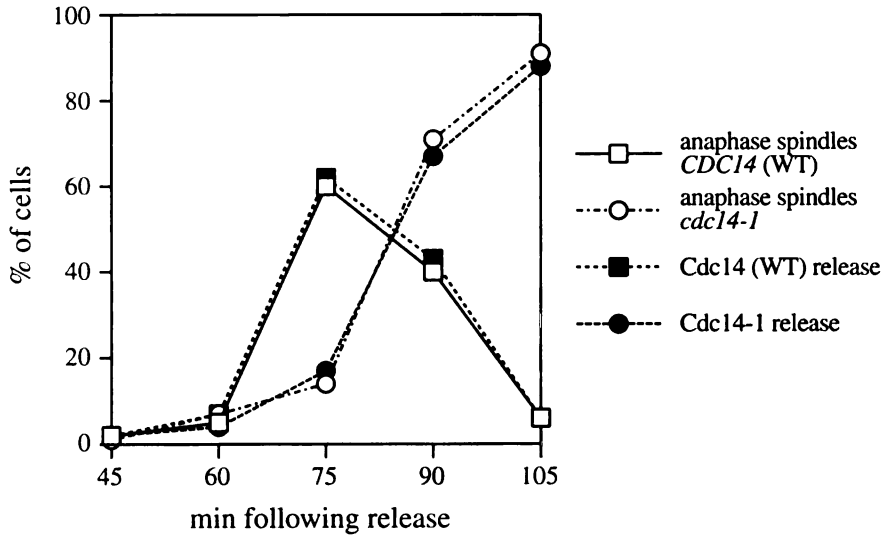
Cells in which the endogenous wild-type *CDC14* (*Mat***a** *bar1 CDC14::CDC14-GFP*) or mutant *cdc14-1* (*Mat***a** *bar1 cdc14-1::cdc14-1-GFP*) gene is tagged at the C-terminus with GFP were synchronized in G1 by treatment with 1 μg/ml α-factor for 3 hours at 23°C, then released into fresh media at 23°C. Thirty minutes following release, cultures were shifted to 34°C to inactivate *cdc14-1*, and 50 minutes following release, 1 μg/ml α-factor was added to re-arrest wild-type cells in the subsequent G1. Samples withdrawn every 15 minutes were fixed and analyzed by indirect immunofluorescence microscopy to determine the morphology of the mitotic spindle and the localization of Cdc14-GFP and Cdc14-1-GFP at each time point. Images from wild-type *CDC14* cells (A-D) and mutant *cdc14-1* cells (E-H) 90 minutes following release from α-factor are shown. In panels A & E, DNA was stained with DAPI; in B & F, the mitotic spindle was detected using anti-tubulin antibodies; and in C & G, Cdc14 was visualized using polyclonal anti-GFP antibodies. (I-L) To verify that the nucleolus was still intact in *cdc14-1-GFP* cells at the mutant arrest point, mutant cells from the 90 minute time point were double-labeled with antibodies that recognize the nucleolar resident protein, Nop1 (J), and with anti-GFP antibodies to detect Cdc14-1-GFP (K). Note that Cdc14-1-GFP is nucleolar and colocalizes with Nop1 in cells that have not reached anaphase (left cell, I-L). Like wild-type Cdc14, Cdc14-1-GFP is efficiently released from the nucleoulus once cells undergo anaphase (K, right cell). No anti-GFP signal was detected in cells lacking the tagged Cdc14 (data not shown). We were also able to observe Cdc14-GFP and Cdc14-1-GFP release in similar experiments performed on living cells (data not shown). (M) The percentage of cells containing anaphase spindles (open symbols) and delocalized Cdc14 (closed symbols) at each time point was quantitated. Greater than 200 cells were counted at each time point. (N) At the indicated times, samples were taken and the levels of Cdc14, Clb2, and Cdc28 were

determined by western blotting with C163 anti-GFP, anti-Clb2, and anti-Cdc28 antibodies, respectively. In parallel experiments (data not shown), we confirmed that Cdc15 is highly phosphorylated in *cdc14-1* cells arrested at 34°C, as it is at 37°C (Figure 4-2B).

Figure 4-7


DNA
tubulin
Cdc14
merge
A
B
C
D
CDC14 (WT)
E
F
G
H
cdc14-1
DNA
Nop1
Cdc14
merge
I
J
K
L
cdc14-1

M


100
80
60
40
20
0
% of cells
45
60
75
90
105
min following release
anaphase spindles CDC14 (WT)
anaphase spindles cdc14-1
Cdc14 (WT) release
Cdc14-1 release

