Title
Cdc28 activates the exit from mitosis in budding yeast

Author
Rudner, Adam Daniel

Publication Date
2000

Peer reviewed|Thesis/dissertation

Cdc28 activates the exit from mitosis in budding yeast

by

Adam Daniel Rudner

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

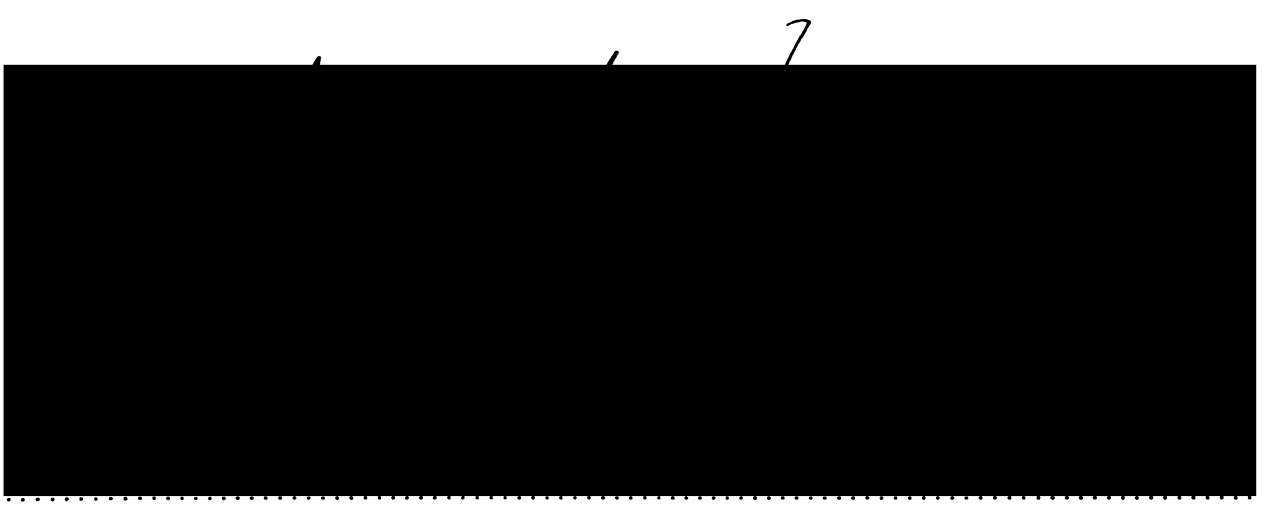
Genetics

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA SAN FRANCISCO



Date University Librarian

Degree Conferred: .....

Cdc28 activates the exit from mitosis in budding yeast

Adam D. Rudner

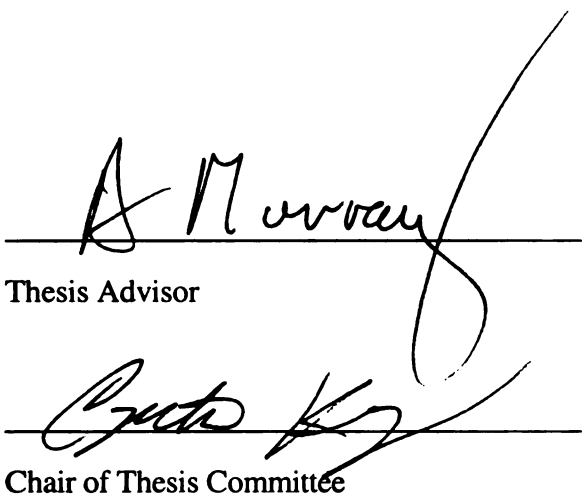
Abstract

Cdc28 regulates all key cell cycle transitions in the budding yeast,*Saccharomyces cerevisiae*. Cdc28 activity is required both to enter mitosis and to exit from mitosis. To exit mitosis, cells must accomplish two tasks: chromosome segregation and inactivation of complexes between mitotic cyclins and Cdc28, which allows the cell cycle to progress into G1. Both steps require the activity of the anaphase promoting complex (APC). The first step requires activation of the Cdc20-dependent APC, which then triggers the activation of the Hct1-dependent APC and the second step. This thesis utilizes genetic and biochemical analyses to show that Cdc28 activates the Cdc20-dependent APC and therefore is required to induce the metaphase to anaphase transition. It also explores the role of inhibitory phosphorylation on Cdc28 in mitotic progression.

CDC28-T18V, Y19F (*CDC28-VF*), a mutant that lacks inhibitory phosphorylation sites, delays the exit from mitosis. This behavior is not due to a lack of inhibitory phosphorylation or increased kinase activity, but reflects reduced Cdc20-dependent APC activity, a defect shared with other mutants that lower Cdc28 activity in mitosis. This defect can be explained by a defect in APC phosphorylation, which depends on mitotic Cdc28 activity in vivo and can be catalyzed by purified Cdc28 in vitro. APC phosphorylation is required for the proper association of Cdc20 with the APC.

Mitotic exit is inhibited by the spindle checkpoint, which monitors the attachment of chromosomes to the spindle. *cdc55* mutants are defective in the spindle checkpoint. This checkpoint defect correlates with both increased inhibitory phosphorylation on Cdc28 and increased phosphorylation of the APC.

Similar inhibitory phosphorylation on Cdc28 homologues prevents mitotic entry in many organisms. In budding yeast this phosphorylation does not slow mitotic entry, but instead slows mitotic exit. Inhibitory phosphorylation on Cdc28 occurs while cells are in mitosis and may function to inhibit Cdc28-dependent phosphorylation of the APC.



Thesis Advisor

Chair of Thesis Committee

Table of Contents

List of Figures and Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Appendix 1

Appendix 2

Appendix 3

Appendix 4

Appendix 5

Introduction

Regulating mitotic exit in budding yeast

The exit from mitosis occurs in two steps

Two major events must occur for a successful mitosis; separation of sister chromatids during the metaphase to anaphase transition, and the inactivation of Cdk1/cyclin complexes (Cdk1 is Cdc28 in budding yeast, and Cdc2 in other organisms). Sister chromatid separation is the major irreversible physical transition that occurs as cells exit mitosis (Nasmyth et al., 2000). There is no example of any cell type that is able to "turn back the clock" and re-cohese their sister chromatids. Cells only have one chance to get this step right, and as a result, many levels of regulation impinge on the timing of this event. Inactivation of Cdk1-associated kinase activity is the major regulatory transition during the exit from mitosis (Morgan, 1999). Mitotic Cdk1 activity drives entry into mitosis, and either directly or indirectly is responsible for all the physical changes that prepare a cell for mitosis: nuclear envelope breakdown (Heald and McKeon, 1990; Peter et al., 1990), changes in microtubule dynamics (Belmont et al., 1990; Verde et al., 1990), fragmentation of the ER/golgi (Lowe et al., 1998), changes in cell shape and growth (Lew and Reed, 1993), chromosome condensation and building of the mitotic spindle (Kimura et al., 1998). Once sister separation has occurred and cells begin to enter the subsequent cell cycle, all these changes in cell architecture must return to their interphase state. This is accomplished by inactivating Cdk1/cyclin complexes at the end of mitosis (Morgan, 1999). Throughout this introduction, I will refer to the exit from mitosis as the completion of both sister chromatid separation and the inactivation of mitotic Cdk1 activity. This introduction will focus primarily on the regulation of the exit from mitosis.

Work on the budding yeast, *Saccharomyces cerevisiae*, has provided the most complete descriptions of the events of mitosis. Mitosis is remarkably conserved, and therefore principles drawn from budding yeast informs research in other organisms. However, comparisons between budding yeast and other experimental organisms highlight how mitotic regulation varies to best meet the needs of an organism's physiology.

Cdc28 activates and inhibits the APC

Both sister chromatid separation and inactivation of Cdk1/cyclin complexes are primarily regulated by the anaphase promoting complex (APC) or cyclosome (Morgan, 1999; Zachariae and Nasmyth, 1999). The APC is a multi-protein complex that functions as an E3 ubiquitin ligase which catalyzes the transfer of ubiquitin onto proteins (Hershko et al., 1994; Sudakin et al., 1995). Once ubiquitinated, these proteins are then targeted for destruction by the 26S proteasome (Ciechanover, 1994). The APC triggers sister separation by degrading the anaphase inhibitor, Pds1 (Cut2 in fission yeast) (Cohen-Fix et al., 1996; Funabiki et al., 1996b; Yamamoto et al., 1996a), and promotes Cdk1/cyclin inactivation by the degradation of the cyclin subunit of this complex, which is essential for the activity of Cdk1 (Glotzer et al., 1991; Murray et al., 1989). Once cells exit mitosis, the APC remains active throughout G1, ensuring that Cdk1 activity remains low through the early part of the cell cycle, and that key regulators of mitosis (like Pds1) do not accumulate (Amon et al., 1994; Irniger et al., 1995; Zachariae and Nasmyth, 1996). In embryonic cycles, which don't have a G1 phase, the APC is turned off as soon as cells exit mitosis, leading to rapid cycling between S and M phases (King et al., 1995; Sudakin et al., 1995). Proteolysis lends itself well to the rapid and irreversible events that occur during mitosis, and in recent years a primary focus of research has been how the APC gets turned on during mitosis.

The APC is activated by two different WD40 proteins, Cdc20 and Hct1 (also known as Cdh1). Overexpression of both in budding yeast can inappropriately activate the APC, and cause degradation of APC targets (Schwab et al., 1997; Visintin et al., 1997). Initially these two activators were proposed to be specificity subunits of the APC, with Cdc20 responsible for the degradation of Pds1 and Clb5 (one of the six B-type cyclins in budding yeast) (Lim et al., 1998), while Hct1 was thought to be critical for degradation of Clb2 (the major mitotic cyclin in budding yeast) and other substrates of the APC which get

degraded late in mitosis like Ase1, Cdc5, and Cdc20 itself (Charles et al., 1998; Juang et al., 1997; Prinz et al., 1998; Shirayama et al., 1998) However, it has been shown that Cdc20 is responsible for some of the degradation of Clb2 in mitosis (Yeong et al., 2000), and Hct1 is critical for degrading Pds1 during G1 (see Chapter 1). These two observations suggest that the apparent differences in specificity are probably due to localization of the targets, competition between the two activators or differences in the efficacy of the two activators towards particular substrate (Yeong et al., 2000). The genetic analysis of *CDC20* and *HCT1* in budding yeast has been backed up by biochemical data showing Cdc20 and Hct1 are sub-stoichiometric activators of the APC in vitro (Fang et al., 1998b; Kramer et al., 1998; Kramer et al., 2000; Lorca et al., 1998).

The primary difference between Cdc20 and Hct1 in budding yeast is when each is active. Cdc20 is active early during the exit from mitosis and *cdc20* mutants arrest in metaphase with sister chromatids still cohesed and the Pds1 protein stable, showing that Cdc20 activity is required to trigger sister separation (Shirayama et al., 1998; Sigrist et al., 1995). Hct1 is active after Cdc20 triggers sister separation, and is responsible for the complete destruction of mitotic cyclins and inactivation of Cdc28 (Schwab et al., 1997; Sigrist and Lehner, 1997; Visintin et al., 1997). Hct1 remains active in G1 and prevents any accumulation of Clb proteins or Pds1. Hct1 activity, however, is not required for mitotic exit because the CDK inhibitor, Sic1, can inhibit Cdc28 activity enough to push cells out of mitosis (Schwab et al., 1997). Sic1 is regulated by the same mechanisms as Hct1, further supporting the idea that Sic1 collaborates with Hct1 at the end of mitosis (Visintin et al., 1998).

What causes the sequential activation of Cdc20 and then Hct1 in budding yeast? Oscillation of Cdc28 activity itself is the prime determinant of these two waves of activation. Cdc28 activates the Cdc20-dependent APC (Amon, 1997; Felix et al., 1990), and then Cdc28 inhibits the Hct1-dependent APC (Jaspersen et al., 1999; Kramer et al., 2000; Zachariae et al., 1998). The specific mechanisms of this regulation will be discussed

in detail below and in Chapters 1 and 2, but the rationale for such regulation is quite logical. In step 1, as Cdc28 activity accumulates in mitosis, it turns off the Hct1-dependent APC, allowing further accumulation of Cdc28 activity. Once Cdc28 activity reaches a critical threshold, it triggers the exit from mitosis and its own destruction by activating the Cdc20-dependent APC (see Chapter 1 and 2). Active Cdc20-dependent APC mediates Pds1 destruction, triggers sister separation and initiates cyclin degradation. Step 2 begins once cyclin destruction is initiated. The drop in Cdc28 activity relieves the repression of the Hct1-dependent APC and Sic1 triggering a positive feedback loop that causes the complete destruction of mitotic cyclins and inhibition of Cdc28 activity (Shirayama et al., 1999; Visintin et al., 1998). In addition, sister separation is thought to reinforce this positive feedback (Cohen-Fix and Koshland, 1999; Tinker-Kulberg and Morgan, 1999). This two step model highlights the dependency of Hct1 activation on prior Cdc20 activation.

These two steps in the exit from mitosis and in APC activation provide the frame work for the rest of this introduction. What activates and inhibits these two forms of the APC? And what is the molecular nature of the dependency?

Step1: Activation of the Cdc20-dependent APC

Sister chromatid separation

In the past few years enormous advances have been made in understanding the regulation of sister chromatid separation and the proteins that may physically hold sister chromatids together (Nasmyth et al., 2000). The key conceptual discovery that opened the search for such proteins was the finding that a protein (or proteins) needs to be degraded by the APC to trigger sister separation (Holloway et al., 1993; Sigrist et al., 1995; Surana et al., 1993). Initially it had been thought that cyclin proteolysis, which is essential for

exiting mitosis, triggered sister separation (Ghiara et al., 1991; Glotzer et al., 1991). But addition of non-degradable cyclin in frog egg extracts, or expression of a non-degradable cyclin in budding yeast causes an anaphase arrest, not a metaphase arrest, indicating that sister chromatids had separated normally (but see Appendix 3). And treatments that slow cyclin destruction delay sister chromatid separation, suggesting that the same machinery that degrades cyclin, also degrades either a protein that physically holds sister chromatids together, or an inhibitor of sister separation.

The Pds1 protein in budding yeast was discovered in a screen for mutants that prematurely separated their sister chromatids (Yamamoto et al., 1996a; Yamamoto et al., 1996b). Pds1 is degraded during the metaphase to anaphase transition, and contains a potential destruction box motif, the degenerate sequence found in most cyclins, that is required for their ubiquitination by the APC (Cohen-Fix et al., 1996). The initial characterization of *pds1* mutants revealed multiple defects in addition to premature sister separation, including sensitivity to DNA damage (see below) and sensitivity to agents that depolymerize microtubules, and oddly, *pds1Δ* cells were viable (Yamamoto et al., 1996a; Yamamoto et al., 1996b). Another attractive candidate for a sister chromatid cohesion protein is the Cut2 protein from fission yeast. Cut2, like Pds1 is degraded at the metaphase to anaphase transition, contains destruction box motifs, and a truncation of the protein that removes the two destruction boxes, renders the protein stable and prevents sister chromatid separation (Funabiki et al., 1996b; Funabiki et al., 1997). However mutants in *cut2*, rather than cause premature sister chromatid separation, prevent sister chromatid separation (Funabiki et al., 1996a). An elegant model has been proposed that Cut1, the binding partner of Cut2, carries out the act of sister chromatid separation, and Cut2 both inhibits and activates Cut1.

This model has been confirmed by work in budding yeast. Mutants in Pds1 that remove its destruction box are stable and when overexpressed cause arrest in metaphase with sister chromatids held together, and in frog egg extracts, Pds1 is a substrate of the

APC (Cohen-Fix et al., 1996). Weak homology exists between Pds1 and Cut2, but only when the budding yeast protein Esp1, which has weak homology to fission yeast Cut1, was found to bind Pds1, has it become clear that Pds1/Esp1 and Cut2/Cut1 are functional homologues (Ciosk et al., 1998). *esp1-1* mutants are unable to separate their sister chromatids, and as in fission yeast, Pds1 is required for the localization and activity of Esp1 (Funabiki et al., 1996a; Kumada et al., 1998). Enough Esp1 activity, however, remains in *pds1Δ* cells that sister chromatids can separate, though with delays. Homologues of both these proteins have been found in frog egg extracts, suggesting that this regulation of sister chromatid separation is conserved (Zou et al., 1999).

Scc1 (also known as Mcd1) is another protein that is essential for sister chromatid cohesion and is the best candidate for the protein that holds sister chromatids together. Scc1 binds to chromosomes after it is synthesized during S-phase, but then as sister separate, it falls off chromosomes (Michaelis et al., 1997). Scc1 is required for both the establishment of sister cohesion during S-phase, and the maintenance of the cohesion until metaphase (Uhlmann and Nasmyth, 1998). Scc1 is cleaved as sister chromatids separate, and this cleavage is necessary for sister chromatid separation (Uhlmann et al., 1999). Cleavage of Scc1 depends on Esp1, which has recently been shown to have protease activity in vitro (Uhlmann and Nasmyth, personal communication).

Scc1 is a member of a complex of proteins which have been named the cohesin complex. Scc1 has been proposed to bridge two cohesin complexes, one binding on each sister chromatid, and when cleaved, allows the separation of sister chromatids (Nasmyth et al., 2000). The cohesin complex has homology to the condensin complex, which is required for chromosome condensation in frog egg extracts and dosage compensation in worms (Hirano et al., 1997). The biochemical characterization of these two complexes are leading to a detailed description of the chemistry of chromosome compaction and association, and have been reviewed in detail (refs) (Hirano et al., 1995; Nasmyth et al., 2000).

Cdc28 activates the Cdc20-dependent APC

Activation of the Cdc20-dependent APC triggers the metaphase to anaphase transition. How then is the Cdc20-dependent APC turned on? Before Cdc20 or the APC had been identified as the machinery that ubiquitinates cyclin and other substrates, it was shown that addition of active Cdc2/cyclin B to frog egg extracts turned on cyclin proteolysis (Felix et al., 1990). This seemed logical, because what better way of causing oscillations of Cdc2 activity than by having Cdc2 trigger its own inactivation? However, for this scheme to work, there must be a lag between activation of Cdc2, and activation of cyclin proteolysis.

This in vitro data complemented the analysis of mutants in *cdc28* in budding yeast and *cdc13* in fission yeast (the cyclin B homologue). Although most alleles of *CDC28* arrest in G1, one allele, *cdc28-1N*, was isolated that arrested in mitosis, with high Cdc28-associated kinase activity, after DNA replication, and with a short spindle, indicating that the cells were in mitosis, but were unable to initiate the exit from mitosis (Piggott et al., 1982; Surana et al., 1991). A similar phenotype is seen in fission yeast *cdc13* mutants. *cdc13-117* arrests in mitosis, with condensed chromatin, though with interphase arrays of microtubules (Hagan et al., 1988; Moreno et al., 1989). In addition, mutations in the small CDK binding protein, Cks1 (in budding yeast) or Suc1 (in fission yeast), also arrests in mitosis (Moreno et al., 1989; Tang and Reed, 1993). The work in budding and fission yeast suggest that a critical level of Cdk1-associated kinase activity is required to get cells out of mitosis and that this level may be greater than the amount needed to get cells into mitosis (Stern and Nurse, 1996). But how does Cdk1 activity trigger mitotic exit?

Once the APC was isolated, and numerous APC subunits were shown to be phospho-proteins, it seemed likely that the APC was activated by phosphorylation (King et al., 1995; Peters et al., 1996). APC phosphorylation only occurs during mitosis, and Cdc2/cyclin B can activate a partially purified APC from clam extracts, though with a

reproducible lag (Hershko et al., 1994; Lahav-Baratz et al., 1995). The activation of the mouse polo-like kinase, Plk1 (Cdc5 in budding yeast and Plx1 in frogs), by Cdc2 was proposed to explain this lag (Kotani et al., 1998). Plk1 can phosphorylate and activate the mammalian APC in vitro, and can do this far better than Cdc2. In addition, depletion of Plx1 from mitotic frog egg extracts blocks cyclin destruction (Descombes and Nigg, 1998). Lastly, mutation of the budding yeast Cdc5 kinase blocks cyclin proteolysis, while overexpression of Cdc5 activates the APC (Charles et al., 1998).

More recent evidence has made a stronger case for Cdc2 being the kinase that activates the APC. Cdc2 can phosphorylate frog APC, but only in the presence of the small CDK binding protein, Suc1 or Cks1 (Kotani et al., 1999; Patra and Dunphy, 1998). Cks1 has been proposed to be a specificity factor that targets Cdc2 to the APC. The presence of Cks1 also eliminates the lag in APC activation seen in clam extracts, and in this crude system, it was proposed that phosphorylation of the APC by Cdc2/cyclin B/Cks1 promotes Cdc20 binding to the APC (Shteinberg and Hershko, 1999; Shteinberg et al., 1999). This finding has also been confirmed in frog egg extracts (Kramer et al., 2000). However, none of these experiments have examined the in vivo requirements for APC phosphorylation. In addition the relative contribution of Cdc2 versus Plk1 has remained murky.

Despite the compelling evidence that Cdc2/Cdc28 activity is required to exit mitosis, recent work in budding yeast has focused instead on the role of Cdc28 inhibiting the exit from mitosis (discussed more below) (Amon, 1997; Li and Cai, 1997) . Chapter 1 and 2 of this dissertation present genetic and biochemical evidence that Cdc28 is required to phosphorylate and activate the Cdc20-dependent APC in budding yeast. Phosphorylation of the APC is required for efficient binding of Cdc20, and thus confirms the proposal made by researchers working in clam and frog egg extracts. Mitotic APC phosphorylation, however, has no consequence on the activation of the Hct1-dependent APC.

Work in Chapter 2 of this dissertation also begins to determine the relative contribution of Cdc28 and Cdc5 (the Plk1 homologue in budding yeast) to APC activation. Both Cdc28 and Cdc5 regulate APC phosphorylation, but it has been very difficult to find conditions that reveal a role for Cdc5 in activating the Cdc20-dependent APC and the metaphase to anaphase transition. Cdc5 is primarily involved in activating the Hct1-dependent APC at the end of mitosis (see below) (Charles et al., 1998; Shirayama et al., 1998). Cdc2 and Plx1 (the Cdc5 homologue in frogs) have also been proposed to work together to activate the entry into mitosis in frog egg extracts (Karaiskou et al., 1999; Kumagai and Dunphy, 1996). Entry into mitosis requires the activation of the Cdc25 phosphatase, and both Cdc2 and Plx1 are essential for this activation (Dunphy, 1994; Kumagai and Dunphy, 1996). It is thought that Cdc2 starts off this phosphorylation and causes linear activation of Cdc25. Plx1 then phosphorylates Cdc25, and this phosphorylation allows the Cdc2 phosphorylation to proceed more rapidly, leading to exponential rather than linear activation of Cdc25 (Karaiskou et al., 1999; Russell and Nurse, 1986a). A similar three step model for APC activation might occur in vivo.

Is there any regulation of APC dephosphorylation? This has not been studied as extensively as phosphorylation of the APC, but it has been shown that an okadaic acid (OA) sensitive phosphatase co-purifies with an inhibitory activity of the APC in clam extracts (Lahav-Baratz et al., 1995). Treatment of a partially purified active clam APC with PP2A inhibits in vitro APC activity. In addition, data from Chapter 3 of this dissertation suggests that PP2A may regulate the APC in budding yeast. In fission yeast, dephosphorylation of APC subunits by protein phosphatase 1 (PP1) is thought to activate the APC (Yamada et al., 1997; Yanagida et al., 1999).

One of the remaining challenges in this field is to understand what causes the lag between the accumulation of Cdc28/Clb (Cdc2/Cyclin B), and the activation of the Cdc20-dependent APC. Dual control of APC phosphorylation by Cdc28 and Cdc5 provides a

possible explanation, but phosphorylation of the APC, although important, is only one of many ways that Cdc20-dependent APC activity is regulated. The many positive and negative influences on Cdc20-dependent APC activity that might contribute to this lag are discussed below.

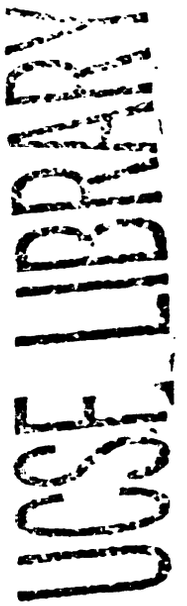
Activation of Cdc20

In budding yeast Cdc20 protein and RNA levels fluctuate throughout the cell cycle, reaching their peak in mitosis (Prinz et al., 1998; Shirayama et al., 1998). *CDC20* transcription is thought to be regulated coordinately with transcription of *CLB2* and other mitotic regulators, whose transcription peaks in mitosis. Transcription of these genes is positively regulated by Cdc28-associated kinase activity, so this serves as one way in which Cdc20 accumulation might lag behind activation of Cdc28/Clb complexes (Amon et al., 1993). Constitutive transcription of *CDC20* driven by a heterologous promoter, however, does not change the timing of mitosis, suggesting that fluctuations in *CDC20* transcription are not essential for the timing of the exit from mitosis (Prinz et al., 1998).

Cdc20 protein is also regulated by APC-dependent proteolysis (Shirayama et al., 1998). Cdc20 protein is unstable throughout the cell cycle, with the instability in G1 being dependent on the APC and destruction boxes in Cdc20. During mitosis its instability is dependent on the APC, but not its destruction boxes, suggesting that the way the APC recognizes Cdc20 varies through the cell cycle. Although Cdc20 is thought to be a target of the Hct1-dependent APC in late mitosis and G1, it is unclear what regulates Cdc20 stability during the early stages of mitosis. One possibility is that Cdc20 auto-regulates itself and is responsible for its own degradation. This model, however, creates a paradox: if Cdc20 is responsible for its own degradation, how does it ever accumulate to levels high enough to trigger the metaphase to anaphase transition?

The regulated phosphorylation of Cdc20 might provide an answer to this question. Cdc20 is phosphorylated in mammalian cells, and both an Aurora kinase and Cdc2 have been shown to be Cdc20 kinases (Farruggio et al., 1999; Weinstein et al., 1994). The phosphorylation of Cdc20, unlike that of Hct1, has no effect on APC activity (see below) (Kotani et al., 1999; Kramer et al., 2000). Phosphorylation of Cdc20, however, protects the protein from ubiquitination in an in vitro ubiquitination assay (Jami Nourse and David Morgan, personal communication). The in vivo relevance of this finding is unclear, but in budding yeast, Cdc20 protein levels are surprisingly low in the *cdc28-1N* mutant , which has altered mitotic Cdc28 activity (see Appendix 3). This finding could reflect either an increased turnover rate, or decreased transcription (Loy et al., 1999).

Cdc20 levels don't fluctuate in embryonic cycles of frogs, which either reflects a difference in Cdc20 regulation during embryonic cycles and somatic cycles, or Cdc20 is present in excess, and only a small pool of Cdc20 is degraded in each cycle (Lorca et al., 1998). This would be similar to what has been found for cyclin B in early cycles of the fly embryo (Lehner and O'Farrell, 1990; O'Farrell et al., 1989).



The spindle checkpoint inhibits Cdc20

Because activation of the Cdc20-dependent APC triggers mitotic exit, all that is needed to prevent exit from mitosis is to inhibit Cdc20. The major inhibitor of Cdc20 activity is the spindle checkpoint (Amon, 1999; Hardwick, 1998). The spindle checkpoint monitors whether kinetochores, the site on chromosmes that bind to spindle microtubules, have correctly attached to the mitotic spindle, and delays exit from mitosis until this event has occurred successfully. Mutations in *MAD1-3*, *BUB1* and *3*, and *MPS1* in budding yeast are defective in this checkpoint (Hoyt et al., 1991; Li and Murray, 1991; Weiss and Winey, 1996). These mutants were discovered because they are unable to arrest in mitosis when treated with the microtubule depolymerizing drug benomyl, which causes spindle

disassembly and thus detachment of chromosomes from the spindle. The spindle checkpoint is activated by unattached kinetochores and an activity on the kinetochore is essential for the generation of the inhibitory signal that then halts cell cycle progression (Nicklas et al., 1995; Pangilinan and Spencer, 1996; Rieder et al., 1995; Wells and Murray, 1996). Once all the kinetochores attach to the spindle, the signal decays, and cell cycle progression continues (Rieder et al., 1995).

Checkpoint activation blocks both sister chromatid separation and cyclin proteolysis, and quite sensibly the downstream target of the spindle checkpoint is the inhibition of the Cdc20-dependent APC (Minshull et al., 1996). This was shown to be a target by the discovery of mutations in *CDC20* (or *slp1* in fission yeast) that are checkpoint defective and have defects binding the Mad2 checkpoint protein (Hwang et al., 1998; Kim et al., 1998). Appendix 4 presents some additional data that confirms that the APC is the target of the checkpoint. Although simple binding of Mad2 to Cdc20 is an attractive model for how the checkpoint inhibits Cdc20, binding is not sufficient, because some of the Cdc20 checkpoint mutants are still able to bind to Mad2 at wild type levels (Hwang, 1998). In addition, Cdc20 and Mad2 are bound to each other at times when the checkpoint is not activated. Therefore, some other activity of Mad2 must be required to prevent Cdc20 from activating the APC. This activity is more complicated than simply preventing Cdc20 from binding to the APC because Mad2 and Cdc20 are found bound to the APC during times of a checkpoint arrest in human cells (Fang et al., 1998a; Kallio et al., 1998).

So what is the checkpoint activity of Mad2? The most compelling model proposed to date is that the oligomerization state of Mad2 is regulated by the checkpoint. Recombinant frog Mad2 purifies as monomers or tetramers, and tetramers are more effective at causing a checkpoint arrest in frog egg extracts than monomers (Fang et al., 1998a). The Mad2 tetramers also have a limited half-life, which provides a mechanism for shutting off the checkpoint. It is still unclear, however, if Mad2 tetramers exist in vivo, and whether other components of the checkpoint regulate tetramer assembly.

The biochemical dissection of the checkpoint has been complemented by very beautiful localization studies which have shown that most of the known checkpoint proteins localize to kinetochores, and many of them are recruited only to unattached kinetochores (Basu et al., 1998; Chan et al., 1998; Chen et al., 1996; Taylor and McKeon, 1997) The target of the checkpoint, Cdc20, has also been localized to kinetochores (Gorbsky et al., 1998). Mad1 is required for Mad2 recruitment to the kinetochore in frogs (Chen et al., 1998), and Bub3 may be required for localization of Bub1 to kinetochores in mammallian cells (Taylor et al., 1998). Recently two complexes that contain both Mad and Bub proteins have been characterized. One, in budding yeast, contains Mad1, Bub1 and Bub3 (Brady and Hardwick, 2000), and the other, called the mitotic checkpoint complex (MCC), from HeLa cells contains Mad2, Mad3 (also known as Bub1R), Bub3 and Cdc20 (V. Sudakin and T. Yen, personal communication)(Brady and Hardwick, 2000).

Once all the checkpoint proteins get to the kinetochore, what do they do there? An attractive model is that the chemistry of the checkpoint can only occur on the kinetochore (Rieder et al., 1997). For example, the checkpoint proteins might be only able to catalyze Mad2 tetramer formation on the kinetochore, and once the Mad2 tetramer is formed, it binds to Cdc20, and then released into solution to inhibit the APC. There is little data for this model other than the finding that Mad2 does visit and then leave the kinetochore with very rapid kinetics, suggesting that binding and release of Mad2 may be important for propagation of the checkpoint signal (T. Salmon, personal communication).

The MCC, which contains Mad2, Mad3, Bub3 and Cdc20, may also form on the kinetochore and be released into solution (V. Sudakin and T. Yen, personal communication). The MCC was purified as a soluble activity that inhibits active APC. This activity can be purified from either interphase or mitotic cells, but can only inhibit a mitotic APC. This is not so surprising because the MCC contains Cdc20, and Cdc20 can only bind to a mitotic phosphorylated APC. The activity of this complex is enhanced by the presence of purified chromosomes, consistent with finding in frog egg extracts that

chromosomes are required for spindle checkpoint activation (A. Szidon and A.W. Murray, personal communication). The oligomerization state of Mad2 in the MCC was not determined, but the presence of Mad3 and Bub3 in this complex suggests that Mad2 tetramers may not act alone to inhibit the Cdc20-dependnet APC. The absence of Mad1 from this complex suggests that Mad1 primarily functions to localize Mad2 to kinetochores, where Mad2 may assemble into the MCC, but is not required for the direct inhibition of the Cdc20-dependent APC. The existence of a soluble checkpoint activity is exciting, because studying a kinetochore bound activity has been very difficult, and may now allow an understanding of the biochemistry of the checkpoint.

The checkpoint and the normal timing of mitosis

The spindle checkpoint is the major inhibitor of Cdc20 in cells, and if the checkpoint were active in every cycle this inhibition could help set the normal timing of mitosis. In budding yeast it is clear that the checkpoint is not strongly activated in every cell cycle. Mutations in *MAD1-3* are viable and are defective in the checkpoint, but don't have any obvious growth defects (Li and Murray, 1991). However, careful physiological experiments have shown that mitosis occurs slightly earlier (10 minutes, see Appendix 4) in these cells, suggesting that although this small advancement in mitosis is not lethal, the checkpoint does contribute to the normal timing of mitosis.

The spindle checkpoint in vertebrate cells, however, appears to function in every cell cycle, and clearly contributes to the normal timing of mitosis. Deletion of Mad2 in mice causes embryonic lethality, caused primarily by premature passage through mitosis (P. Sorger, personal communication). It has also been shown that microinjection of Mad2 antibodies into mitotic PtK cells causes rapid induction of anaphase (Gorbsky et al., 1998), and that microinjection of a dominant negative Bub1 into mouse cells also hastens mitotic exit (Taylor and McKeon, 1997). In contrast, the spindle checkpoint does not operate in

early embryonic cycles of frogs, and cell cycle progression can occur in the absence of a spindle, or even of chromosomes (Minshull et al., 1994; Murray and Kirschner, 1989).

Because the spindle checkpoint is meant to preserve genomic integrity one would expect that mutations in the checkpoint might cause genomic instability and therefore be associated with cancers. This has been shown in a study that found mutations in human Bub1 in many colorectal tumors (Cahill et al., 1998). Loss of the checkpoint probably affects later stages of tumorigenesis, because until cells are induced to enter the cell cycle from quiescence, a mutation in the spindle checkpoint will have no phenotype.

Other checkpoints in budding yeast that prevent mitotic exit

In addition to the spindle checkpoint, two other checkpoints in budding yeast prevent mitotic exit, the DNA damage checkpoint and the morphogenesis checkpoint. These two checkpoints appear to have different targets than the spindle checkpoint, but eventually must have the same consequence, either inhibition of the Cdc20-dependent APC, or a stabilization of its substrates.

The DNA damage checkpoint

The DNA damage checkpoint has been studied in many organisms, and at first glance it seems quite different in budding yeast (Russell and Nurse, 1986b). In all other organisms studied the major target of the checkpoint is the regulation of inhibitory phosphorylation on Cdk1 (Enoch and Nurse, 1990; Gould and Nurse, 1989; Jin et al., 1996; Norbury et al., 1991; Rhind et al., 1997).. This inhibitory phosphorylation occurs on residues tyrosine 15, and threonine 14 of fission yeast Cdc2, and these residues are conserved in all Cdk1 homologues. In fission yeast, Wee1 and Mik1 phosphorylate these residues, and Cdc25 dephosphorylates them (Baber-Furnari et al., 2000; Featherstone and

Russell, 1991; Gould and Nurse, 1989; Russell and Nurse, 1986a; Russell and Nurse, 1987). DNA damage causes inhibition of Cdc25, and perhaps activation of Wee1 and Mik1, which inhibits Cdc2 and prevents mitotic entry. This pathway is not only the target for the DNA damage checkpoint, but also the checkpoint that senses unreplicated DNA, and in fission yeast regulates the cell size needed for entry into mitosis (Enoch and Nurse, 1990; Nurse, 1975).

This pathway does not seem to be important for the DNA damage checkpoint in budding yeast (though see the discussion of the morphogenesis checkpoint below, and Chapter 4) (Amon et al., 1992a; Sorger and Murray, 1992). This finding has been explained as being the result of the unusual coupling of S-phase and mitosis in budding yeast. S-phase and mitosis are both regulated by Cdc28/Clb complexes and events of both occur immediately after budding. Thus spindle pole body (SPB) duplication and mitotic spindle formation occur simultaneously with S-phase. In budding yeast similar amounts of Cdc28 activity can induce both events, while in fission yeast it has been proposed that different quantities of Cdc2-associated activity promote passage through the subsequent steps of the cell cycle (Stern and Nurse, 1996). In budding yeast if inhibitory phosphorylation prevented mitotic entry it might also prevent S-phase, which would not be a particularly useful checkpoint. In fact massive overexpression of Swe1 (the budding yeast homologue of *wee1*) does prevent both S-phase and mitosis, though eventually both S-phase and mitotic entry occur, and the cells arrest in mitosis (Lim et al., 1996; Lin and Arndt, 1995) Thus, because budding yeast has a mitotic S-phase, the checkpoint must prevent mitotic exit, not mitotic entry.

During a DNA damage checkpoint arrest in budding yeast, Pds1 is stabilized and Clb proteins remain stable, just as is seen in arrest by the spindle checkpoint (Cohen-Fix and Koshalnd, 1997). Pds1 has been shown is critical for this arrest, not only because sisters separate prematurely in *pds1Δ* cells which have been damaged, but because cell cycle progression occurs, causing the damaged cells to progress to the next G1 and die

(Yamamoto et al., 1996b). This contrasts to the spindle checkpoint, where spindle damage in a *pds1Δ* still causes cell cycle arrest, but sister chromatids separate. This result argues that Pds1 may be the target of the checkpoint. Although overexpression of Cdc20 can override a DNA damage checkpoint arrest, Cdc20 is not thought to be the target of the checkpoint because if it were, damaged *pds1Δ* cells would not progress in the cell cycle, but instead arrest in anaphase like *pds1Δ cdc20Δ* cells (Hwang et al., 1998; Lim and Surana, 1996; Visintin et al., 1997).