N

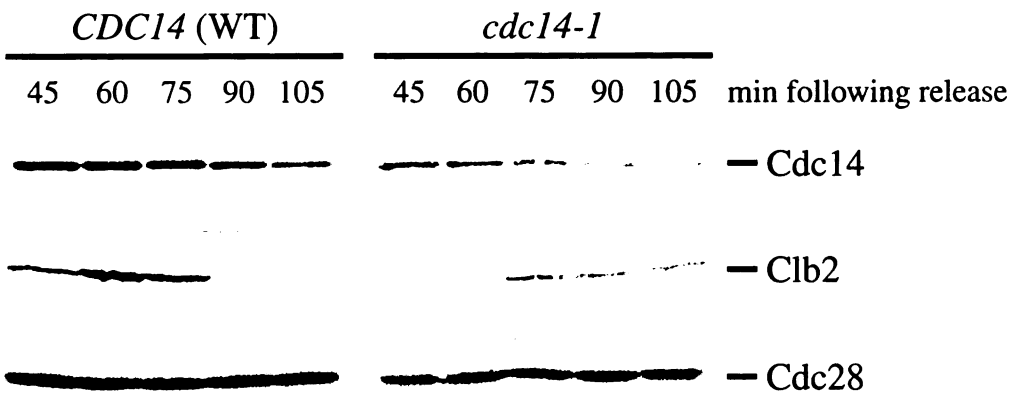
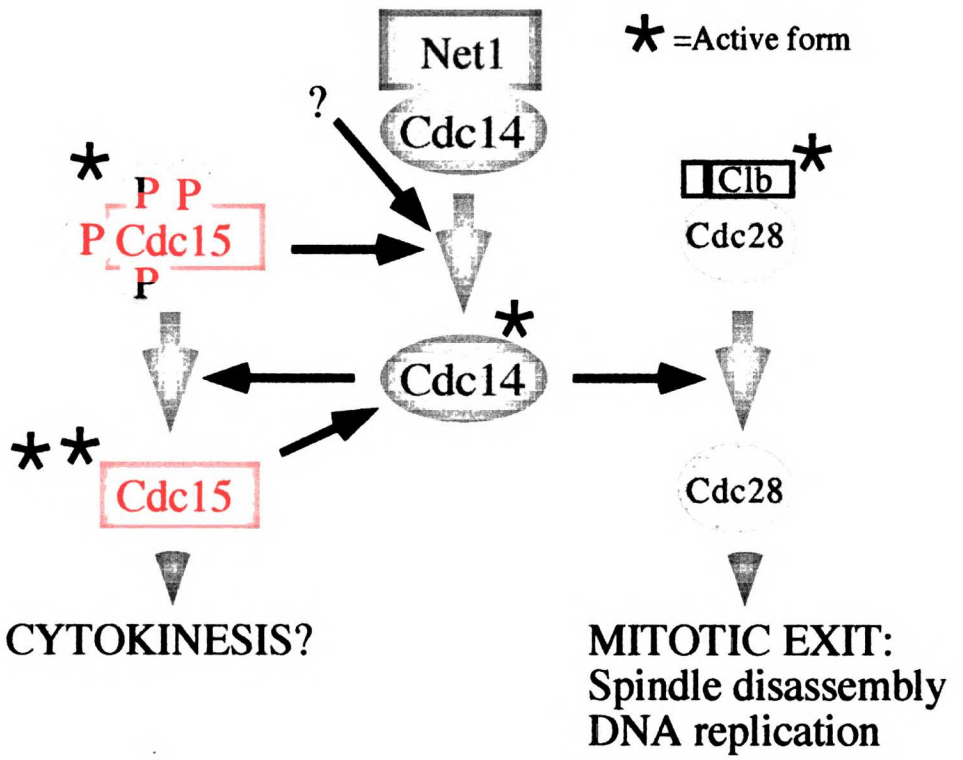

CDC14 (WT)
cdc14-1
45 60 75 90 105
45 60 75 90 105
min following release
Cdc14
Clb2
Cdc28

Figure 4-8. Model of the regulatory pathways governing mitotic exit and cytokinesis.

At the end of anaphase, phosphorylated Cdc15 helps promote the activation of Cdc14, which then dephosphorylates and activates proteins involved in Cdk inactivation, leading to spindle disassembly and other events of mitotic exit. Activation of Cdc14 also leads to dephosphorylation and activation of Cdc15. Although this relationship appears to set up a positive feedback loop, our evidence argues that this loop is not essential for Cdc14 activation *in vivo*. Instead, we speculate that dephosphorylation of Cdc15 by Cdc14 promotes a second Cdc15 function in cytokinesis, as suggested by recent studies in budding yeast (Jiménez et al., 1998; Lippincott and Li, 1998) and fission yeast (Fankhauser and Simanis, 1994; Sohrmann et al., 1998).

Figure 4-8


Net1
Cdc14
?
* =Active form
* P P
P Cdc15
P
Clb
*
Cdc28
*
Cdc14
**
Cdc15
Cdc28
CYTOKINESIS?
MITOTIC EXIT:
Spindle disassembly
DNA replication

Chapter Five

Conclusions

The transition from M phase to G1 requires inactivation of mitotic cyclin-dependent kinases (Cdks). Regulation of Cdk inactivation must be coupled to mitotic progression to ensure that cell division does not occur prior to the completion of chromosome segregation. In the budding yeast *Saccharomyces cerevisiae*, Cdc28-Clb kinase activity is inhibited following anaphase by at least two mechanisms: anaphase-promoting complex (APC)-mediated destruction of mitotic cyclins and binding of the Clb-specific Cdk inhibitor, Sic1 (Morgan, 1999; Zachariae and Nasmyth, 1999). Cdc28 inactivation by these two mechanisms is negatively regulated throughout mitosis by Cdc28-dependent phosphorylation (Moll et al., 1991; Schwob et al., 1994; Amon, 1997; Zachariae et al., 1998a; Jaspersen et al., 1999). Following completion of anaphase, activation of the late mitotic network increases Cdc14 phosphatase activity, and Cdc14 reverses the effects of Cdc28 to trigger activation of the APC and accumulation of Sic1 (Visintin et al., 1998; Jaspersen et al., 1999; Shou et al., 1999; Visintin et al., 1999). Inactivation of Cdc28 is required for initiation of cytokinesis (Ghiara et al., 1991; Lew and Reed, 1993); however, the mitotic exit network may also positively regulate cell division through mechanisms distinct from Cdc28 regulation (discussed below).

Hct1 phosphorylation

A non-phosphorylatable mutant form of Hct1 triggers premature APC activation and cyclin destruction, arguing that Cdc28-Clb phosphorylation of Hct1 is one important mechanism used to regulate Cdc28 inactivation (Zachariae et al., 1998a; Jaspersen et al., 1999). During mitosis, Hct1 activation occurs following Clb5 destruction by APC-Cdc20 at the metaphase-to-anaphase transition (Shirayama et al., 1999). Interestingly, Hct1 is most effectively phosphorylated by Cdc28-Clb5 *in vitro* (Zachariae et al., 1998a), suggesting that regulation of Hct1 by Clb5 may ensure that mitotic cyclin destruction does not occur prior to chromosome segregation.

However, Hct1 phosphorylation cannot be the only mechanism regulating the timing of cyclin destruction. In the early embryonic cell cycles of *Xenopus* and *Drosphilia*, cyclin proteolysis occurs in perfectly timed waves despite the fact that Hct1 is not present (Sigrist and Lehner, 1997; Lorca et al., 1998). Even in yeast and vertebrate somatic cells, careful analysis of the timing of cyclin destruction reveals that B-type cyclin levels decline much earlier in mitosis than previously believed; destruction of one pool of cyclin B begins in metaphase and is likely catalyzed by APC-Cdc20 (Clute and Pines, 1999; Yeong et al., 2000). These results tell us two important things about the regulation of cyclin proteolysis. First, Hct1 and Cdc20 are more than just substrate specificity factors. Second, the timing of cyclin destruction during the cell cycle is regulated a multiple levels. Regulation of the APC, Cdc20, Hct1, and even the substrate likely contribute to the timing of mitotic cyclin proteolysis.