Although Pds1 was initially characterized as an inhibitor of sister separation, it also can inhibit cyclin proteolysis, and this property is probably essential for its role in the DNA damage checkpoint. Overexpression of non-degradable Pds1 (Pds1-dbΔ) blocks both sister chromatid separation and inactivation of Cdc28-associated kinase activity, and both activities of Pds1 are thought to be mediated via Esp1 (Cohen-Fix and Koshland, 1999; Tinker-Kulberg and Morgan, 1999). *esp1-1* mutants cannot separate their sister chromatids, but also have severe defects in cyclin proteolysis, and overexpression of Esp1 activates the APC and inappropriate cyclin proteolysis during a spindle checkpoint and DNA damage arrest (Ciosk et al., 1998; Tinker-Kulberg and Morgan, 1999). Esp1 appears to act on both the Hct1-dependent APC via the mitotic exit network (see below) but also on the Cdc20-dependent APC, in a mechanism that is not well understood. However, the role of Esp1 in promoting cyclin proteolysis has been questioned, and instead it has been suggested that Esp1 activates the Bub2 checkpoint, which is distinct from the spindle and DNA damage checkpoints (see below, especially discussion of whether *bub2Δ* mutants might prematurely activate the APC) (Shirayama et al., 1999). This interpretation might explain why *esp1-1* mutants delay cyclin proteolysis, but offers no explanation for why overexpression of Esp1 activates the APC.

Additional evidence for Pds1 being the target of the checkpoint has been the examination of checkpoint induced phosphorylation of Pds1. This phosphorylation has been shown to depend on *RAD9* and *MEC1*, two components of the checkpoint, but not

the protein kinase Rad53 (Cohen-Fix and Koshalnd, 1997; Gardner et al., 1999). Pds1 phosphorylation has been proposed to protect Pds1 from APC-dependent degradation. The kinase responsible for phosphorylating Pds1 has been proposed to be the budding yeast homologue of the fission yeast *chk1* (Furnari et al., 1997; Sanchez et al., 1999). This is a curious result because Chk1 has been conserved in many organisms and its role is primarily to phosphorylate Cdc25 and inhibit its activity . Has Chk1 simply adopted a new role in budding yeast? No data exists showing whether Chk1 homologues phosphorylate Pds1 homologues, and whether budding yeast Chk1 phosphorylates Mih1 (the budding yeast Cdc25 homologue). Although *chk1Δ* cells cannot induce phosphorylation of Pds1 in response to DNA damage, they have only a mild checkpoint defect. This mild phenotype can become a severe phenotype when *chk1Δ* is combined with *rad53* mutants. This data has suggested that the DNA damage checkpoint bifurcates into a Chk1/Pds1 branch and a Rad53 branch (Gardner et al., 1999; Sanchez et al., 1999).

Rad53 has been proposed to act on the Cdc20-dependent APC directly, via the protein kinase Cdc5, which along with Cdc28 may be required to activate the Cdc20-dependent APC (see above) (Kotani et al., 1998; Sanchez et al., 1999). This model is largely based on the finding that *cdc5-1* mutants, which have previously been shown to arrest only in anaphase (Charles et al., 1998), can stop cell cycle progression at metaphase in a *rad53* mutant that has suffered DNA damage (Sanchez et al., 1999). A role for Cdc5 in the metaphase to anaphase transition has been proposed in other organisms, but little evidence exists for this in budding yeast. The only other evidence for a role of Cdc5 in the DNA damage checkpoint is the finding that a special allele of *CDC5*, *cdc5-ad*, has been found that block adaptation to DNA damage checkpoint arrest (Toczyski et al., 1997). This allele has a very specific role in response to DNA damage and shows no defects in anaphase, the time when the *cdc5-1* allele has its primary defects. The specific defect of *cdc5-ad* is unknown, but its phenotype strengthens the proposal that Cdc5 plays a role in the DNA damage checkpoint and the metaphase to anaphase transition.

Because S phase and M-phase occur simultaneously in budding yeast, the checkpoint that senses unreplicated DNA also prevents mitotic exit. Cells treated with hydroxyurea, which slows DNA replication, arrest in mitosis with a short spindle and separated SPB's, but with unreplicated DNA. This checkpoint does not operate through Pds1, and so it remains unclear how it halts cell cycle progression (Cohen-Fix and Koshalnd, 1997; Yamamoto et al., 1996b). A possible target might be Clb5, a cyclin important for S-phase progression (Epstein and Cross, 1992; Schwob and Nasmyth, 1993), whose stabilization might prevent mitotic exit (see Appendix 3).

The morphogenesis checkpoint.

Although inhibitory phosphorylation is not required for the DNA damage checkpoint in budding yeast, it is required for what has been termed the bud emergence or morphogenesis checkpoint (Lew, 2000; Lew and Reed, 1995) Initially characterized as a checkpoint that monitors whether cells have budded, this checkpoint is also triggered by osmotic shock and actin depolymerization caused by the drug latrunculin A (McMillan et al., 1998). Cell cycle delays caused by this checkpoint are abolished in *swe1Δ* cells, suggesting that inhibition of Cdc28 activity is the target of this checkpoint. Although checkpoint activation causes delays in the accumulation of Clb2 protein and the accumulation of Clb2-associated kinase activity, this arrest is not a G2/M arrest, because cells are able to build a short bipolar spindle, a characteristic of mitotic arrest. So, like the DNA damage checkpoint, this checkpoint prevents mitotic exit. This checkpoint is not as robust as the spindle or DNA damage checkpoint, because at most, it delays cell cycle progression by an hour or two.

The Swe1 dependence of the checkpoint raises some interesting questions about the role of Swe1 in delaying passage through mitosis. As mentioned above, if this inhibition were very strong, cells would not enter mitosis and DNA replication would be delayed.

This checkpoint delays the cell cycle later, suggesting that mild inhibition of Cdc28 can prevent APC activation. How this occurs is unclear, but Chapters 1 and 2 of this dissertation show mutations that lower mitotic Cdc28 activity have defects in activating the APC, and this is due primarily to a reduction in APC phosphorylation. Therefore, the target of inhibitory phosphorylation of Cdc28 may indirectly be APC phosphorylation. This model has not been tested directly, but preliminary evidence in Chapter 4 suggests that this might be true

One interesting way to think about the morphogenesis checkpoint is that it is the equivalent of G2 size control studied in fission yeast, which is also regulated by inhibitory phosphorylation of Cdc2 (Nurse, 1975). In fission yeast the size requirement is satisfied in G2 after DNA replication and allows entry into mitosis. The size requirement is important so that there is enough room in the cell for the two daughter nuclei after cytokinesis. If cells are too small (as in *wee1* cells), there is a greater chance of chromosome damage or loss. In budding yeast, the problem of cell size is dealt with in an entirely different manner. Budding yeast buds, and the bud neck creates a spatial barrier for the two nuclei during cytokinesis. The bud only needs to grow large enough to fit a daughter nucleus inside. This clever solution may allow the G2 size control to be satisfied very early, right after budding during the beginning of S-phase and mitotic entry. However, when budding yeast exit from mitosis, they are faced with the problem of making sure the daughter nucleus actually makes it into the bud before they begin cytokinesis. The Bub2 checkpoint, described below, may solve this problem by monitoring delivery of the daughter nucleus to the daughter bud.

Other targets of Cdc20

Activation of the Cdc20-dependent APC is the trigger for mitotic exit. But is Pds1 the only substrate that needs to be degraded by the Cdc20-dependent APC? In budding

yeast *cdc20Δ pds1Δ* cells separate their sisters, but are unable to turn on Clb proteolysis, and arrest in an anaphase-like state (Lim et al., 1998). This suggests that another protein (or proteins) has to be degraded in order for cell cycle progression to proceed. Degradation of this substrate has been proposed to be the molecular explanation of the dependency of Hct1-dependent APC activation on prior Cdc20-dependent APC activation (Visintin et al., 1998).

Mutations in the S-phase cyclin Clb5 suppress the anaphase arrest and lethality of *cdc20Δ pds1Δ*. The triple mutant *cdc20Δ pds1Δ clb5Δ* is quite healthy, and sister separation occurs on schedule, as does de-localization of Cdc14 from the nucleolus (see below) (Shirayama et al., 1999). This phenotype is only seen with deletions of *CLB5*, not *CLB1,3,4* or *6*. Deletions of *CLB2*, the cyclin which is responsible for the majority of the Cdc28-associated activity in mitosis, are synthetically lethal in combination with *pds1Δ*. A recent paper, however, suggests that Clb2 may play a similar role as Clb5, because a small drop in Clb2 protein levels, that is *CDC20* dependent, is seen as cells transit form metaphase to anaphase (Yeong et al., 2000). The lethality of *clb2Δ* and *pds1Δ* suggests that although Clb2 and Clb5 may have some overlapping functions, Clb2 has an additional role, one which becomes essential when *PDS1* is deleted. Although Cdc20 is dispensable in budding yeast, APC-dependent proteolysis appears not to be. *cdc20Δ pds1Δ clb5Δ* cells still require Hct1 function to carry out Clb2 proteolysis.

Although this result is very interesting in budding yeast, how universal is the finding? In embryonic cycles where all APC-proteolysis depends on Cdc20, and no Hct1 is even present, loss of Cdc20 will block all APC dependent proteolysis, and would likely cause a permanent mitotic arrest (Lorca et al., 1998; Sigrist et al., 1995). In addition, the spindle checkpoint appears to be essential in organisms other than yeast, and therefore Cdc20 function would also be essential.

cdc20Δ pds1Δ clb5Δ cells are viable, but contain no checkpoints because the target of both the spindle checkpoint (Cdc20) and the DNA damage checkpoint (Pds1) have been

deleted. The Cdc20/Pds1/Clb5 module may therefore provide the link from these extrinsic controls onto the free running cell cycle. It remains a mystery, however, how sister separation is triggered roughly on schedule in the absence of Cdc20 and Pds1 (Shirayama et al., 1999). It was already known that sister separation occurred on time (or late) in *pds1Δ* cells (Ciosk et al., 1998), but *cdc20Δ pds1Δ clb5Δ* viability also eliminates Cdc20 and Clb5 as possible candidate regulators of sister separation.

The simplest explanation for what controls the timing of sister separation is that either the activity of Esp1 or the accessibility of Scc1 to Esp1 cleavage is regulated by Cdc28 (Uhlmann et al., 1999). This would link sister chromatid separation directly to oscillations of Cdc28 activity, and may also explain why *clb2Δ* cells are synthetically lethal in combination with *pds1Δ*. The absence of Pds1 lowers Esp1 activity, and these cells may have a greater reliance on high Cdc28/Clb2 activity to promote sister separation (Ciosk et al., 1998; Kumada et al., 1998). Activating sister chromatid separation, along with the APC, would provide a second key role for Cdc28 in promoting the exit from mitosis.

Step 2: Activation of the Hct1-dependent APC

Inactivation of Cdc28

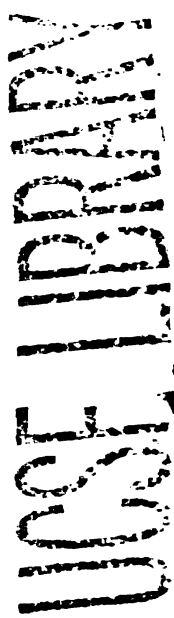
The biggest recent advance in the understanding of exit from mitosis in budding yeast has been the analysis of the activation of the Hct1-dependent APC and the final inactivation of Cdc28 activity as yeast cells transit form anaphase to G1. Hct1 is the primary effector in the inactivation of Cdc28 (Schwab et al., 1997; Visintin et al., 1997). However *hct1Δ* cells are viable, and Sic1, the Cdc28/Clb inhibitor that is critical for restraining S-phase in G1, is thought to act with Hct1 to inhibit Cdc28 activity at the end of mitosis (Nugroho and Mendenhall, 1994; Schwab et al., 1997). Cdc28/Clb phosphorylation of Hct1 prevents its binding to the APC, and phosphorylation of Sic1

targets it for degradation (Jaspersen et al., 1999; Verma et al., 1997; Zachariae et al., 1998). Therefore, the initial drop in Cdc28 activity mediated by the Cdc20-dependent APC probably begins to relieve the inhibition on Hct1 and Sic1 (Shirayama et al., 1999; Yeong et al., 2000).

The activation of the Cdc14 phosphatase, however, is required to remove the phosphorylation on Hct1, Sic1 and Swi5 (a transcriptional activator of Sic1, that is inhibited by phosphorylation) and cause activation of Hct1, transcription of Sic1 and stabilization of Sic1 (Jaspersen et al., 1999; Toyn et al., 1997; Visintin et al., 1998). Mutation of *CDC14* arrests cells in anaphase with separated sister chromatids, and Hct1 remains phosphorylated and inactive, and Sic1 is absent. Cdc14 phosphatase activity doesn't fluctuate through the cell cycle, but its localization does. Cdc14 is held in the nucleolus by Net1 (also known as Cfi1), and is rapidly released from the nucleolus when cells enter anaphase (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999). Degradation of Pds1 is required for release of Cdc14 from the nucleolus, and degradation of Clb5 allows Cdc14 to dephosphorylate Hct1, Sic1 and Swi5 (Shirayama et al., 1999). It is not clear if the nucleolus is simply acting as a storage sight for Cdc14, or if some aspect of nucleolar function or structure is providing an input to cell cycle progression (Visintin and Amon, 2000). It is also unclear what changes in the nucleolus to cause the release of Cdc14.

The dual control by Hct1 and Sic1 is probably only essential in organisms that have an extended G1 phase. Embryonic cycles, which cycle rapidly between S and M phases, only express Cdc20, which is sufficient to catalyze sister separation and complete cyclin proteolysis (Lorca et al., 1998; Sigrist et al., 1995). Hct1 is only is expressed when cells acquire a G1-phase and as in budding yeast, is probably essential for preventing accumulation of Cdc2 activity in G1, which could trigger S-phase inappropriately (Sigrist and Lehner, 1997).

The viability of *cdc20Δ pds1Δ clb5Δ* cells highlight the importance of Hct1 and Sic1 in budding yeast mitotic exit. In other organisms, the site of cytokinesis is largely determined by spindle placement, and is dependent on a drop in Cdc2 activity (Cerutti and Simanis, 2000). Therefore in most organisms, there is no reason to delay the inactivation of Cdc2, and cyclin proteolysis initiates with sister separation, and both events are Cdc20 dependent. In budding yeast, cytokinesis is pre-determined by the bud site, and cells risk diploidizing or losing chromosomes if the spindle does not align properly (see below). Budding yeast, therefore, has an extended period of high Cdc28-associated kinase activity during anaphase, and Hct1 and Sic1, not Cdc20, are the major regulators of Cdc28 inactivation. Budding yeast provides an extreme example where the efficiency of DNA replication, spindle formation and chromosome attachment to the spindle may have reduced selective pressures on mitotic checkpoints, and thus the Cdc20-dependent APC, but its unusual way of performing cytokinesis, has increased its reliance on the Hct1-dependent APC and Sic1.



The mitotic exit network activates Cdc14

Release of Cdc14 from the nucleolus is regulated by a large collection of genes termed the mitotic exit network (MEN) (Jaspersen et al., 1998). These genes are *TEM1*, *LTE1*, *CDC15*, *CDC5*, *DBF2*, *DBF20*, and *MOB1*, and were identified because conditional mutations in the genes arrest yeast cells in anaphase (Balasubramanian et al., 2000; Luca and Winey, 1998; Shirayama et al., 1994; Toyn and Johnston, 1994). These genes encode primarily kinases (Cdc15, Cdc5, Dbf2 and Dbf20), a ras related GTPase (Tem1) and a guanine nucleotide exchange factor (Lte1). Mutations in these genes prevent release of Cdc14 from the nucleolus, but the MEN may also regulate Cdc14 at a step after release from the nucleolus (Jaspersen and Morgan, 2000). Mutation of *NET1*, which

causes premature release of Cdc14 from the nucleolus, rescues loss of function mutations in many of these genes (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999).

The individual activities of many of the proteins encoded by the MEN genes have been studied in detail in budding yeast, but how these proteins function in a pathway is still poorly understood. Work in fission yeast, which contains homologues of many of these proteins (although they effect cytokinesis, not cell cycle progression, see below), has helped order the pathway and has also shown that many of these components localize to the spindle pole body (SPB) (Balasubramanian et al., 2000; Cerutti and Simanis, 2000). In fission yeast Spg1, the Tem1 homologue, localizes to both SPBs but as cells enter anaphase, GTP bound Spg1 is found only on one of the SPBs (Cerutti and Simanis, 1999; Sohrmann et al., 1998). This asymmetry leads to the asymmetric localization of Cdc7, the Cdc15 homologue, which also binds the SPB. Sid2, the Dbf2 homologue, and Plo1 and Mid2, polo kinases, are also both localized to the SPBs, though not asymmetrically, in fission yeast (Bahler et al., 1998; Mulvihill et al., 1999; Sparks et al., 1999). Recently it has been shown that Cdc5, Tem1 and Cdc15 localize to the spindle pole body in budding yeast (Shirayama et al., 1998) (S. Jaspersen and D. Morgan, and A. Toh-E, personal communications).

Although this collection of genes is called the mitotic exit network (MEN) in budding yeast, the name is unfortunate, because the fission yeast homologues do not seem to regulate mitotic exit, but cytokinesis. Mutations in MEN homologues block cytokinesis, though cell cycle progression continues (Cerutti and Simanis, 2000; Gould and Simanis, 1997). Once Cdc7 localizes to the Spg-GTP containing SPB, Sid2 kinase is thought to relocalize to the site of septum formation, and thus may be one of the downstream targets of the pathway (Sparks et al., 1999). Recently it has been found that *zfs1* mutations reduce the requirements for much of this pathway in fission yeast. *zfs1* is likely to be an inhibitor of the downstream target of this pathway, and formally plays a role similar to Net1 (Bel[illegible]aminelli et al., 1999).

Although the MEN in budding yeast regulates cell cycle progression, it also has some role in cytokinesis. Mutations in *cdc15-2* and other MEN components have defects in cytokinesis, though *cdc14* mutants do not . In addition, *net1* suppression of MEN mutations only suppress cell cycle progression defects, not cytokinesis defects (Shou et al., 1999). Cdc14, therefore, may be only one of the targets of the MEN in budding yeast.

Does the MEN regulate cell cycle progression in fission yeast? Minor defects in mitotic exit are seen in mutants of fission yeast MEN homologues, but it seems that this pathway is used primarily to regulate cytokinesis (Balasubramanian et al., 2000). Fission yeast, like animal cells, do not have a prolonged period during anaphase with high Cdc2 activity, and there is no reason to think it is necessary. It seems more likely that a signaling pathway designed to regulate cytokinesis was co-opted by budding yeast to also regulate cell cycle progression.

Substrates of Hct1

The consequence of Cdc14 release from the nucleolus is the activation of Hct1 and Sic1. Hct1 is required for the complete destruction of the Clb proteins, but also mediates the degradation of Ase1, Cdc5, Cdc20, and mediates destruction of Pds1 in G1 (Charles et al., 1998; Pellman et al., 1995; Prinz et al., 1998; Shirayama et al., 1998). Non-degradable versions of Cdc5 and Ase1 have only minimal defects, and the viability of *hct1Δ* cells suggests that the destruction of these other proteins is not critical. *hct1Δ* cells have a greater requirement on Cdc20 function, so Cdc20 may play a role degrading other Hct1 substrates. Likewise, Hct1 may compensate for loss of Cdc20 function, because *HCT1* is essential in a *cdc20Δ pds1Δ clb5Δ* triple mutant (Shirayama et al., 1999). In both cases the essential role of Cdc20 and Hct1 may be to activate Clb degradation, but this has not been tested directly. If this were true, than cells that express high levels of Sic1 might be able to live in the absence of Pds1, Clb5 and all APC function.