The late mitotic network controls Cdc28 inactivation and mitotic exit

In *S. cerevisiae*, the timing of cyclin destruction and exit from mitosis are also regulated by a group of proteins known as the mitotic exit network or the late mitotic family. A complex series of genetic interactions between the late mitotic mutants indicate that they function in a common pathway to promote mitotic exit (Kitada et al., 1993; Shirayama et al., 1994b; Shirayama et al., 1996; Grandin et al., 1998; Jaspersen et al., 1998; Luca and Winey, 1998). Multiple lines of evidence suggest that the primary defect in mitotic exit mutants is an inability to inactivate Cdc28. Late mitotic mutants all arrest with high levels of mitotic cyclins due to an inability to activate APC-Hct1 (Surana et al., 1993; Shirayama et al., 1994b; Toyn and Johnston, 1994; Charles et al., 1998; Jaspersen et al., 1998). Accumulation of Sic1 is also blocked in the mutants, and increasing Sic1 levels by overexpression of *SIC1* is sufficient to rescue the growth defect of most of the mutants (Donovan et al., 1994; Toyn et al., 1996; Charles et al., 1998; Jaspersen et al., 1998). Cdc28 inactivation by the mitotic exit network may occur through the late mitotic protein,

Cdc14. The fact that release of Cdc14 from the nucleolus requires the activities of all the other late mitotic mutants and that overexpression of Cdc14 is able to rescue the growth defect of most mutants suggest Cdc14 is the downstream effector of the network (Shirayama et al., 1996; Grandin et al., 1998; Jaspersen et al., 1998; Visintin et al., 1998; Shou et al., 1999; Visintin et al., 1999). How the late mitotic network leads to Cdc14 activation is currently not understood: what triggers activation by the network, does the network form a classical signaling pathway or are there multiple parallel pathways leading to Cdc14 activation, how is the Net1-Cdc14 complex disrupted, does Cdc14 require additional activation? In addition, the late mitotic network might also promote Cdk inactivation at other steps; Cdc5 homologs in higher eukaryotes directly phosphorylate and activate the APC core (Descombes and Nigg, 1998; Kotani et al., 1998; Kotani et al., 1999).

The phosphatase Cdc14 is the only component of the mitotic exit network that has been shown to directly control Cdc28 inactivation. Cdc14 reverses the inhibitory effects of Cdc28 on Sic1, Swi5, and Hct1 to promote cyclin destruction and Sic1 accumulation at the end of mitosis (Visintin et al., 1998; Jaspersen et al., 1999). Increasing Cdc14 activity in anaphase is required to trigger Cdc28 inactivation, but is this the only function of Cdc14? Many additional aspects of mitotic progression, including bipolar spindle formation and inhibition of cytokinesis, are regulated by Cdc28 (Lew et al., 1997). Exit from mitosis must require dephosphorylation of these Cdc28 substrates. The ability of Cdc14 to remove phosphates from Cdk sites and its activation in late mitosis make it an ideal candidate to initiate spindle disassembly and cytokinesis by reversing the effects of Cdc28. Cdc14 may also have additional indirect roles in exit from mitosis, by controlling the phosphorylation state of other mitotic regulators such as Cdc15 or Mob1 (Chapter 4, R. Menssen & W. Seufert, personal communication, F. Luca & M. Winey, personal communication).

Regulation of Cdc14

Despite the fact that activation of Cdc14 is one of the rate-limiting steps in Cdk inactivation, little is known about its regulation. Association with Net1 is currently the only known mechanism of Cdc14 regulation (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999). Net1 binding keeps Cdc14 sequestered in the nucleolus during most of the cell cycle. Following anaphase, the Net1-Cdc14 complex dissociates and Cdc14 is redistributed into the nucleus and cytoplasm. A plausible mechanism of Cdc14 regulation by Net1 might be that a late mitotic kinase or Cdc28 phosphorylates Net1 to disrupt the Cdc14-Net1 interaction and cause Cdc14 relocalization. Evidence for this mechanism of regulation comes from observations that the activities of the late mitotic kinases and Cdc28 are high, Net1 is highly phosphorylated, and Cdc14 has been redistributed in cells lacking Cdc14 catalytic function (Grandin et al., 1998; Shou et al., 1999; Jaspersen and Morgan, 2000). In this scheme, a drop in kinase activity after cells exit mitosis allows re-accumulation of hypophosphorylated Net1, which re-sequesters Cdc14 in the nucleolus. It is currently not know if Net1 binding simply regulates the accessibility of Cdc14 to its substrates, or if it also directly inhibits Cdc14 activity (Shou et al., 1999).

Several preliminary experiments suggest that localization of Cdc14 to the nucleolus by Net binding may not be the only mechanism of Cdc14 regulation. Delocalization of Cdc14 in *cdc20Δ pds1Δ* arrested cells is not sufficient to cause an increase in Cdc14 activity, measured *in vitro*; these cells have comparable amounts of Cdc14-associated phosphatase activity to *cdc20Δ* cells, which arrest in metaphase with inactive, nucleolar Cdc14 (Shirayama et al., 1999) (J. Charles & D. Morgan, personal communication). Comparisons of phosphatase activity from various cell cycle arrests show that Cdc14 activity is reduced 2-5 fold in *cdc15-2* or *cdc5-1* arrested cells even though its localization remains the same in all the cells analyzed (J. Charles & D. Morgan, personal communication). One interpretation of these results is that changes in the affinity of the Net1-Cdc14 interaction during the cell cycle result in differences in recovery of Net1 (a

putative Cdc14 inhibitor) during the *in vitro* phosphatase assay. Alternatively, these results might suggest that Cdc14 activity is positively regulated at the end of mitosis by some additional mechanism, perhaps phosphorylation. Cdc14 is a phospho-protein *in vivo*, and interestingly, Cdc15 is able to phosphorylate Cdc14 *in vitro* (J. Charles, S. Jaspersen, & D. Morgan, unpublished results). However, the importance of phosphorylation on Cdc14 activity and the significance of this mechanism of regulation *in vivo* have not been determined. Additional mechanisms of Cdc14 regulation might be particularly important in higher eukaryotes where the nucleolus breaks down early in mitosis.