The Bub2 checkpoint inhibits Cdc14

Why are the components of the MEN localized to the SPB, and what is the purpose of the asymmetric localization of Spg1 and Cdc7 in fission yeast? Studies on the checkpoint gene *cdc16* in fission yeast suggest that the localization and asymmetry might be important for checkpoint signaling. During periods of mitotic arrest, *cdc16* mutants go through repeated rounds of septation (Fankhauser et al., 1993). Cdc16 along with the Byr4 protein form a two component GTPase activating protein (GAP) that also localizes to the spindle pole body (Sohrmann et al., 1998). *cdc16* mutants localize GTP-bound Spg1 and Cdc7 to both spindle pole bodies at inappropriate times, suggesting that Cdc16/Byr4 normally keeps Spg1 inactive.

BUB2, the budding yeast *cdc16* homologue, was initially isolated as a mutant defective in the spindle checkpoint (Hoyt et al., 1991). But in later studies, *bub2Δ* mutants behaved differently than *mad1-3*, and *bub1* and *3* mutants (Hardwick et al., 1996; Wang and Burke, 1995). Four recent papers clearly demonstrated that Bub2 functions in a checkpoint that is independent from the Mad spindle checkpoint (Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999; Li, 1999). The Mad checkpoint regulates the Cdc20-dependent APC and sister separation, and although it has been reported that *mad* mutants and *mps1* mutants degrade Clb proteins and enter the next cell cycle (and see Chapter 3) (Li and Murray, 1991; Minshull et al., 1996; Weiss and Winey, 1996), these recent reports failed to see this. Deleting *BUB2* in addition to *MAD2*, however, caused robust Clb proteolysis and re-replication, suggesting that Bub2 might directly regulate inactivation of Cdc28-associated kinase activity via Cdc14. Dbf2 hyperphosphorylation, which can be triggered by spindle depolymerization, was also shown to depend on *BUB2*, but not the *MAD* genes, further connecting Bub2 to the MEN (Fesquet et al., 1999). Lastly, mutants in *BFA1*, the *byr4* homologue, were shown to have phenotypes similar to

bub2Δ, and overexpression of Bfa1 caused an anaphase arrest similar to mutants in the MEN genes (Li, 1999).

If the spindle checkpoint has two branches, one that regulates the Cdc20-dependent APC and another that regulates the Hct1-dependent APC, do they sense different defects? Dbf2 hyperphosphorylation is induced in conditions that activate the Mad checkpoint, suggesting that the Bub2 checkpoint might sense similar defects. But because the Bub2 checkpoint does not influence sister separation, it seems unlikely that it monitors kinetochore attachment to the spindle.

An attractive model, based largely on the work done in fission yeast, is that the Bub2 checkpoint monitors spindle elongation by monitoring the position of the future daughter spindle pole body (Balasubramanian et al., 2000; Sohrmann et al., 1998). In this model, Bub2 would keep Tem1 in the GDP bound state on the daughter SPB until the SPB made it past the bud neck during anaphase B. Once in the daughter, Tem1 would shift to the GTP bound form and activate the MEN. This model depends on the asymmetric localization of an activator of Tem1, and Lte1, the guanine nucleotide exchange factor (GEF) that is part of the MEN, has recently been shown to localize only to the cortex of the bud (T. Carrol and D. Morgan, personal communication). Delivery of the spindle pole body would trigger release of Cdc14, inactivation of Cdc28 and final exit from mitosis.

No Lte1 homologue has been found in fission yeast, but if Lte1 function is conserved, such a homologue would be expected to localize to tip of the fission yeast cell that receives the daughter SPB, and activation of Spg1 on that SPB would then trigger cytokinesis. Cdc16 function would be required throughout the cell cycle to prevent re-activating Spg1 on the SPBs and causing septation. Because fission yeast are symmetric, there is no clear reason why there should be any bias to which SPB bound Spg1-GTP.

In budding yeast spindle elongation into the daughter and thus delivery of the daughter SPB depends on cytoplasmic microtubules (Sullivan and Huffaker, 1992; Yang et al., 1997; Yeh et al., 1995). Movement of the daughter nucleus also depends on

cytoplasmic dynein, and the protein products of the *KAR9* and *BIK1* genes (Berlin et al., 1990; Miller and Rose, 1998). Kar9 localizes to the daughter cell cortex, and binds to the Bik1 protein, which binds microtubules (Korinek et al., 2000; Lee et al., 2000). These two proteins are thought to function as the cortical anchor for cytoplasmic microtubules and mutation in either of these genes causes problems with spindle elongation and migration of the daughter nucleus into the bud. Mutants in *DHC1*, which encodes the budding yeast dynein hevay chain, have similar, but more severe defects, and combination of *dhc1Δ* and *kar9Δ* mutants is synthetically lethal. It has therefore been argued that Dhc1 and Kar9/Bik1 are two independent pathways responsible for spindle placement, but since this process is mediated by microtubules, it is hard to know just how independent the two pathways are. These mutants delay the proteolysis of Clb proteins suggesting that they may activate the Bub2 checkpoint, and in support of this idea, *kar9Δ bub2Δ* mutants are barely viable (D. Thompson and A.W. Murray). It is possible that Bik1 may play a role in the Bub2 checkpoint because *bik1Δ* mutants abolish the delays in mitotic exit seen in *dhc1* and *act5* mutants (*ACT5* the budding yeast dynactin gene, which is critical for dynein function (Muhua et al., 1994)) mutants (Muhua et al., 1998). One possibility is that Bik1 is involved in localizing Lte1 to the daughter cell, and therefore in a *bik1Δ* mutant, Tem1 becomes prematurely activated.

Interestingly, *bub2Δ* cells appear to have little phenotype on their own, which raises the question of how *BUB2* mutants were discovered in the first place (Hoyt et al., 1991). Presumably the relative importance of the Mad and Bub2 checkpoint might vary depending on the experimental protocol or strain background (Alexandru et al., 1999). These differences might also explain the differences seen in the extent of Clb proteolysis in *mad* mutants in different studies. In the first studies of *mad* and *mps1* mutants, Cdc28 inactivation and Clb2 proteolysis clearly occurs when cells inappropriately exit mitosis (see chapter 3). Given the recent studies on Bub2, this result would suggest that the Bub2 checkpoint arrest is not permanent and can easily be overridden. The Bub2 checkpoint

might be influenced by cell size or nutritional status which may vary in different experiments, or may be due to the eventual delivery of the daughter SPB to the daughter bud due to incomplete depolymerization of cytoplasmic microtubules.

Other inputs

Two recent pieces of data suggest that signaling from Bub2 to Cdc14 is not a simple linear pathway. Overexpression of non-degradable Pds1 (Pds1-dbΔ) blocks Clb proteolysis, and this is not simply due to a delay in delivering the daughter SPB to the daughter cell, because overexpression of Pds1-dbΔ in anaphase also delays Clb proteolysis (Cohen-Fix and Koshalnd, 1997). This result suggests that Pds1 inhibits the MEN downstream of Bub2 signaling. As mentioned above, overexpression of Esp1 during mitosis can trigger APC activation and Clb proteolysis, and *esp1-1* mutants delay activating Clb proteolysis (Cohen-Fix and Koshland, 1999; Tinker-Kulberg and Morgan, 1999). The effects of Esp1 are dependent on Cdc20 function and also on the function of the MEN. A complicated feedback mechanism has been proposed whereby Esp1 activates the MEN, which then stimulates Cdc20 activity. Another possibility is that Esp1 might act in opposition to Clb5 inhbition of cell cycle progression. This might explain why the effects of overexpressing Esp1 are substantially weaker in a *cdc20* mutant, in which Clb5 is stable. The role of Esp1 in activating Clb proteolysis has been questioned by the finding that *bub2Δ* rescues the Clb proteolysis defect of *esp1-1*, suggesting that *esp1-1* simply activates the Bub2 checkpoint. This explanation does not explain the Esp1 overexpression phenotype, and it also ignores the fact that *bub2Δ* mutants alone appear to cause an increase in Clb proteolysis, which might explain the rescue of *esp1-1* mutants (see Appendix 1). The increased Clb proteolysis of *bub2Δ* cells also suggests that Bub2 may function throughout the cell cycle to keep Cdc14 in the nucleolus, and may explain why *bub2Δ* has been found as a suppressor of the *cdc20-1* mutant (Tavormina and Burke, 1998).

The second result has been a careful analysis of the phosphorylation of Cdc15. Cdc15 is phosphorylated throughout the cell cycle, though as cells exit mitosis, it is briefly de-phosphorylated (Jaspersen and Morgan, 2000). This dephosphorylation is mediated by Cdc14, and is thought to activate the protein kinase activity of Cdc15. De-phosphorylation of Cdc15, however, is not required for Cdc14 release from the nucleolus. Phosphorylation of Cdc15 may be mediated in part by Cdc28, and would be a potential inhibitory input into the MEN from Cdc28. De-phosphorylation does promote Cdc15 activity because overexpression of a non-phosphorylatable Cdc15 can activate the APC and suppress mutations in the MEN pathway. Thus it appears that Cdc14 feeds back onto the MEN, and that Cdc15 may regulate Cdc14 after its release from the nucleolus.

Budding and fission yeast use different solutions for the same problem

Although the MEN has different targets in budding and fission yeast, the end result is quite similar. In fission yeast, it is critical that the two daughter nuclei move far away from the site of septation to prevent the septum from severing a chromosome. In budding yeast the site of cytokinesis is fixed at the bud neck, so a successful cytokinesis depends on the passage of the daughter nuclei through the bud neck and into the daughter bud. Anaphase B is thus a bit more difficult in budding yeast, so it is critical to keep cells in mitosis by maintaining Cdc28 activity during that time. Once the daughter nucleus is correctly delivered, cell cycle progression continues. Cell cycle progression can occur in the absence of proper cytokinesis, and frequently does in cells that have been arrested in mitosis, but because the bud neck provides a physical constriction between mother and daughter, this does not cause the problems that it causes in fission yeast and animal cells.

Chapter 1

Cdc28 activates exit from mitosis in budding yeast

Abstract

The activity of the cyclin dependent kinase, Cdc28 (Cdk1), inhibits the transition from anaphase to G1 in budding yeast. *CDC28-T18V, Y19F* (*CDC28-VF*), a mutant that lacks inhibitory phosphorylation sites, delays the exit from mitosis and is hypersensitive to perturbations that arrest cells in mitosis. Surprisingly, this behavior is not due to a lack of inhibitory phosphorylation or increased kinase activity, but reflects reduced activity of the anaphase promoting complex (APC), a defect shared with other mutants that lower Cdc28/Clb activity in mitosis. *CDC28-VF* has reduced Cdc20-dependent APC activity in mitosis, but normal Hct1-dependent APC activity in the G1 phase of the cell cycle. The defect in Cdc20-dependent APC activity in *CDC28-VF* correlates with reduced association of Cdc20 with the APC. The defects of *CDC28-VF* suggest that Cdc28 activity is required to induce the metaphase to anaphase transition and initiate the transition from anaphase to G1 in budding yeast.

Introduction

To exit mitosis, cells must accomplish two tasks: chromosome segregation and inactivation of complexes between mitotic cyclins and Cdk1 (known as Cdc28 in budding yeast and Cdc2 in other eukaryotes), which allows the cell cycle to progress into G1. Both steps require the activity of the anaphase promoting complex (APC)[1] or cyclosome, a multi-protein complex that is required for the ubiquitination of cyclin and other unstable substrates (King et al., 1995; Sudakin et al., 1995; Yanagida et al., 1999; Zachariae and Nasmyth, 1996; Zachariae and Nasmyth, 1999). The activity of the APC depends on its interaction with two WD-40 proteins, Cdc20 (Lorca et al., 1998; Sethi et al., 1991; Sigrist et al., 1995; Visintin et al., 1997) and Hct1 (Kitamura et al., 1998; Schwab et al., 1997; Sigrist and Lehner, 1997; Visintin et al., 1997; Yamaguchi et al., 1997). Cdc20 initiates the metaphase to anaphase transition by inducing ubiquitination of the anaphase inhibitor Pds1 (Cohen-Fix et al., 1996; Funabiki et al., 1997). This reaction causes Pds1 degradation and sister chromatid separation (Ciosk et al., 1998; Funabiki et al., 1996b). Cdc20-dependent APC activity is inhibited by the spindle checkpoint, which senses defects in chromosome attachment to the spindle and delays the onset of anaphase until these defects are corrected (Fang et al., 1998a; Hardwick, 1998; Hwang et al., 1998; Kim et al., 1998; Rieder et al., 1995).

The second WD-40 protein, Hct1, also activates the APC (Kramer et al., 1998; Schwab et al., 1997; Sigrist and Lehner, 1997; Visintin et al., 1997). In budding yeast, Hct1-dependent APC activity is necessary for the ubiquitination and degradation of Clb2, the major mitotic cyclin, which causes the sudden drop in the protein kinase activity of Cdc28 at the end of mitosis and keeps the APC active throughout G1 (Jaspersen et al., 1999; Zachariae et al., 1998). The activation of the Hct1-dependent APC depends on the prior activation of the Cdc20/APC, and this dependency helps ensure that the events of

mitosis occur in the proper sequence (Lim et al., 1998; Shirayama et al., 1999; Visintin et al., 1998; Yeong et al., 2000).

In budding yeast, active Cdc28 inhibits the transition from anaphase to G1 (Amon, 1997; Li and Cai, 1997). One of the primary targets that Cdc28/Clb2 complexes inhibit is Hct1. Phosphorylation of Hct1 by Cdc28/Clb complexes prevents it from binding to and activating the APC (Jaspersen et al., 1999; Zachariae et al., 1998). This inhibition is opposed by the phosphatase Cdc14, which de-phosphorylates Hct1, initiating a positive feedback loop that drives the cell into G1 (Jaspersen et al., 1999; Visintin et al., 1998). As Hct1 activity rises, the rate of Clb destruction increases, reducing the kinase activity of Cdc28 and further activating Hct1. The cyclin dependent kinase (CDK) inhibitor Sic1 (Donovan et al., 1994; Mendenhall, 1993), which inhibits Cdc28/Clb complexes, also participates in this feedback loop, since both its transcription and stability are inhibited by Cdc28/Clb activity (Amon, 1997; Toyn et al., 1997; Visintin et al., 1998).

Does Cdc28 also play a role in inducing anaphase? In frog and clam egg extracts, activation of the APC depends on active Cdc2/CyclinB complexes (Felix et al., 1990; Hershko et al., 1994; Minshull et al., 1994; Shteinberg and Hershko, 1999). This activating role might be explained by the finding that Cdc2/Cyclin B can phosphorylate subunits of the APC (Kotani et al., 1999; Patra and Dunphy, 1998), and this phosphorylation is correlated with activating the APC and binding of Fizzy, the Cdc20 homolog in frogs (Fang et al., 1998b; Kotani et al., 1998; Peters et al., 1996; Shteinberg et al., 1999). In embryonic cell cycles, no Hct1 homolog is present (Lorca et al., 1998; Sigrist and Lehner, 1997), suggesting that Cdc20-dependent APC activity targets both Pds1 homologs and mitotic cyclins for destruction. In budding yeast, the inability of Cdc20 to catalyze the complete destruction of mitotic cyclins means that Hct1 or Sic1 are required for cells to exit from mitosis (Schwab et al., 1997; Visintin et al., 1997). Since both Hct1 and Sic1 are inhibited by Cdc28, it has been difficult to ask whether a previous step in the exit from mitosis requires Cdc28.

Cdc28/Clb activity is also regulated by inhibitory phosphorylation on residues tyrosine 19, and possibly threonine 18 (Amon et al., 1992b; Booher et al., 1993; Sorger and Murray, 1992). Tyrosine 19 is phosphorylated by Swe1 (the homolog of Wee1 in fission yeast) and dephosphorylated by Mih1 (the homolog of Cdc25 in fission yeast) (Booher et al., 1993; Russell et al., 1989). Phosphorylation on the homologous sites of fission yeast and vertebrate Cdc2 controls the timing of entry into mitosis and can also be induced by the checkpoints that detect unreplicated or damaged DNA (Enoch and Nurse, 1990; Gould and Nurse, 1989; Jin et al., 1996; Norbury et al., 1991; Rhind et al., 1997). In budding yeast, tyrosine 19 is phosphorylated during S-phase, but *CDC28-T18A, Y19F* (*CDC28-AF*) cells respond normally to the DNA damage checkpoint (Amon et al., 1992a; Sorger and Murray, 1992). Inhibitory phosphorylation of Cdc28 is required for the bud emergence or morphogenesis checkpoint, which delays mitosis in cells that have not budded (Lew and Reed, 1993).

Our previous work suggested that inhibitory phosphorylation of Cdc28 might aid in mitotic exit. *cdc55Δ* cells, which lack a B subunit of protein phosphatase 2A (PP2A) (Healy et al., 1991), are spindle checkpoint defective and have increased phosphorylation on tyrosine 19 of Cdc28 (Minshull et al., 1996; Wang and Burke, 1997). The premature exit from mitosis in *cdc55Δ* cells with damaged spindles is suppressed by *CDC28-T18V, Y19F* (*CDC28-VF*), suggesting that inhibitory phosphorylation of Cdc28 might work in concert with Hct1 and Sic1 to reduce Cdc28-associated activity at the end of mitosis.

Here we show that this hypothesis is incorrect. Although *CDC28-VF* has defects in leaving mitosis, these are not due to a lack of inhibitory phosphorylation but reflect a second defect of *CDC28-VF*. This defect slows the normal activation of the Cdc20-dependent APC and reveals that Cdc28-associated activity is essential for the activation of the APC in mitosis.

Results

CDC28-VF impairs mitotic exit

Since *CDC28-VF* suppresses the checkpoint defect of *cdc55Δ* (Minshull et al., 1996), we wondered if the *CDC28-VF* mutant alone might have difficulty leaving mitosis. Figure 1A shows that progression through mitosis is delayed in *CDC28-VF*. Wild type and *CDC28-VF* cells were arrested in G1 by the mating pheromone alpha factor and released into the cell cycle. Once cells had budded, alpha factor was re-added to arrest cells that had completed the cycle. This regimen allows us to look clearly at one synchronous cell cycle. *CDC28-VF* cells show a 30 minute delay in the degradation of the anaphase inhibitor Pds1 and sister chromatid separation. Clb2 proteolysis and the fall in Clb2-associated kinase activity are delayed by more than 30 minutes. These delays can be partially attributed to a 15 minute delay in Clb2 accumulation, but the persistence of peak levels of Clb2 for at least 60 minutes clearly reflects an additional defect in *CDC28-VF*. In addition, mitotic entry is not delayed because short spindles, a marker for mitotic entry, appear at the same time in *CDC28-VF* and wild type cells (data not shown). *CDC28-VF* does not delay exit from G1, since the CDK inhibitor Sic1 (Schwob et al., 1994) disappears at the same time (30 minutes) in wild type and *CDC28-VF*.

Although *CDC28-VF* delays passage through mitosis, the doubling time of *CDC28-VF* cells is nearly identical to that of wild type cells (data not shown). This apparent paradox can be explained by the fact that *CDC28-VF* cells exit mitosis at a larger cell size than wild type cells, and therefore have to grow less in G1 to reach the critical cell size needed to pass Start, the cell cycle transition that commits them to replicating their DNA (Johnston et al., 1977). Thus the increase in time spent in mitosis is made up by a decrease in time spent in G1.