A late mitotic signaling pathway

Components of late mitotic network include the protein kinases Cdc5, Cdc15, and Dbf2, the GTPase Tem1, a putative two component GTPase activating protein (GAP) Bub2-Byr4, and a predicted guanine-nucleotide exchange factor (GEF) Lte1 (Johnston et al., 1990; Schweitzer and Philippsen, 1991; Kitada et al., 1993; Shirayama et al., 1994a; Shirayama et al., 1994b; Li, 1999). Based on their biochemical activities, it is tempting to speculate that mitotic exit network proteins comprise a signaling pathway that triggers Cdc14 release and Cdk inactivation. However, efforts to order the proteins into a pathway based on classical genetic epistasis analysis have been inconclusive (Kitada et al., 1993; Shirayama et al., 1994b; Shirayama et al., 1996; Grandin et al., 1998; Jaspersen et al., 1998; Luca and Winey, 1998). Overexpression studies have been equally unsuccessful, often leading to conflicting results--the order of the pathway often reflects the activity that is being assayed (Figure 5-1) (Grandin et al., 1998; Jaspersen et al., 1998; Visintin et al., 1998) (J. Charles & D. Morgan, personal communication; F. Luca & M. Winey, personal communication).

The most compelling evidence suggesting that the late mitotic proteins form part of a signaling pathway comes for work in *S. pombe*. Catalysis of Spg1 nucleotide exchange (the Tem1 homolog) *in vitro* and *in vivo* requires the activity of Cdc16 (the Bub2 homolog)

and Byr4, which form a two component GAP (Furge et al., 1998; Jwa and Song, 1998; Cerutti and Simanis, 1999). Spg1-GTP, but not Spg1-GDP, physically binds to the Cdc15 homolog, Cdc7 (Sohrmann et al., 1998). Nucleotide hydrolysis of Spg1 during mitosis results in the asymmetric localization of Cdc7 to one spindle pole body, leading to cytokinesis. Based on homology between the fission and budding yeast proteins, it seems likely that certain members of the late mitotic network form a single signaling cascade (Figure 5-1A). Whether this signaling pathway is utilized to trigger all mitotic exit functions, or simply to initiate cytokinesis (see below) is not known. Other late mitotic proteins such as Dbf2, Mob1, and Cdc5 may function upstream, downstream, or in parallel to this predicted signaling cascade. Understanding the details of the signaling pathways within the budding yeast mitotic exit network will require in-depth biochemical analysis, as well as binding and localization studies *in vivo*.

Cdk inactivation and cytokinesis

In *S. pombe*, the primary function of the mitotic exit network is septum formation and cytokinesis (Gould and Simanis, 1997; LeGoff et al., 1999). In contrast, the *S. cerevisiae* late mitotic network is required for Cdk inactivation (Morgan, 1999; Zachariae and Nasmyth, 1999). This apparent discrepancy in function raises two important and interesting questions: does the mitotic exit network regulate different aspects of the cell cycle in the two different organisms, or does the mitotic exit network have a dual function in Cdk inactivation and cytokinesis that is emphasized differently based on the physiology of the two yeasts? Addressing this issue in *S. cerevisiae* is complicated by the fact that Cdc28 inactivation triggers cytokinesis (Ghiara et al., 1991; Lew and Reed, 1993). However, several recent pieces of evidence suggest that the late mitotic network in budding yeast not only regulates Cdc28 inactivation, but also promotes cytokinesis. Cell division is defective in *tem1Δ net1-1* cells despite the fact that Cdc28 inactivation occurs normally (Shou et al., 1999). Consistent with a role for Tem1 in cytokinesis, a physical interaction

between Tem1 and components of the actomyosin ring was recently reported (Shannon and Li, 1999). Our data in Chapter 4 suggests that dephosphorylation of Cdc15 by Cdc14 promotes a cytokinesis function that may include regulating the localization of components of the actin cytoskeleton to the site of cleavage (Jiménez et al., 1998; Lippincott and Li, 1998) (R. Menssen & W. Seufert, personal communication). Cytokinesis may also require other components of the late mitotic network, including Dbf2 and Mob1, which transiently localize to the bud neck at the time of cell division (F. Luca & M. Winey, personal communication). It is currently unclear how the mitotic exit proteins promote cytokinesis. Perhaps they modify the septins, proteins in the actomyosin ring, or other cytoskeletal components. Identification of Cdc5, Cdc15, and Dbf2 substrates will inevitably help us understand how the mitotic exit network regulates Cdc28 inactivation, cytokinesis, and other aspects of late mitosis.

Timing mitotic exit

Involvement of the late mitotic network at multiple steps of mitotic exit makes sense: by coordinating Cdc28 inactivation with cytokinesis, cells ensure that cell division occurs only after the completion of chromosome segregation. But what triggers activation of the mitotic exit network following anaphase, and what prevents it from activating cyclin destruction, Sic1 accumulation, and cytokinesis in other parts of the cell cycle?

Part of the answer to these questions may be that individual components of the mitotic exit network are cell cycle regulated. Binding to Net1 sequesters Cdc14 in the nucleolus until anaphase (Shou et al., 1999; Visintin et al., 1999). Control of Dbf2 and Cdc5 transcription and phosphorylation restricts their peak of kinase activity to mitosis (Johnston et al., 1990; Kitada et al., 1993; Toyn and Johnston, 1994; Charles et al., 1998; Cheng et al., 1998; Shirayama et al., 1998). The proteins levels of Cdc15 and Mob1 do not change, but their sub-cellular localization appears to be regulated by cell cycle

dependent changes in phosphorylation (Jaspersen et al., 1998; Komarnitsky et al., 1998; Jaspersen and Morgan, 2000) (R. Menssen & W. Seufert, personal communication; F. Luca & M. Winey, personal communication). Nothing is known about the regulation of Tem1 or Lte1 activity, but Lte1 has recently been shown to localize to the bud (A. Amon, personal communication). Currently, we do not understand the mechanisms that contribute to cell cycle regulation of the mitotic exit network, nor do we know how these regulatory influences are integrated to control the timing of Cdc28 inactivation.