To see if the mitotic delay in *CDC28-VF* is due to difficulty exiting mitosis, we examined cells that were recovering from activation of the spindle checkpoint. Wild type and *CDC28-VF* cells were arrested in mitosis by treating them with nocodazole (an inhibitor of microtubule polymerization) for three hours and then released from this arrest into fresh medium containing alpha factor to arrest them in G1 as they left mitosis. Wild type cells degrade Clb2, inactivate Clb2-associated kinase, and separate their sisters within 90 minutes of removing nocodazole (Figure 1B). *CDC28-VF* cells, however, take 150 to 180 minutes to fully escape from the nocodazole arrest.

Since *CDC28-VF* cells are delayed in exiting mitosis, we investigated how they responded to a prolonged mitotic arrest caused by the spindle checkpoint. We examined the response to overexpressing the protein kinase Mps1, which arrests cells in mitosis by activating the checkpoint, but does not damage the spindle (Hardwick et al., 1996). After about eight hours, wild type cells overcome the arrest, divide and resume proliferating. In contrast, *CDC28-VF* cells overexpressing Mps1 cannot proliferate (Figure 2A); when individual cells are followed microscopically, many never divide, and the remainder go through only one or two divisions (Figure 2B and data not shown). The cell cycle arrest and eventual lethality are completely suppressed by the *mad3Δ* mutation, which inactivates the spindle checkpoint (Hardwick et al., 1998; Li and Murray, 1991). *mad3Δ* and *mad3Δ CDC28-VF* cells divide as if there were no activation of the checkpoint. These results show that *CDC28-VF* cells, unlike wild type, cannot escape from mitosis in the presence of constant stimulation of the spindle checkpoint. *CDC28-VF* cells are also sensitive to other perturbations that activate the spindle checkpoint, including the presence of short linear chromosomes (Wells and Murray, 1996) and mutations that damage the spindle (Hardwick et al., 1999). Like Mps1 overexpression in *CDC28-VF*, these treatments are lethal and cause long delays in mitosis (data not shown).

The mitotic defect of *CDC28-VF* is not caused by a lack of inhibitory phosphorylation.

We initially observed that *CDC28-VF* and *cdc55Δ* have opposite effects on the exit from mitosis, consistent with the idea that inhibitory phosphorylation of Cdc28 aids exit from mitosis. More careful examination reveals that the mitotic exit defect in *CDC28-VF* is not due to effects on Cdc28 phosphorylation. Figure 3A shows serial dilutions of a panel of mutants, spotted onto galactose-containing plates, which induce Mps1 overexpression, or glucose-containing plates, which do not. *swe1Δ* cells lack the tyrosine kinase that phosphorylates Cdc28 (Booher et al., 1993). Like *CDC28-VF* cells, they have no phospho-tyrosine present on Cdc28 (Figure 3B), but unlike *CDC28-VF* or *CDC28-F* they are no more sensitive to Mps1 overexpression than wild type cells. Since another kinase might phosphorylate threonine 18 in *swe1Δ* cells, we investigated the behavior of *swe1Δ CDC28-V* cells, which should lack all inhibitory phosphorylation. *swe1Δ CDC28-V* resemble *CDC28-V* cells, both being only slightly more sensitive to Mps1 overexpression than wild type cells. *mih1Δ* cells, which like *cdc55Δ* cells have increased inhibitory phosphorylation on Cdc28 (Figure 3B), do not have a spindle checkpoint defect as judged by their sensitivity to Mps1 overexpression or to microtubule depolymerizing agents (Figure 3A and data not shown).

In principle, the relative insensitivity of *swe1Δ* to Mps1 overexpression could be explained by the existence of other kinases that phosphorylate tyrosine 19 of Cdc28. We do not believe such a kinase exists. We have never detected phospho-tyrosine on Cdc28 in *swe1Δ* or *swe1Δ mih1Δ* cells (Figure 3B and data not shown) and *mih1Δ* cells show a 15 minute delay in entering mitosis that is completely suppressed by deleting *SWE1*, suggesting that Swe1 is the only kinase responsible for inhibiting Cdc28 (data not shown). In addition, if such a kinase existed, we would expect *CDC28-AF* (Amon et al., 1992a; Sorger and Murray, 1992), which substitutes alanine at position 18 of Cdc28 rather than

valine, to behave identically to *CDC28-VF*. However, *CDC28-AF* behaves like *CDC28-F*, both of which are less sensitive to Mps1 overexpression than *CDC28-VF* (Figure 3A).

We favor the idea that the T18V, Y19F substitution causes a phosphorylation-independent defect in Cdc28. Based on the crystal structure of human Cdk2, tyrosine 19 of Cdc28 is adjacent to the gamma phosphate of bound ATP (De Bondt et al., 1993). Thus, *CDC28-VF*, and to a lesser extent the *CDC28-F* and *CDC28-AF* mutations, might affect ATP binding, substrate binding, catalytic activity, or substrate specificity of Cdc28.

Mitotic Cdc28 kinase activity is required for proper response to the spindle checkpoint.

Because the *CDC28-VF* defect is not due to a lack of inhibitory phosphorylation, we asked if other mutations that affect mitotic Cdc28 activity might share phenotypes with *CDC28-VF*. *cdc28-1N*, *clb2Δ* and *cks1-38* (a mutant in Cks1, a Cdc28-binding protein required for passage through Start and mitosis) are all more sensitive to Mps1 overexpression than wild type (Figure 4A) (Hadwiger et al., 1989; Piggott et al., 1982; Surana et al., 1991; Tang and Reed, 1993). This phenotype is not seen in *cdc28-4*, a temperature sensitive allele of Cdc28 which prevents passage through Start and is primarily defective in the G1 function of Cdc28 (Reed, 1980).

Does a reduction in mitotic Cdc28 activity cause the *CDC28-VF* phenotype? We have observed that both Clb2- and Clb3-associated kinase activity and total Cdc28-associated kinase activity of *CDC28-VF* cells is lower than wild type (Figure 4B and data not shown). This is seen both in synchronously cycling cells (Figure 1, Clb2-associated kinase activity) and in cells arrested by the spindle checkpoint (Figure 4B). We estimate the specific activity of a Cdc28-VF/Clb complex is roughly half that of a wild type Cdc28/Clb complex. Because the difference between wild type and *CDC28-VF* is small, it is difficult to know if it is biologically significant. However, an independent substitution in

the ATP binding site of Cdc28, *CDC28-F88G*, has a reduced specific activity in vitro (Ubersax et al., submitted) and is as sensitive to overexpression of Mps1 as *CDC28-VF* (Figure 4C).

The mitotic defect of *CDC28-VF* is semi-dominant. The heterozygote *CDC28/CDC28-VF* has an intermediate sensitivity to Mps1 overexpression (data not shown) and overexpression of Cdc28-VF in otherwise wild type cells creates cells which are fully sensitive to overexpressed Mps1 (data not shown and Figure 5). These results suggest that Cdc28-VF is a dominant negative mutant, which competes with the wild type kinase for substrates, mitotic cyclins, and Cks1. In support of this idea, multiple copies of the *CDC28* gene on a *2μ* plasmid suppress the lethality of overexpressing Mps1 in a *CDC28-VF* strain (Figure 4D). The semi-dominant phenotype of *CDC28-VF* does not reflect haploinsufficiency of Cdc28, since *CDC28/cdc28Δ* diploids do not have a phenotype like *CDC28-VF* (data not shown).

CDC28-VF is defective in activating the APC

Because *CDC28-VF* impairs the exit from mitosis, crippling other pathways involved in this process might kill *CDC28-VF* cells. Our inability to make double mutants between *CDC28-VF* and mutants in the APC supports this idea (Table II). *CDC28-VF* is synthetically lethal in combination with mutations in components of the APC (*cdc23-1*, *cdc16-1*, *apc10-1* (formerly *doc1-1*)) (Hwang and Murray, 1997; Irniger et al., 1995; Lamb et al., 1994) and positive regulators of the APC (*cdc5-1*, *hct1Δ* and *cdc20-1*) (Charles et al., 1998; Schwab et al., 1997; Shirayama et al., 1998; Visintin et al., 1997). These double mutants are inviable at all temperatures, and although the double mutant spores are able to germinate, they die in microcolonies of large budded cells (data not shown), indicating a terminal arrest in mitosis. These genetic interactions are specific to the APC and regulators of the APC, because two other mutants that arrest in mitosis, *cdc13-1*

(which activates the DNA damage checkpoint) and *cdc15-2* (which arrests cells in anaphase) are both viable in combination with *CDC28-VF*.

The interactions of *CDC28* alleles and *clb2Δ* with Mps1 overexpression and the *cdc23-1* mutant are correlated with each other: *cdc28-1N*, *CDC28-F88G* and *clb2Δ* are synthetically lethal in combination with *cdc23-1* and cannot proliferate when overexpressing Mps1, whereas *cdc28-4* is viable in combination with *cdc23-1* or Mps1 overexpression (Table II and (Irniger et al., 1995)). *CDC28-F* and *CDC28-AF*, which have milder phenotypes than *CDC28-VF*, are synthetically lethal with *cdc23-1*, but viable in combination with *cdc16-1*, a weaker APC mutant (Table II). Lastly, *swe1Δ* is viable in combination with *cdc23-1* and *cdc16-1* (Table I) demonstrating that Swe1 is unlikely to have a role in promoting the exit from mitosis.

The genetic interactions between *CDC28-VF* and the APC and its regulators are a mixed blessing. Although they suggest that Cdc28 may help activate the APC, they prevent us from examining the phenotype of a *CDC28-VF apc*$^-$ double mutant, since it is impossible to create such a mutant. We overcame this difficulty by exploiting the fact that overexpressing Cdc28-VF creates cells that behave phenotypically like *CDC28-VF*. This overexpression in *cdc23-1* cells at the permissive temperature of 23°C is toxic and no cells survive even a brief 1 hour pulse of *CDC28-VF* expression driven by the *GAL1* promoter (data not shown).

We overexpressed *CDC28* and *CDC28-VF* in *cdc23-1* cells that had been arrested by alpha factor and then released them from the arrest into fresh medium at 23°C , adding alpha factor after budding so that cells will re-arrest when they reach the next G1. Overexpression of *CDC28-VF* causes a permanent large budded arrest with high levels of Clb2 and Clb3 (Figure 5A). Sic1 levels never rise and Clb2-associated kinase activity never falls. Sister separation and spindle elongation are delayed by 2 hours in cells expressing *CDC28-VF* (Figure 5B and data not shown). These observations show that the induction of anaphase is delayed and the transition from anaphase to G1 is completely

blocked in *cdc23-1 CDC28-VF* cells. These phenotypes are not due to activating the spindle checkpoint because neither *mad2Δ* nor *mad2Δ bub2Δ* bypass the arrest (data not shown) (Alexandru et al., 1999; Hoyt et al., 1991; Li and Murray, 1991). We therefore conclude that *CDC28-VF* is defective in activating both the Cdc20-dependent APC, which triggers anaphase, and the Hct1-dependent APC, which completes cyclin proteolysis.

CDC28-VF has normal G1 Hct1-dependent APC activity

The failure to induce mitotic cyclin proteolysis in *cdc23-1* mutants overexpressing Cdc28-VF could reflect the requirement for Cdc20 activity to activate the Hct1-dependent APC (Shirayama et al., 1999; Visintin et al., 1998; Yeong et al., 2000), or it could reflect a Cdc20-independent defect in the activity of Hct1. To distinguish between these possibilities, we asked if *CDC28-VF* has normal APC activity in G1, a time when all APC activity is Hct1 dependent and Cdc20 is absent (Charles et al., 1998; Prinz et al., 1998; Shirayama et al., 1998) and see below). We immunoprecipitated the APC from alpha factor arrested cells with antibodies raised against Cdc26, a non-essential component of the APC (Hwang and Murray, 1997), and measured its ability to ubiquitinate an iodinated fragment of sea urchin Cyclin B in a reconstituted ubiquitination assay (Charles et al., 1998). We detected no differences in APC activity in wild type and *CDC28-VF*, and the activity of immunoprecipitates from both wild type and *CDC28-VF* could be increased by adding recombinant Hct1 (Figure 6) (Jaspersen et al., 1999). In addition, we have shown that Hct1-dependent proteolysis of Clb2 and Pds1 in G1 is normal in *CDC28-VF* (data not shown and Figure 7C). These experiments are consistent with the idea that the *CDC28-VF* mutant has no direct effect on the activity of the Hct1-dependent APC.

Cdc28 activates the Cdc20-dependent APC

Cdc20 is required for sister chromatid separation (Shirayama et al., 1998). *CDC28-VF* shows delays in sister separation (Figures 1 and 5), suggesting that Cdc20-dependent APC activity is defective in these cells. We have approached this issue in more detail by examining the half-life of Pds1, a substrate of the Cdc20-dependent APC (Shirayama et al., 1999; Tinker-Kulberg and Morgan, 1999; Visintin et al., 1997) during anaphase, a time when the Cdc20-dependent APC is thought to be active (Jaspersen et al., 1998). First, we needed to confirm that degradation of Pds1 in anaphase is due to Cdc20 and not Hct1. We arrested wild type, *cdc20-3* and *hct1Δ* strains in anaphase by overexpressing a non-degradable Clb2 (*pGAL-CLB2-Δ176*) (Surana et al., 1993). These strains also contained an epitope tagged form of Pds1 replacing the endogenous gene. When all cells had reached anaphase (t=0 in Figure 7A), the cultures were shifted to 37°C to see if Pds1 levels would rise in either the *cdc20-3* or *hct1Δ* strain, as an indication that Pds1 had become more stable. Pds1 levels rose in the *cdc20-3* strain, but not in the *hct1Δ* strain, showing that the stability of Pds1 in anaphase is controlled by the Cdc20- rather than the Hct1-dependent APC (Figure 7A).

We next examined the half life of Pds1 during an anaphase arrest caused by the *cdc15-2* mutation. Pds1 is unstable during this arrest and Cdc20 is required to exit from the arrest (Jaspersen et al., 1998; Tinker-Kulberg and Morgan, 1999). *cdc15-2*, *cdc15-2 CDC28-VF* and *cdc15-2 clb2Δ* cells were arrested in anaphase, an epitope tagged *PDS1* gene driven by the *GAL1* promoter was induced by adding galactose for 1 hour, and its expression was terminated by adding glucose. The half life of Pds1 in *cdc15-2* cells in this experiment was less than 15 minutes, but was greater than 1.5 hr in *CDC28-VF cdc15-2* and *clb2Δ cdc15-2* cells (Figure 7B) showing that the *CDC28-VF* and *clb2Δ* mutations compromise Cdc20-dependent APC activity.

Earlier studies argued that Hct1 and Cdc20 were specificity factors for the APC, with Cdc20 directing the ubiquitination of Pds1 and Hct1 directing that of mitotic cyclins (Visintin et al., 1997). The instability of Pds1 in G1 cells, which lack detectable Cdc20 (Prinz et al., 1998), prompted us to re-examine this issue. Wild type, *CDC28-VF* and *cdc20-3* cells were arrested in G1 by alpha factor, or in the case of *hct1Δ*, which is resistant to alpha factor (Schwab et al., 1997), by the *cdc28-13* mutation (Reed, 1980). Once arrested, wild type, *CDC28-VF* and *cdc20-3* cells were shifted to 37°C (for *cdc28-13* and *cdc28-13 hct1Δ*, asynchronous cultures were transferred to 37°C), *pGAL-PDS1* was induced by adding galactose for two hours and then expression was shut off by adding glucose. Pds1 was equally unstable in wild type, *CDC28-VF*, *cdc20-3* and *cdc28-13* cells, but was completely stable in the *hct1Δ cdc28-13* cells. This control shows that the defect in Pds1 stability in *CDC28-VF* is specific to anaphase and, together with Figure 7A, shows that Pds1 is targeted for destruction by the Cdc20-dependent APC in mitosis and by the Hct1-dependent APC in G1. In addition, this experiment reinforces the conclusion derived from in vitro experiments in Figure 6 that *CDC28-VF* has no defects in Hct1-dependent APC activity in G1. Our results differ from those of Visintin et al.(1997), who found that stability of Pds1 in G1 is regulated by Cdc20. Their results may have been due to incomplete alpha factor arrest of the *cdc20-1* allele used in their study. Our results agree with the recent observation that Clb2 is targeted for destruction by both the Cdc20 and Hct1 dependent forms of the APC (Yeong et al., 2000).

The stabilization of Pds1 in mitotic *CDC28-VF cdc15-2* cells suggests that *CDC28-VF* is defective in the Cdc20-dependent APC. As a first step in investigating the biochemical defect of *CDC28-VF*, we examined the interaction between Cdc20 and the APC in anaphase, a time when the Cdc20-dependent APC is active. We arrested *cdc15-2* and *cdc15-2 CDC28-VF* cells in anaphase, immunoprecipitated the APC with anti-Cdc26 antibodies, and examined the amount of associated Cdc20. Equal amounts of APC was immunoprecipitated from all three strains, but in *CDC28-VF* cells there was less associated

Cdc20 (Fig 8), even though the total level of Cdc20 was similar in wild type and *CDC28-VF*.

Discussion

Cdc28 activates the APC

We have shown that the *CDC28-VF* mutant is defective activating the Cdc20-dependent APC. *CDC28-VF* cells show a short delay in exiting mitosis, but this delay becomes more severe when the APC is compromised by spindle checkpoint activation or defects in the APC or its regulators. These phenotypes allowed us to show a requirement for Cdc28 in exiting mitosis. The defect in the exit from mitosis in *CDC28-VF* is correlated with reduced binding of Cdc20 to the APC and lower Cdc20-dependent APC activity. *CDC28-VF* cells have normal Hct1-dependent APC activity in G1, suggesting that their failure to exit mitosis is the result of interrupting the chain of events that normally leads from mitosis to G1: reduced Cdc20-dependent APC activation prevents the activation of Cdc14, which normally leads to activation of Hct1 and increased levels of Sic1, the key steps in inactivating the mitotic activity of Cdc28 (Jaspersen et al., 1999; Lim et al., 1998; Shirayama et al., 1999; Visintin et al., 1998).

It has been shown previously that Cdc28 inhibits exit from mitosis. High levels of Cdc28-associated kinase activity cause arrest in anaphase (Holloway et al., 1993; Surana et al., 1993), and inhibiting Cdc28 in mitotically arrested cells activates the APC, driving them through cytokinesis and into G1 (Amon, 1997; Li and Cai, 1997). These observations led to the conclusion that the major role for mitotic Cdc28 in regulating the APC was to inhibit the activity that drove cells out of mitosis. Our results show that Cdc28 has different effects on APC activity at different stages of mitosis. Cdc28/Clb activity activates the Cdc20-dependent APC to induce the metaphase to anaphase transition, but inhibits the Hct1-dependent APC, thus inhibiting cytokinesis and the transition from anaphase to G1. This pattern of regulation helps ensure chromosome segregation precedes

cell division and allows budding yeast to regulate these events separately - a useful feature in an organism that specifies the site of cytokinesis long before spindle assembly.

Cdc28 is required for recovery from checkpoint-dependent arrest

Proteins that were identified as members of the spindle checkpoint have two roles in keeping cells with depolymerized microtubules from leaving mitosis. Six of them, Mps1, Mad1-Mad3, Bub1, and Bub3, detect kinetochores that are not attached to the spindle (Hoyt et al., 1991; Li and Murray, 1991; Weiss and Winey, 1996), and prevent sister chromatid separation by inhibiting the Cdc20-dependent APC (Hwang et al., 1998; Kim et al., 1998). In contrast, Bub2, also identified as part of the spindle checkpoint (Hoyt et al., 1991), detects an unknown lesion and arrests cells in anaphase, probably by preventing the activation of Cdc14 (Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999; Li, 1999). The opposition between the inhibition of the Cdc20-dependent APC by the spindle checkpoint and activation by Cdc28 explains why *CDC28-VF* cells, which are defective in activating the Cdc20-dependent APC, have difficulty overcoming a checkpoint-dependent arrest.

We do not know how wild type cells eventually escape from mitosis despite continued Mps1 overexpression, a process that could be described as adaptation to the spindle checkpoint (Minn et al., 1996; Rieder and Palazzo, 1992; Rudner and Murray, 1996). In particular we cannot distinguish between constitutive mechanisms, such as stabilization of mitotic cyclins leading to a slow rise in Cdc28/Clb activity that eventually leads to APC activation, and induced ones, such as a slow process initiated by components of the checkpoint that leads to reduced inhibition of Cdc20. Although we initially thought of *CDC28-VF* as an adaptation mutant (Minshull et al., 1996), we now believe that it achieves its effect by reducing the ability of Cdc28 to activate the Cdc20-dependent APC whether or not the checkpoint is active. It is only when the checkpoint is active, however,

that this reduction is sufficient to keep cells from leaving mitosis. This discussion highlights a general difficulty in studying adaptation mutants: the ability to overcome prolonged checkpoint-dependent arrests appears to be very sensitive to small, checkpoint-independent defects in basic cell cycle machinery (Toczyski et al., 1997).

Other mutants share phenotypes with *CDC28-VF*

Genetic analysis of *CDC28* has revealed two types of mutants, those that primarily affect G1 (*cdc28-4, cdc28-13*) (Reed, 1980) and those that primarily affect exit from mitosis (*CDC28-VF, cdc28-1N*) (Piggott et al., 1982; Surana et al., 1991). The mitotic mutants share phenotypes with *clb2Δ* and *cks1-38*. Do these four mutants, *CDC28-VF, cdc28-1N, clb2Δ* and *cks1-38*, have a common biochemical defect? *clb2Δ* cells contain no Clb2, and therefore have reduced levels of mitotic Cdc28 activity (Grandin and Reed, 1993). The defects of *cdc28-1N* and *cks1-38,* which arrest cells in mitosis, may have more to do with altering the substrate specificity of Cdc28. When assayed by immunopreciptation of Clb2-associated kinase, *cdc28-1N* strains have similar kinase activity to wild type, but they have no kinase activity associated with Cks1-coupled beads (Kaiser et al., 1999; Surana et al., 1991). These results suggest that the primary defect in *cdc28-1N* is its failure to bind Cks1, which has been shown in frog and clam extracts to be essential for exit from mitosis and APC phosphorylation by Cdc2/Cyclin B (Patra and Dunphy, 1996; Patra and Dunphy, 1998; Shteinberg and Hershko, 1999).

Our only clue to the biochemical defect of Cdc28-VF is that it appears to have a small reduction in its specific activity (Figure 4D). We think this defect may be important because *CDC28-F88G*, a mutant in the ATP binding site of Cdc28, also has reduced specific activity (Ubersax et al., submitted), and shares phenotypes with *CDC28-VF* (Figure 4C). The *cdc28-1N* and *cks1-38* mutations may produce their effects by reducing the level of the Cdc28/Clb/Cks1 complexes, whereas in *clb2Δ* cells, the complexes of

Cdc28 with the remaining Clb proteins may be less capable of activating the Cdc20-dependent APC.

Although the Cdc28 activity is lower in *CDC28-VF* cells, we do not think that the defect in *CDC28-VF* is simply due to lower total Cdc28 activity per cell. Overexpression of Clb2 and Clb3, which raises Cdc28 activity in cells (Stueland et al., 1993), does not suppress the mitotic defect of *CDC28-VF* (data not shown). This result is consistent with the idea that the specific activity per Cdc28 molecule, not the total Cdc28 activity per cell, is critical for activating the exit from mitosis. An alternative explanation is that the different mutants change the substrate specificity of Cdc28, preventing phosphorylation of important mitotic substrates.

A cautionary tale

Protein phosphorylation is a common way of regulating protein activity. Mutating putative phosphorylation sites to non-phosphorylatable residues is a widely used technique for assessing the biological function of phosphorylation of specific proteins (Jaspersen et al., 1999; Li et al., 1995; Zachariae et al., 1998). Our analysis of *CDC28-VF* shows that such mutations can have unanticipated effects that are independent of phosphorylation.

It is difficult to tell how common such effects are. Our observations of *CDC28-VF* suggest that the studies conducted with the *CDC28-AF*, *CDC28-VF* and *CDC28-F* mutants in budding yeast (as well as experiments with the corresponding mutants in Cdc2) should be reexamined to exclude the possibility that the observed effects of these mutants were due to phosphorylation independent defects. A simple control is to ask whether the phenotype of the *CDC28* mutants is exactly mimicked by deletion of *SWE1*. If so, the conclusions of the original experiments are secure. If not, phosphorylation-independent effects due to mutating the inhibitory residues may contribute to the observed phenotypes. In the general case, the ideal control is to show that inactivating the kinase that phosphorylates a particular

protein produces a similar effect on the substrate's activity as do the phosphorylation site mutants.

Does Cdc28 phosphorylate the APC?

How does Cdc28 promote anaphase? Experiments in frogs, clams and mammalian cell culture have all suggested that phosphorylation activates the APC by modifying four of its subunits: Cdc16, Cdc23, Cdc27 and Apc1 (BimE) (Hershko et al., 1994; Kotani et al., 1999; Kotani et al., 1998; Patra and Dunphy, 1998; Peters et al., 1996). These proteins are phosphorylated during mitosis and the phosphorylated APC has greater Cdc20-dependent activity in vitro, whereas dephosphorylation of purified APC causes a loss of activity (Lahav-Baratz et al., 1995; Shteinberg et al., 1999). Studies in clams have suggested that this phosphorylation is required for proper Cdc20 binding (Shteinberg et al., 1999). In the accompanying paper, we show that the APC is phosphorylated by Cdc28 in budding yeast, and that a defect in this phosphorylation causes reduced Cdc20-dependent APC activity and contributes to the *CDC28-VF* phenotype.

Figure 1 *CDC28-VF* delays in exit from mitosis

(A) Wild type (—□—, ADR1859) and *CDC28-VF* (—O—, ADR1857) were grown overnight at 23°C in YPD to mid log phase, arrested in G1 with alpha factor (1μg/ml) and at t=0 the cells were released from the G1 arrest. At t=90, alpha factor (1.5μg/ml) was added back to the cultures to re-arrest the cells in the next G1. (left) Samples were taken at the indicated times and processed for Western blots and histone H1 kinase assays. (right) Sister chromatid separation was scored by counting the number of fluorescent spots (one or two) of GFP-lacI bound to 256 tandem repeats of *lacO* integrated at the *TRP1* locus.

(B) Wild type (—□—, ADR548) and *CDC28-VF* (—O—, JM477) were grown overnight at 23°C in YEP+2% glucose to log phase, arrested in mitosis with nocodazole (10μg/ml) for 3 hours and at t=0 the cells were released from the arrest into fresh medium containing alpha factor (10μg/ml). Samples were taken at the indicated times and processed for western blots and histone H1 kinase assays. Sister chromatid separation was scored by counting the number of fluorescent spots (one or two) of GFP-lacI bound to 256 tandem repeats of lacO integrated at the *LEU2* locus.

Figure 1

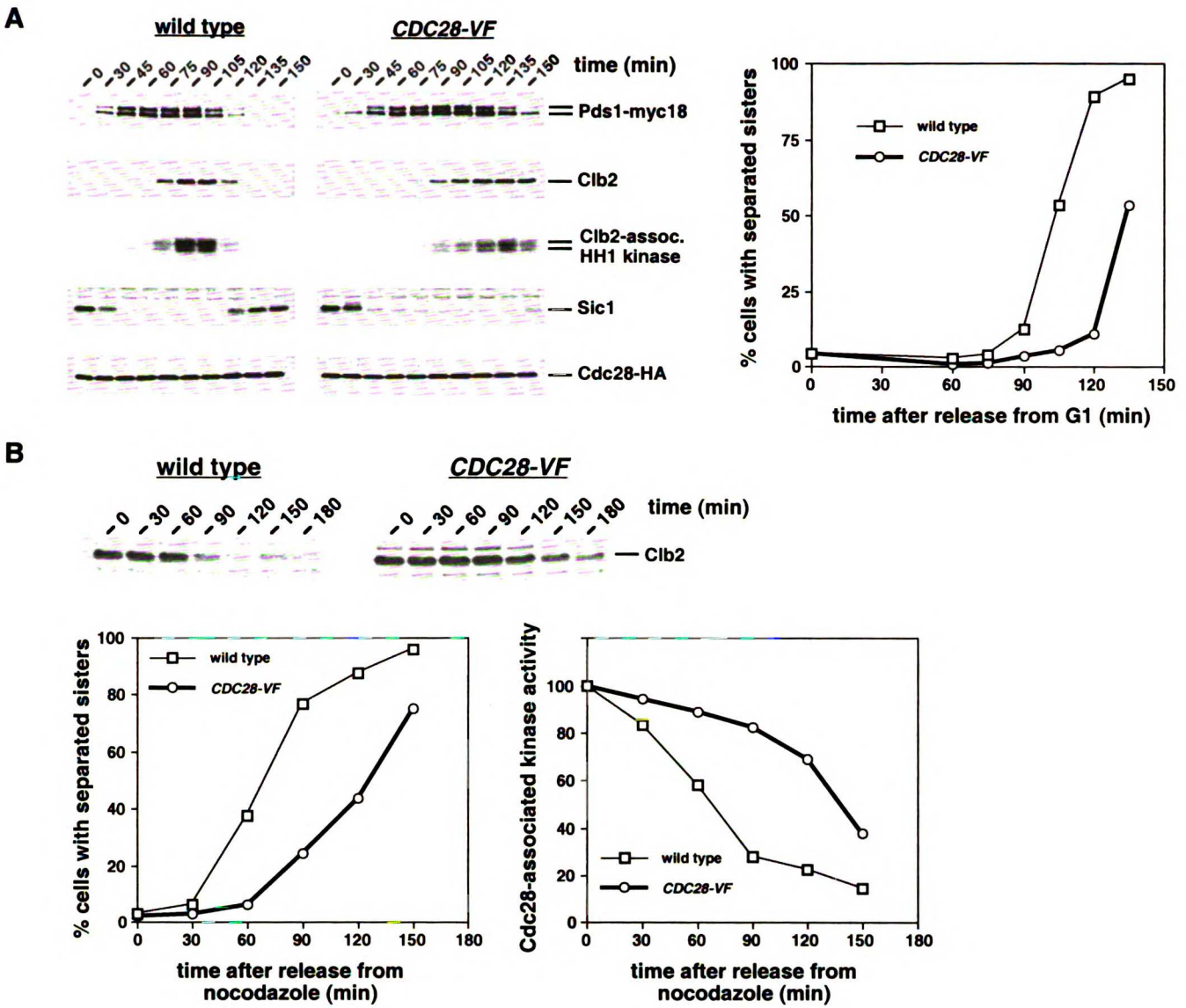

A
wild type
CDC28-VF
0 30 45 60 75 90 105 120 135 150
time (min)
Pds1-myc18
Clb2
Clb2-assoc. HH1 kinase
Sic1
Cdc28-HA
% cells with separated sisters
wild type
CDC28-VF
time after release from G1 (min)
B
wild type
CDC28-VF
0 30 60 90 120 150 180
time (min)
Clb2
% cells with separated sisters
time after release from nocodazole (min)
Cdc28-associated kinase activity
time after release from nocodazole (min)

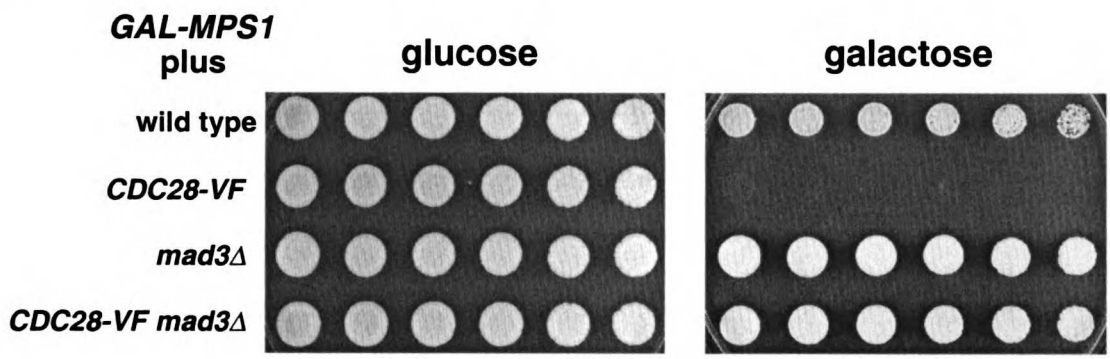
Figure 2 *CDC28-VF* cannot overcome a spindle checkpoint-dependent arrest

(A) Serial dilution assay. All strains contain *pGAL-MPS1*. wild type (—□—, KH153), *CDC28-VF* (—○—, KH181), *mad3Δ* (—◇—, ADR1248) and *CDC28-VF mad3Δ* (—△—, KH183), were grown overnight in YEP+2% glucose to log phase and adjusted to A_{600} 0.35. Two-fold serial dilutions of each strain were prepared in a multi-well dish and then spotted onto YEP+2% glucose (left) or YEP+2% galactose (right). The plates were incubated at 30°C for 2 days.

(B) Microcolony assay. The same strains as in (A) were grown overnight at 30°C in YEP+2% raffinose to log-phase and at t=0, unbudded cells were picked out onto YEP+2% galactose plates. As each cell divided, the number of cells in each microcolony were counted at the indicated times. Large budded cells were counted as two cells and the original cells which did not bud were not included in the analysis. Each strain has been tested at least three times, following 20-30 cells of each strain in each experiment. In the experiment shown, thirty cells of each strain were followed.

Figure 2

A


GAL-MPS1
plus
glucose
galactose
wild type
CDC28-VF
mad3Δ
CDC28-VF mad3Δ

B

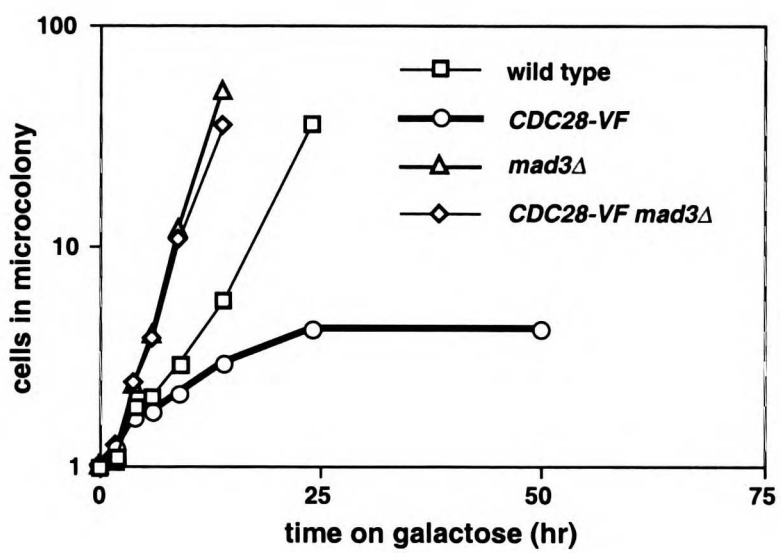

100
10
1
cells in microcolony
0
25
50
75
time on galactose (hr)
wild type
CDC28-VF
mad3Δ
CDC28-VF mad3Δ

Figure 3 Changes in inhibitory phosphorylation on Cdc28 do not affect sensitivity to the spindle checkpoint

(A) Serial dilutions of strains with varying amounts of inhibitory phosphorylation on Cdc28. All strains contain *pGAL-MPS1*. wild type (KH153), *CDC28-VF* (KH181), *CDC28-V* (ADR1105), *CDC28-F* (KH204), *swe1Δ* (KH207), *swe1Δ CDC28-V* (ADR1100), *CDC28-AF* (ADR1506) and *mih1Δ* (ADR1378) were grown to saturation for two days in YEP+ 2% glucose at 30°C , diluted ten-fold and four-fold serial dilutions were prepared in a multi-well dish and spotted onto YEP+2% glucose (left) or YEP+2% galactose (right). The plates were incubated at 30°C for 2 days.

(B) Phosphotyrosine blot. Wild type (JM425), *CDC28-VF* (JM434), *cdc55Δ* (JM445), *swe1Δ* (ADR684) and *mih1Δ* (ADR1314) were grown overnight in YEP+2% glucose at 23°C to log phase. The cells were harvested, lysed and Cdc28-HA was immunoprecipitated with the 12CA5 antibody. The immunoprecipitates were run out on a polyacrylamide gel and probed either with a phosphotyrosine antibody (top) or with 12CA5 (bottom).