In addition, cell cycle periodicity may not be the only mechanism of late mitotic regulation. In cells arrested in metaphase with the microtubule depolymerizing drug nocodazole, all of the components of the mitotic exit network are present, yet cyclin destruction and Sic1 accumulation are inhibited (Fesquet et al., 1999; Fraschini et al., 1999). Since the spindle assembly checkpoint is activated in these cells, these results are easily explained: Mad2 blocks APC-Cdc20 activation, Pds1 and Clb5 remain stable, and Cdc14 stays in the nucleolus (Hwang et al., 1998; Kim et al., 1998; Shirayama et al., 1999). If Mad2 is removed from these cells to prevent inhibition of APC-Cdc20, we would expect cells to proceed into "anaphase", degrading Pds1 and Clb5, releasing Cdc14 from the nucleolus, and eventually initiating Clb2 proteolysis and Cdk inactivation (the lack of microtubules prevents chromosome segregation and spindle elongation, so the cells have not truly completed anaphase). However, deletion of Mad2 does not lead to this phenotype; Clb2 proteolysis and inactivation of its associated Cdk activity are still blocked in *mad2Δ* cells treated with nocodazole, despite the fact that both APC-Cdc20 and components of the late mitotic network are active (Alexandru et al., 1999). These results suggest the possibility of an inhibitor of the late mitotic network that prevents cyclin destruction and Cdk inactivation in cells that have not successfully completed anaphase.

In these experiments, Bub2 and Byr4, the *S. cerevisiae* homologs of the *S. pombe* two component GAP, are required to negatively regulate Cdc28 inactivation, cytokinesis, and DNA re-replication (Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al.,

1999; Li, 1999). Bub2-Byr4 function by inhibiting the activation of at least one component of the late mitotic network, Dbf2, although the fact that the effect of deleting Bub2 can be phenocopied by overproduction of Tem1 suggests Bub2-Byr4 acts upstream of multiple components of the mitotic exit network to prevent its function (Alexandru et al., 1999; Fesquet et al., 1999). Neither Bub2 nor Byr4 seem to be essential for correct cell cycle timing (Fraschini et al., 1999; Li, 1999), indicating that additional factors must regulate the mitotic exit network in its absence. One possible candidate is the GEF Lte1, a protein whose predicted biochemical function counteracts that of Bub2-Byr4 (Shirayama et al., 1994a). In this scenario, the balance of Bub2-Byr4 and Lte1 activity controls the timing of Cdc28 inactivation possibly by regulating the nucleotide state of Tem1. Downregulation of Bub2-Byr4 and/or upregulation of Lte1 promotes accumulation of Tem1-GTP, cyclin destruction, and Sic1 accumulation, while increases in Bub2-Byr4 activity and/or decreases in Lte1 function have the opposite effect (Figure 5-1A). The contributions of Bub2-Byr4 and Lte1 activity to the control of Cdk inactivation are easily tested *in vivo*. Does Lte1 overproduction and/or Bub2-Byr4 loss of function deregulate cyclin destruction? Does this effect depend on Tem1? In *lte1Δ* and/or *GAL-BUB2* cells, is the timing of Cdk inactivation altered? Is this effect exacerbated by Tem1 mutants?

To understand how the mitotic exit network regulates Cdc28 activation, we must explore the mechanisms regulating Bub2-Byr4 and Lte1 function. How does Bub2-Byr4 inhibit the function of the late mitotic network? What regulates the function of this two-component GAP? Are there other GAPs that can substitute for Bub2-Byr4 to ensure the correct timing of mitotic exit? How is Lte1 regulated? Does localization affect its activity? Do Bub2-Byr4 and Lte1 regulate the nucleotide state of Tem1? What does Bub2-Byr4/Lte1 "sense" in the cell to trigger Cdc28 inactivation only following completion of anaphase? In the remainder of this Chapter, I will speculate on the answers to some of these questions and describe how I believe Bub2-Byr4, Lte1, and the rest of the mitotic exit network function to control the events of late mitosis.

A cytokinesis checkpoint

Detailed kinetic analysis of nuclear movements during the yeast cell cycle indicates that cells have a checkpoint to delay cytokinesis until nuclear translocation into the bud is complete (Yeh et al., 1995). In dynein heavy chain or other nuclear positioning mutants, spindle elongation is often uncoupled from nuclear migration in the bud (Muhua et al., 1994; Yeh et al., 1995; Miller and Rose, 1998; Muhua et al., 1998; Miller et al., 1999); a delay in cytokinesis until the spindle has correctly positioned itself between the mother and daughter cell is critical to prevent inaccurate transmission of genetic material. Genetic interactions between Bub2 and mutants that affect various aspects of spindle function suggest that Bub2 might monitor some aspect of spindle elongation or positioning and prevent activation of the mitotic exit network until the defect is corrected (Figure 5-2) (D. Thompson & A. Murray, personal communication). By monitoring spindle dynamics, the Bub2 checkpoint would ensure that Cdk inactivation and initiation of cytokinesis are coupled to successful completion of anaphase. It would help ensure that chromosomes, as well as the spindle pole bodies (SPB), are equally partitioned between the two cells.

One possible signal for the successful completion of anaphase is positioning of the two SPBs at the distal tip of the mother and daughter cell. Components of the mitotic exit network are ideally suited to receive this signal since many, including Bub2 and Tem1, are localized to the SPB (Shirayama et al., 1998; Fraschini et al., 1999; Li, 1999) (R. Menssen & W. Seufert, personal communication; F. Luca & M. Winey, personal communication; A. Amon, personal communication). In addition, localization of Lte1 exclusively to the bud could ensure that upregulation of the late mitotic network occurs only following passage of one SPB into the daughter cell. Localization of Cdc15 to the SPB seems to be cell cycle regulated in a manner somewhat reminiscent of Cdc7. Cdc15 is found on the old/mother SPB throughout the cell cycle; following Cdc14 activation and anaphase, Cdc15 also localizes to the new/daughter SPB (R. Menssen & W. Seufert, personal communication). Interestingly, many subunits of the APC are also found at the centrosomes (the SPB

equivalent) in higher eukaryotes (Tugendreich et al., 1995; Jorgensen et al., 1998), and destruction of cyclin B begins spatially at the spindle pole (Su et al., 1998; Clute and Pines, 1999; Huang and Raff, 1999). So perhaps, elongation of the spindle into the bud results in late mitotic activation by shifting the balance of Bub2-Byr4 and Lte1 activity to a state that favors nucleotide exchange. Binding of Tem1 to GTP on the new SPB recruits Cdc15 to the daughter SPB. Signaling through the late mitotic network results in activation of the APC, Cdk inactivation, and cytokinesis (Figure 5-2).

Figure 5-1. Predicted mitotic exit network signaling pathways.

Analysis of different aspects of the mitotic exit network has suggested the following pathways might contribute to late mitotic signaling. These pathways are not mutually exclusive, and may be used to differentially regulate certain aspects of mitotic exit.

(A) Biochemical and genetic analysis of *S. pombe* homologs of the late mitotic proteins indicates that Bub2-Byr4 forms a two component GAP that regulates the nucleotide state of Tem1. Tem1 overexpression can rescue *Δlte1*, suggesting that Lte1 is the Tem1 GEF. In the GTP-bound form, Tem1 binds to Cdc15 to trigger cytokinesis. Other components of the mitotic exit network may act downstream of Tem1-GTP/Cdc15 or function in a parallel pathway.

(B) Analysis of the pattern of Mob1 phosphorylation and localization during mitosis suggests that Cdc15 functions upstream of Cdc5, Cdc14, and Dbf2. Hypo-phosphorylation of Mob1 in *cdc15-2* cells prevents Mob1 from localizing to the SPB. Intermediate levels of Mob1 phosphorylation are observed in *cdc5-1* and *cdc14-1* arrested cells, and Mob1 is localized to one SPB. In *dbf2-2* mutants, Mob1 is hyperphosphorylated and localized to both SPBs. The function of Mob1 at the SPB is unclear, although it has been hypothesized to regulate SPB duplication.

(C) Cdc14 activation requires the functions of Cdc5, Dbf2, Tem1, and Cdc15. Cdc14 release from the nucleolus may be signaled by these proteins through a cascade similar to that in (A), or multiple different pathways may regulate Cdc14 redistribution. Components of mitotic exit network may function to disrupt the Net1-Cdc14 complex, and/or they may also be required for additional levels of Cdc14 activation (see text).