Figure 3

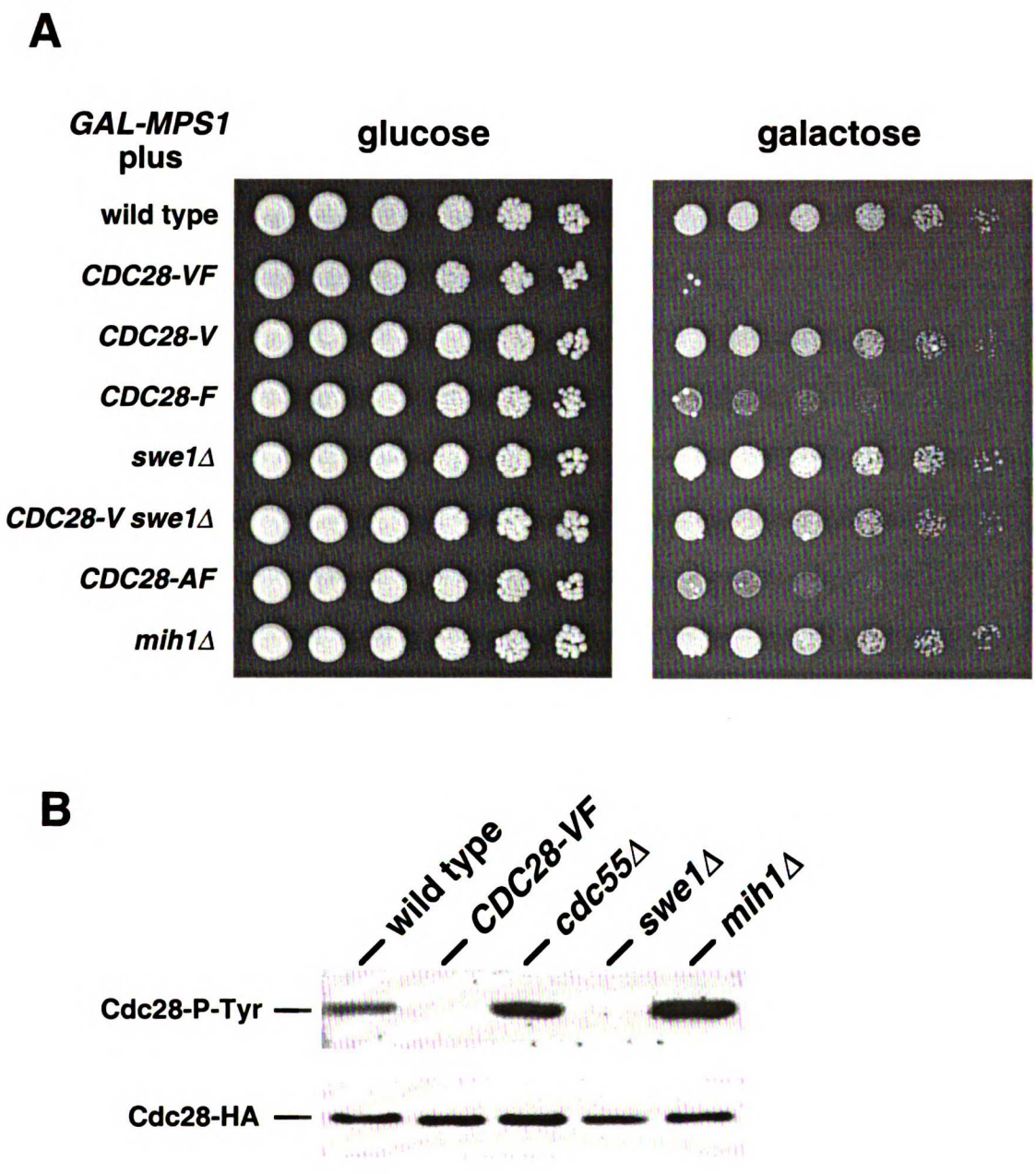

A
GAL-MPS1 plus
glucose
galactose
wild type
CDC28-VF
CDC28-V
CDC28-F
swe1Δ
CDC28-V swe1Δ
CDC28-AF
mih1Δ
B
wild type
CDC28-VF
cdc55Δ
swe1Δ
mih1Δ
Cdc28-P-Tyr
Cdc28-HA

Figure 4 Mutants with defects in mitotic Cdc28 activity resemble *CDC28-VF*

(A) Defects in mitotic Cdc28 activity are sensitive to spindle checkpoint-dependent arrest. All strains contain *pGAL-MPS1*. wild type (KH153), *CDC28-VF* (KH181), *cdc28-1N* (ADR1899), *cdc28-4* (ADR1901), *clb2Δ* (ADR1606) and *cks1-38* (ADR1903) were grown to saturation for two days in YEP+ 2% glucose at 23°C, diluted ten-fold and four-fold serial dilutions were prepared in a multi-well dish and spotted onto YEP+2% glucose (left) or YEP+2% galactose (right). The plates were incubated at 23°C for 2.5 days.

(B) The specific activity of Cdc28-VF is lower than Cdc28. Wild type (JM425) and *CDC28-VF* (JM434) were grown overnight in YEP+2% glucose at 23°C to log phase and arrested in mitosis with nocodazole (10μg/ml) for 3 hours. The cells were then harvested, lysed and Clb2/Cdc28 and Clb3/Cdc28 complexes were immunoprecipitated, and their histone H1 kinase activity was measured. The western blot (bottom) shows that equal amounts of Cdc28 are precipitated in the two strains, although the kinase activity (top) of Cdc28-VF is reduced relative to wild type. The activity of wild type Cdc28 is reported as 100% for both the anti-Clb2 and anti-Clb3 immunoprecipitates.

(C) *CDC28-F88G* behaves like *CDC28-VF*. All strains contain *pGAL-MPS1*. wild type (KH153), *CDC28-VF* (KH181) and *CDC28-F88G* (ADR2034) were grown to saturation for two days in YEP+ 2% glucose at 30°C , diluted ten-fold and four-fold serial dilutions were prepared in a multi-well dish and spotted onto YEP+2% glucose (left) or YEP+2% galactose (right). The plates were incubated at 30°C for 2 days.

(D) *2μ-CDC28* suppresses *CDC28-VF*. All strains contain *pGAL-MPS1*. wild type (KH153) or *CDC28-VF* (KH181) containing either *2μ-CDC28* or an empty *2μ* vector were grown to saturation for two days in CSM-trp+2% glucose at 30°C, diluted five-fold and four -fold serial dilutions were prepared in a multi-well dish and were spotted onto CSM-trp+2% glucose (left) or CSM-trp+2% galactose (right). The plates were incubated at 30°C for 2 days.

Figure 4

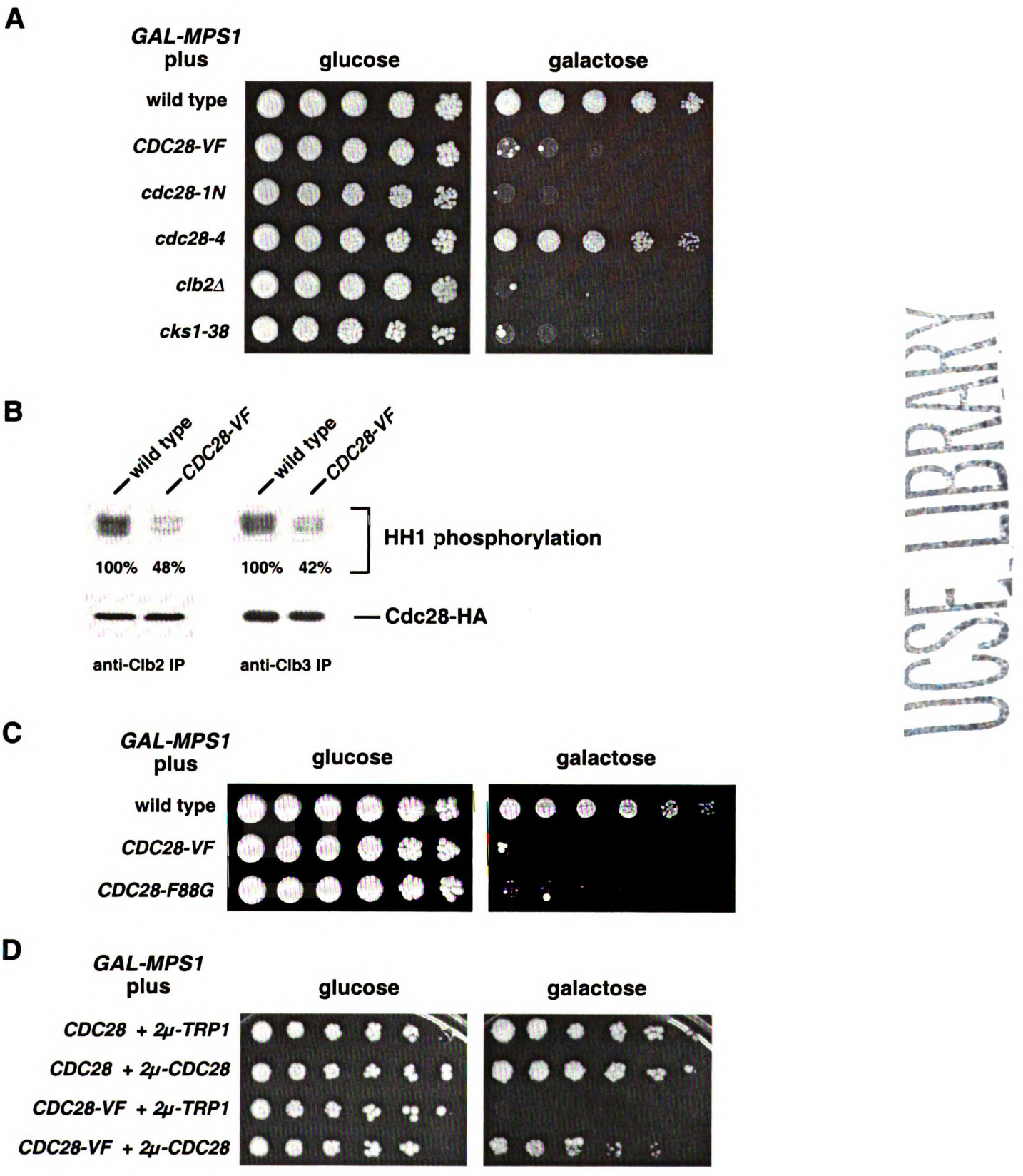


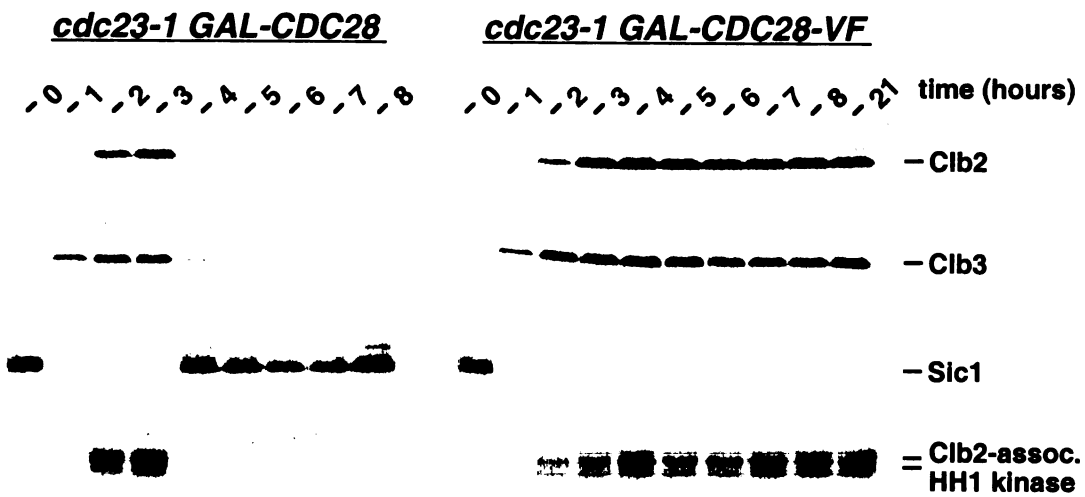
Figure 5 *cdc23-1 GAL-CDC28-VF* arrest in mitosis at the permissive temperature of 23°C

(A) *cdc23-1 GAL-CDC28* (—□—, ADR1685) and *cdc23-1 GAL-CDC28-VF* (—○—, ADR1687) were grown overnight at 23°C in YEP+2% raffinose to log phase, arrested in G1 with alpha factor (1μg/ml) for 3.5 hours and at t=0 released from the G1 arrest into fresh YEP+2% galactose. After cells had budded (t=2), alpha factor (1.5g/ml) was added back to the cultures to re-arrest the cells in the next G1. Samples were taken at the indicated times and processed for western blots and histone H1 kinase assays.

(B) Sister chromatid separation was scored by counting the number of fluorescent spots (one or two) of GFP-lacI bound to 256 tandem repeats of *lacO* integrated at the *TRP1* locus.

Figure 5

A



B

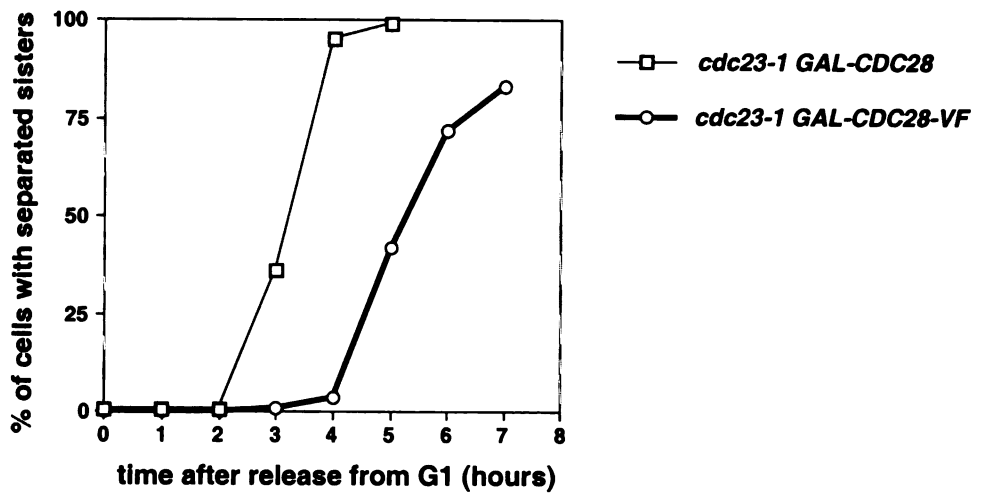


Figure 6 *CDC28-VF* has normal G1 APC activity.

Wild type (ADR1389) and *CDC28-VF* (ADR1252) were grown overnight at 30°C in YEP+2% glucose to log phase and arrested in G1 with alpha factor (1μg/ml) for 3 hours The cells were harvested, lysed and the APC was immunoprecipitated with anti-Cdc26 antibodies. The in vitro ubiquitination activity of the immunoprecipitates was measured in the absence or presence of added Hct1 protein (left). The substrate for the in vitro ubiquitination is an iodinated N-terminal fragment of sea urchin cyclin B (CycB). Western blotting of the immunoprecipitates shows that equal amounts of Cdc16, Cdc23 and Cdc27 are present in the APC isolated from wild type and *CDC28-VF* cells (right).

Figure 6 **G1 APC assay**

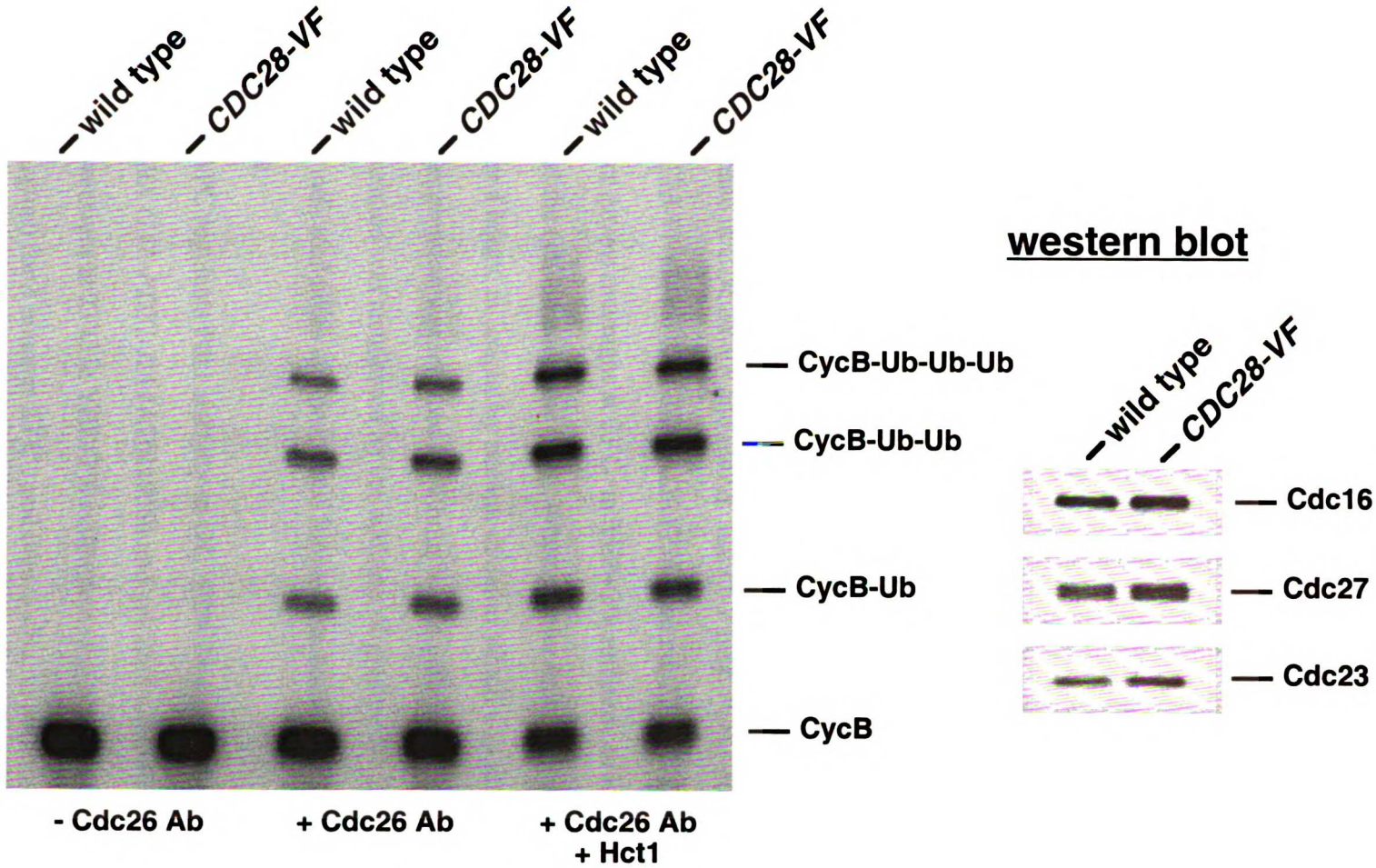


Figure 7 *CDC28-VF* cells have a defective Cdc20-dependent APC

(A) Destruction of Pds1 in anaphase depends on *CDC20*. Wild type (ADR1870), *cdc20-3* (ADR1783) and *hct1Δ* (ADR1786) were grown overnight at 23°C in YEP+2% raffinose to log phase. The three strains contain an epitope tagged Pds1 *(PDS1-myc18)*, can overexpress a truncated form of Clb2 (*pGAL-CLB2-Δ176*) which will arrest cells in anaphase, and express a GFP tagged alpha tubulin (*pHIS3-GFP-TUB1*) which allows the length of the spindle to be easily assessed by microscopy. Wild type and *cdc20-3* were arrested in G1 by alpha factor (1μg/ml) for 3.5 hours, released from the G1 arrest into YEP+2% galactose, and at t=0, when greater than 90% of the cells had reached anaphase (after 5 hours, as judged by spindle length), the cultures were shifted to 37°C . *hct1Δ* , which is resistant to alpha factor, was shifted from YEP+2% raffinose directly to YEP+2% galactose. Samples were taken at the indicated times and processed for western blots.

(B) Pds1 is stable in anaphase in *CDC28-VF* and *clb2Δ*. *cdc15-2 GAL-PDS1-HA* (ADR1743), *cdc15-2 CDC28-VF GAL-PDS1-HA* (ADR1736) and *cdc15-2 clb2Δ GAL-PDS1-HA* (ADR1774) were grown overnight at 23°C in YEP+2% raffinose to log phase and shifted to 37°C to arrest the cells in anaphase (raf). When greater then 90% of the cells had reached anaphase (after 4 hours, as judged by nuclear division which was scored by DAPI staining), Pds1-HA expression was induced for 1 hour by the addition of galactose (to 2%), and at t=0 its expression was terminated by the addition of glucose (to 2%). Samples were taken at the indicated times and processed for western blots. Cdc28-HA is shown as a loading control.