Figure 5-1

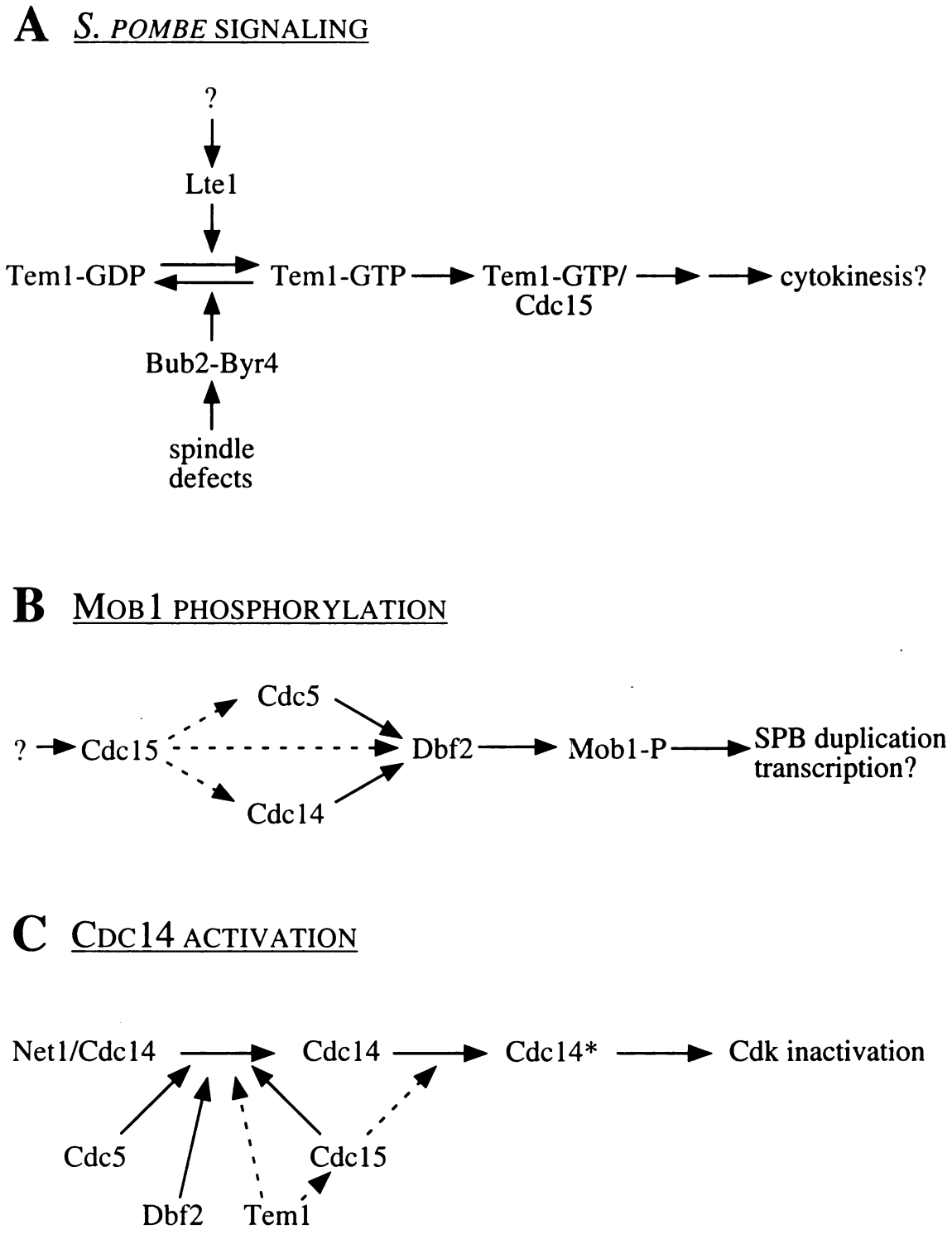
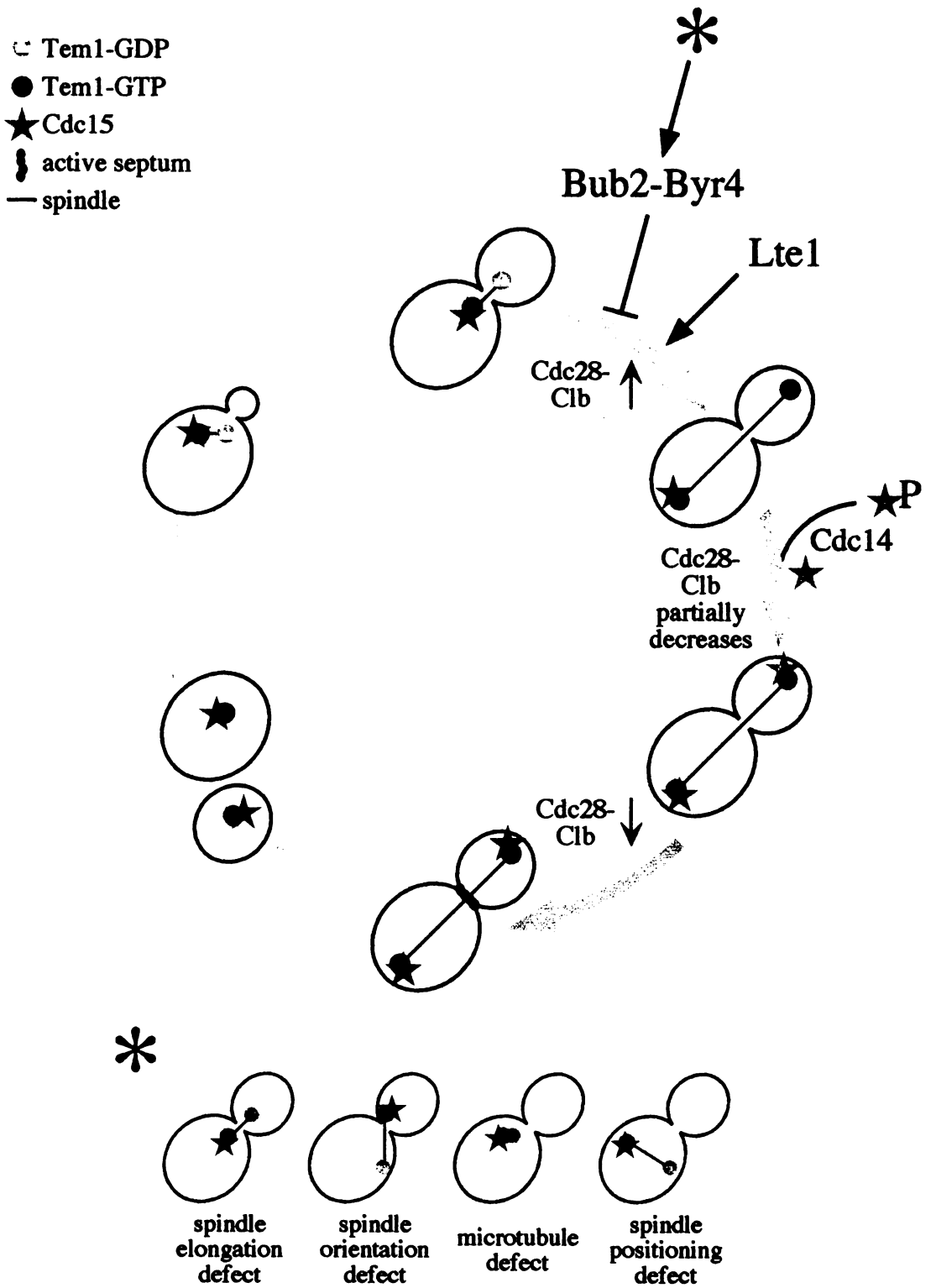

A S. POMBE SIGNALING
?
Lte1
Tem1-GDP
Tem1-GTP
Tem1-GTP/
Cdc15
cytokinesis?
Bub2-Byr4
spindle
defects
B MOB1 PHOSPHORYLATION
Cdc5
?
Cdc15
Dbf2
Mob1-P
SPB duplication
transcription?
Cdc14
C CDC14 ACTIVATION
Net1/Cdc14
Cdc14
Cdc14*
Cdk inactivation
Cdc5
Cdc15
Dbf2
Tem1

Figure 5-2. A speculative model: regulated localization of components of the mitotic exit network controls completion of M phase in *S. cerevisiae*.

Tem1 is localized to the SPB throughout the cell cycle, however Bub2 and Lte1 regulate its nucleotide state. Tem1-GTP and Cdc15 remain on the old SPB from the previous cell cycle, but Tem1-GTP is inhibited from accumulating on the new SPB until mitosis due to high levels of Bub2 activity and/or low levels of Lte1 function. Elongation of the mitotic spindle into both the mother and the daughter cell triggers Bub2 downregulation and/or upregulation of Lte1, leading to Tem1 nucleotide exchange on the new SPB. Dephosphorylation of Cdc15 by Cdc14 increases its affinity for Tem1-GTP on the new SPB. By an unknown mechanism, binding of Tem1-GTP/Cdc15 to the new SPB promotes Cdk inactivation and cytokinesis. Defects in spindle elongation, orientation, or positioning are detected by the Bub2 checkpoint: activation of Bub2 inhibits the mitotic exit network and delays Cdk inactivation and cytokinesis until the defect is corrected.

Figure 5-2


Tem1-GDP
Tem1-GTP
Cdc15
active septum
spindle
Bub2-Byr4
Lte1
Cdc28-
Clb
P
Cdc14
Cdc28-
Clb
partially
decreases
Cdc28-
Clb
spindle
elongation
defect
spindle
orientation
defect
microtubule
defect
spindle
positioning
defect

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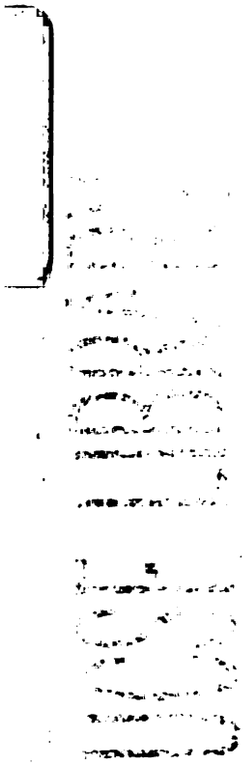

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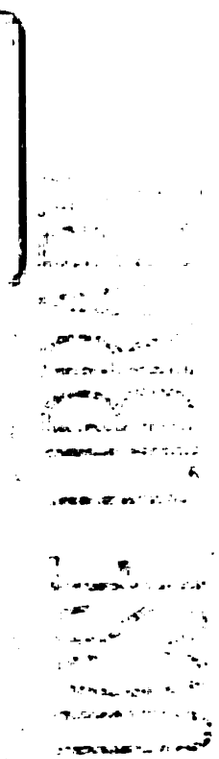

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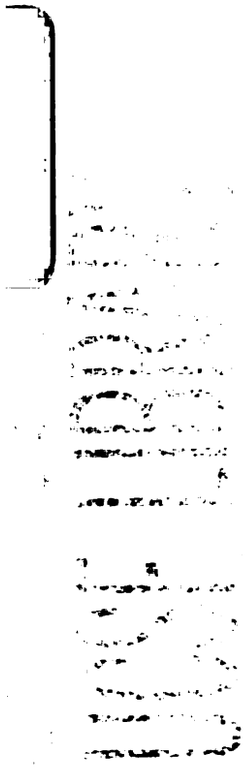

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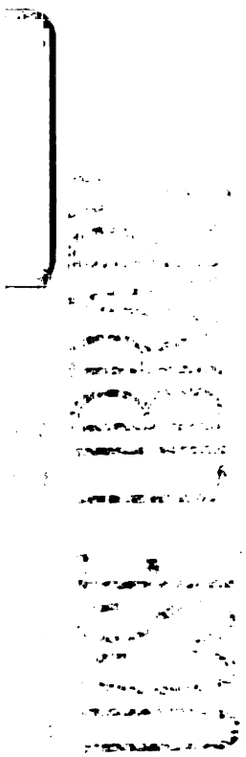

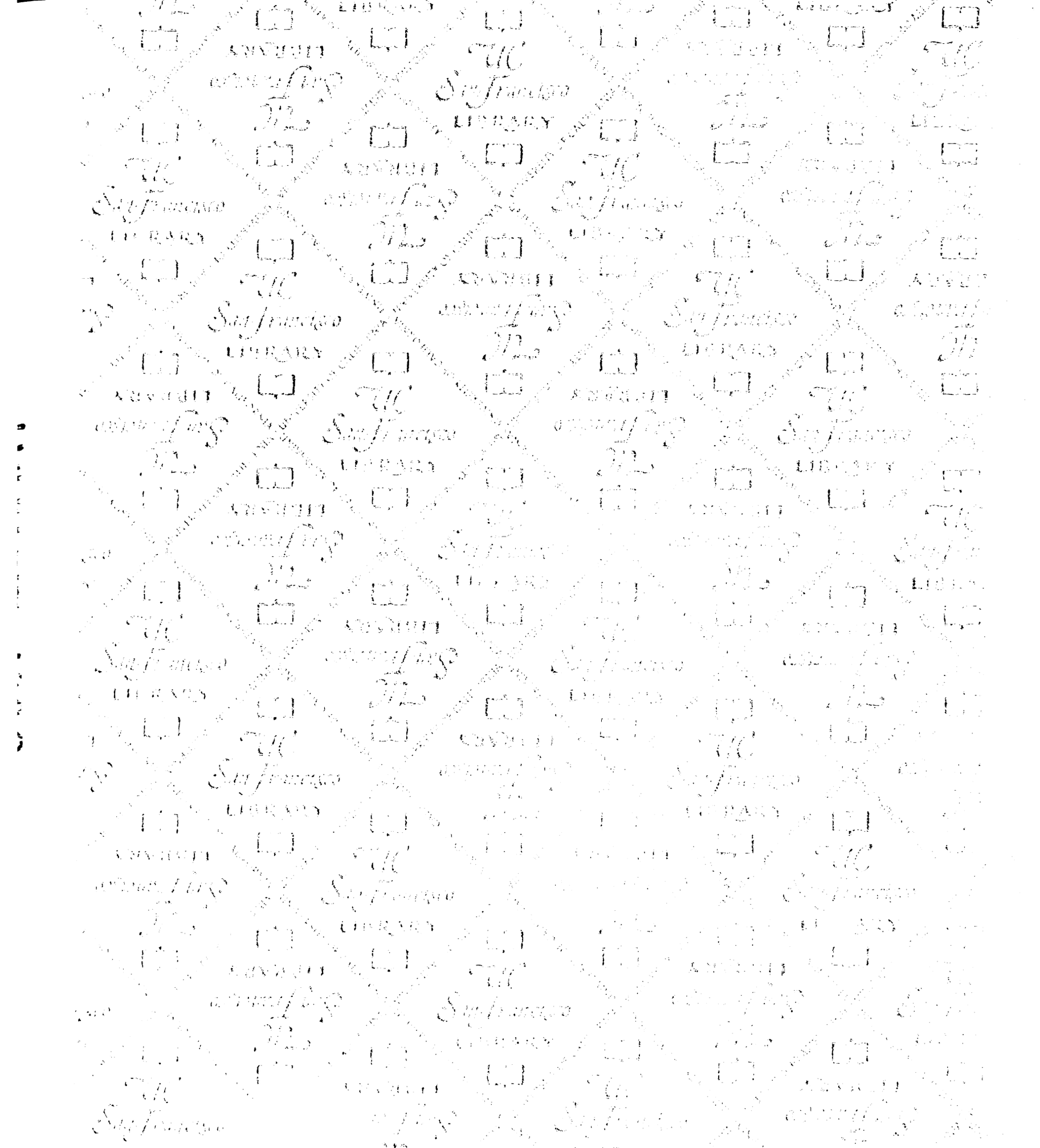
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