(C) The Hct1-dependent APC regulates Pds1 stability in G1. *CDC28-HA GAL-PDS1-HA* (ADR1968), *CDC28-VF-HA GAL-PDS1-HA* (ADR1959) and *cdc20-3 GAL-PDS1-HA* (ADR1921) were grown overnight at 23°C in YEP+2% raffinose to log phase and arrested in G1 with alpha factor (1μg/ml) for 3 hours at 23°C , and then shifted to 37°C for an additional 1 hour (raf). *cdc28-13 GAL-PDS1-HA* (ADR1925) and *cdc28-13 hct1Δ GAL-PDS1-HA* (ADR1928) were grown overnight at 23°C in YEP+2% raffinose to log phase

and arrested in G1 by shifting the cultures to 37°C for 3.5 hours. Pds1-HA expression was induced by addition of galactose (to 2%) for 2 hours, and at t=0 its expression was terminated by the addition of glucose (to 2%). Samples were taken at the indicated times and processed for Western blots. Sic1 is shown as a loading control and as evidence that all cells remain arrested in G1.

Figure 7

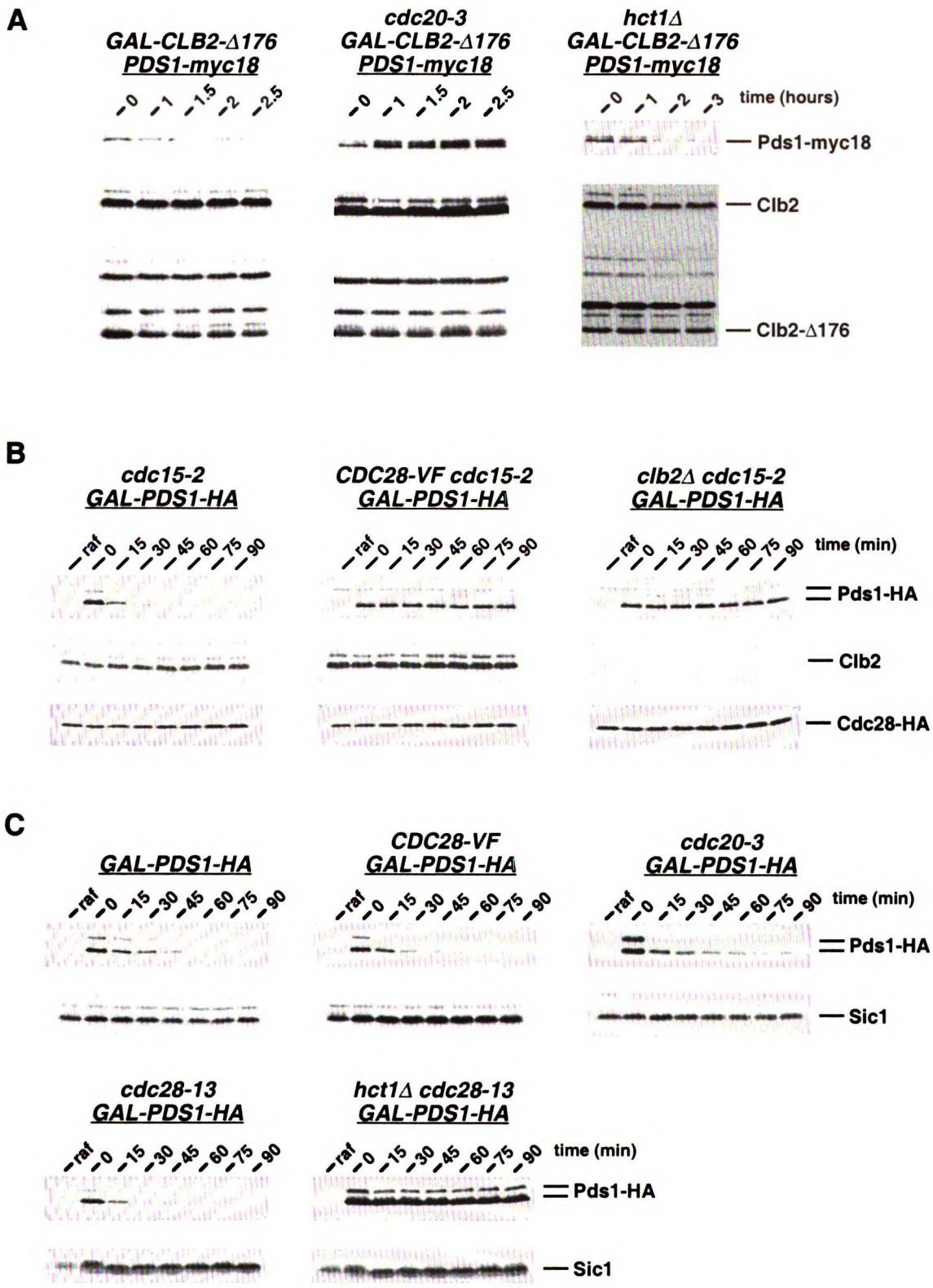

A
GAL-CLB2-Δ176
PDS1-myc18
cdc20-3
GAL-CLB2-Δ176
PDS1-myc18
hct1Δ
GAL-CLB2-Δ176
PDS1-myc18
0 1 1.5 2 2.5
0 1 1.5 2 2.5
0 1 2 3
time (hours)
Pds1-myc18
Clb2
Clb2-Δ176
B
cdc15-2
GAL-PDS1-HA
CDC28-VF cdc15-2
GAL-PDS1-HA
clb2Δ cdc15-2
GAL-PDS1-HA
raf 0 15 30 45 60 75 90
raf 0 15 30 45 60 75 90
raf 0 15 30 45 60 75 90
time (min)
Pds1-HA
Clb2
Cdc28-HA
C
GAL-PDS1-HA
CDC28-VF
GAL-PDS1-HA
cdc20-3
GAL-PDS1-HA
raf 0 15 30 45 60 75 90
raf 0 15 30 45 60 75 90
raf 0 15 30 45 60 75 90
time (min)
Pds1-HA
Sic1
cdc28-13
GAL-PDS1-HA
hct1Δ cdc28-13
GAL-PDS1-HA
raf 0 15 30 45 60 75 90
raf 0 15 30 45 60 75 90
time (min)
Pds1-HA
Sic1

Figure 8 Cdc20 binding to the APC is impaired in *CDC28-VF*

cdc15-2 (ADR1790) and *cdc15-2 CDC28-VF* (ADR1793) were grown overnight in YEP+2% glucose at 23°C to log phase. Both strains contain an epitope tagged Cdc20 *(CDC20-myc12)*. The cultures were shifted to 37°C and after greater than 85% of the cells were arrested in anaphase (4 hours, as judged by nuclear division which was scored by DAPI staining), the cells were harvested, lysed and the APC immunoprecipitated with anti-Cdc26 antibodies. The amount of Cdc20-myc12 bound to the APC was determined by western blotting the immunoprecipitates with the 9E10 antibody. Equal amounts of APC were precipitated with the anti-Cdc26 antibodies as judged by Cdc23 levels (left) and equal amounts of cell lysate were used in the immunoprecipitation as judged by Cdc20-myc12 and Cdc28-HA levels (right, cell lysate).

Figure 8

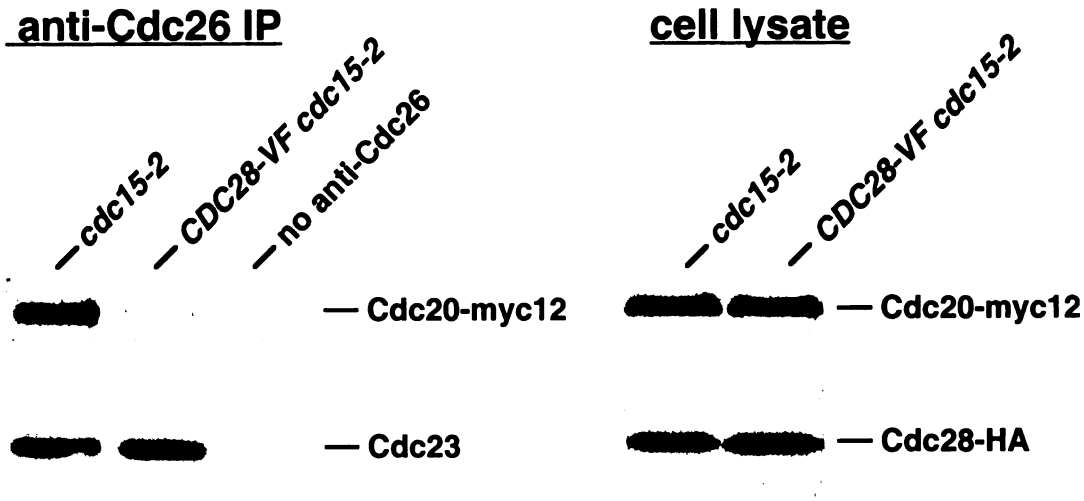

anti-Cdc26 IP
cell lysate
cdc15-2
CDC28-VF cdc15-2
no anti-Cdc26
cdc15-2
CDC28-VF cdc15-2
Cdc20-myc12
Cdc23
Cdc20-myc12
Cdc28-HA

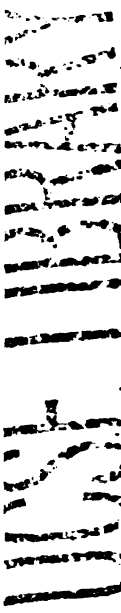
Table 1 Genetic interations of *CDC28-VF*

	CDC28-VF	*CDC28-V*	*CDC28-F*	*CDC28-AF*	*swe1Δ*	*CDC28-F88G*	*cdc28-1N*	*clb2Δ*	*cdc28-4*
cdc23-1	sl	sl	sl	sl	+	sl	sl	sl	+
cdc16-1	sl	+	+	+	+	+	ND	ND	+
doc1-1	sl								
cdc20-1	sl								
hct1Δ	sl								
cdc5-1	sl								
cdc13-1	+								
cdc15-2	+								

synthetic lethal (sl)
not determined (ND)
viable (+)

Chapter 2

Phosphorylation by Cdc28 activates the Cdc20-dependent activity of the anaphase promoting complex



Abstract

Budding yeast initiates anaphase by activating the Cdc20-dependent anaphase promoting complex (APC). The mitotic activity of Cdc28 (Cdk1) is required to activate this form of the APC, and mutants that are impaired in mitotic Cdc28 function have difficulty leaving mitosis. This defect can be explained by a defect in APC phosphorylation, which depends on mitotic Cdc28 activity in vivo and can be catalyzed by purified Cdc28 in vitro. Mutating putative Cdc28 phosphorylation sites in three components of the APC, Cdc16, Cdc23, and Cdc27, makes the APC resistant to phosphorylation both in vivo and in vitro. The non-phosphorylatable APC has normal activity in G1 but its mitotic, Cdc20-dependent activity is compromised. These results show that Cdc28 activates the APC in budding yeast to trigger anaphase. Previous reports have shown that the budding yeast Cdc5 homolog, Plk, can also phosphorylate and activate the APC in vitro. We show that like Cdc28, *cdc5* mutants affect APC phosphorylation in vivo. However, although Cdc5 can phosphorylate Cdc16 and Cdc27 in vitro, this in vitro phosphorylation does not occur on in vivo sites of phosphorylation.

Introduction

Proteolysis plays a critical role in the eukaryotic cell cycle. During the exit from mitosis, ubiquitin mediated proteolysis destroys an inhibitor of sister chromatid separation (Pds1 in budding yeast and Cut2 in fission yeast) (Funabiki et al., 1996b; Holloway et al., 1993; Yamamoto et al., 1996b) and the mitotic cyclins (Clb1-Clb4 in budding yeast) (Ghiara et al., 1991; Glotzer et al., 1991; Yamano et al., 1996). These proteins are targeted for degradation by the anaphase promoting complex (APC)[1] or cyclosome, which is the E3 ubiquitin ligase for cyclins (King et al., 1995; Sudakin et al., 1995; Zachariae et al., 1996), Pds1 (Cohen-Fix et al., 1996; Funabiki et al., 1997) and other substrates (Juang et al., 1997; Prinz et al., 1998; Shirayama et al., 1998), marking them for destruction by the 26S proteasome. The APC is regulated by the binding of two conserved activators, Cdc20 and Hct1 (also known as Cdh1) (Fang et al., 1998b; Kitamura et al., 1998; Lorca et al., 1998; Schwab et al., 1997; Visintin et al., 1997). In budding yeast, Cdc20-dependent APC activity initiates the metaphase to anaphase transition and the series of events that activate the Hct1-dependent APC, which induces complete mitotic cyclin destruction (Lim and Surana, 1996; Shirayama et al., 1999; Visintin et al., 1997). Hct1 acts in conjunction with the cyclin dependent kinase (CDK) inhibitor Sic1 to induce the rapid drop in Cdc28-associated kinase activity that drives cells out of mitosis and into the next G1 (Amon, 1997; Donovan et al., 1994; Li and Cai, 1997; Mendenhall, 1993). The Hct1-dependent and the Cdc20-dependent APC can both target Pds1 for destruction (Visintin et al., 1997), suggesting that the main difference between them is the time during the cell cycle when each is active (Prinz et al., 1998).

Phosphorylation of Hct1 by Cdc28/Clb complexes keeps it from binding or activating the APC (Jaspersen et al., 1999; Zachariae et al., 1998). This phosphorylation is removed by Cdc14, a phosphatase which is activated after Cdc20-dependent destruction of Pds1, Clb2 and the S-phase cyclin, Clb5 (Jaspersen et al., 1999; Shirayama et al., 1999;

Visintin et al., 1998; Yeong et al., 2000). The late activation of Cdc14 ensures that cells do not inactivate Cdc28 and exit mitosis until well after they have initiated sister chromatid segregation.

Cdc20 is regulated in at least three ways: the gene is transcribed only in mitosis, the protein is targeted for destruction by the APC, and Cdc20 activity is inhibited by the spindle checkpoint, which monitors whether chromosomes have attached to the spindle properly (Fang et al., 1998a; Hwang et al., 1998; Kallio et al., 1998; Kim et al., 1998; Kramer et al., 1998; Prinz et al., 1998; Shirayama et al., 1998; Weinstein, 1997).

The Cdc20-dependent APC is regulated by phosphorylation. APC subunits are phosphorylated in fission yeast, frogs, clams and mammalian tissue culture cells (Hershko et al., 1994; Kotani et al., 1998; Peters et al., 1996; Yamada et al., 1997). Phosphorylation correlates with APC activity in vivo, and experiments in vitro have suggested that phosphorylating the APC regulates Cdc20 binding and APC activity (Kotani et al., 1999; Kotani et al., 1998; Shteinberg et al., 1999). Studies in frog egg extracts and mammalian tissue culture cells have shown that the protein kinase Plk (known as Cdc5 in budding yeast, and Plx1 in frogs) and the complex of Cdc2, CyclinB, and Cks1, a small Cdk binding protein, can phosphorylate the APC in vitro. Depletion of either Cks1 or Plx1 from frog extracts blocks cyclin destruction suggesting that both Cdc2 and Plx1 may activate the APC (Descombes and Nigg, 1998; Kotani et al., 1999; Kotani et al., 1998; Patra and Dunphy, 1996), but the relative importance of these two kinases in vivo is unclear. Phosphorylation of the APC by cAMP-dependent protein kinase A inhibits the APC both in vivo and in vitro (Kotani et al., 1998; Yamashita et al., 1996). Lastly, protein phosphatase 2A (PP2A) inhibits the APC (Lahav-Baratz et al., 1995; Shteinberg et al., 1999), whereas protein phosphatase 1 (PP1) activates the APC (Yamada et al., 1997).

In the accompanying paper we show that *CDC28-T18V, Y19F (CDC28-VF)* and other mutants with altered mitotic Cdc28 activity are compromised in activating the Cdc20-dependent APC, revealing a requirement for Cdc28 in APC activation. Here we show that

CDC28-VF is defective in the mitotic phosphorylation of the APC and that this phosphorylation depends on Cdc28 activity both in vivo and in vitro. Mutating potential phosphorylation sites in the APC components Cdc16, Cdc23, and Cdc27 reduces Cdc20 binding to the APC and Cdc20-dependent APC activity in vivo.

Results

Cdc28 phosphorylates the APC in vitro

Mutants that reduce mitotic Cdc28 activity have difficulty activating the Cdc20-dependent APC suggesting that Cdc28 might phosphorylate the APC or Cdc20 (Rudner et al., accompanying paper). We therefore asked if the budding yeast APC is phosphorylated in vitro. We used APC that was isolated by immunoprecipitating cell lysates with antibodies against Cdc26, a non-essential component of the APC (Hwang and Murray, 1997), and used these immunoprecipitates as a substrate for purified recombinant Cdc28/Clb2/Cks1 (a gift of Jeff Ubersax and David Morgan) in the presence of [γ-^{32}P]-ATP. In APC isolated from wild type cells, three major bands and a single minor band were phosphorylated (Figure 1, top). We determined the identity of these four bands by phosphorylating the APC isolated from cells containing epitope tagged subunits that change their molecular weight. If the band shifted up in the epitope tagged APC, we concluded that the phosphorylated protein is the APC subunit. By this criterion, the protein at 97kD is Cdc16, the protein at 85kD is Cdc27, and the minor species at 65kD is Cdc23 (Figure 1, top). Similar experiments have shown the band at 42kD is Apc9 (data not shown).

We do not think the phosphorylation of the APC in these reactions is due to kinases that co-immunoprecipitate with the APC; no labeling is seen in immunoprecipitates lacking added Cdc28/Clb2/Cks1. However, a kinase bound to the APC might need to be activated by Cdc28, as has been reported for Plk phosphorylation of the mammalian APC (Kotani et al., 1998). We therefore tested whether Cdc5, the Plk homolog in budding yeast, was required for in vitro APC phosphorylation (Kitada et al., 1993). We isolated the APC from a *cdc5-1* mutant that had been arrested in G1 by alpha factor at 25°C and then shifted to the restrictive temperature of 37°C for 1 hour. This APC is fully phosphorylated in vitro by Cdc28 (Figure 1), showing that Cdc5 is not required for APC phosphorylation in this in

vitro assay. In addition, Cdc5 is not detectable in alpha factor arrested cells (Charles et al., 1998; Hardy and Pautz, 1996; Shirayama et al., 1998), or in anti-Cdc26 immunoprecipitates of the APC, isolated from mitotic cells which contain Cdc5 (Julia Charles and David Morgan, personal communication, and data not shown).

The APC is phosphorylated in vivo

Is the APC phosphorylated in vivo? Wild type cells were arrested at three points in the cell cycle: during G1 by adding alpha factor (a mating pheromone), during S-phase by adding hydroxyurea (HU, a DNA synthesis inhibitor) and in mitosis with nocodazole (a microtubule polymerization inhibitor). The arrested cells were labeled with $^{32}PO_4$ and the APC was isolated by immunoprecipitating cell lysates with antibodies against Cdc26. Three major proteins of 97kD, 85kD and 65kD were strongly labeled in nocodazole arrested cells, and to lesser extent in HU and alpha factor arrested cells (Figure 2A). These three proteins do not precipitate from *cdc26Δ* cells. The molecular weights of these proteins suggest that they are the APC subunits Cdc16, Cdc27 and Cdc23, and mutating phosphorylation sites in these proteins abolishes in vivo phosphorylation of the APC (see below).

Since Cdc28/Clb complexes are inactive in G1, the differences in APC phosphorylation during different cell cycle stages suggests this reaction depends on Cdc28/Clb complexes. We tested this hypothesis directly by comparing the phosphorylation of the APC in *CDC28-VF*, *clb2Δ* and *cdc28-1N* cells, three mutants which affect the mitotic activity of Cdc28 (Grandin and Reed, 1993; Piggott et al., 1982; Surana et al., 1991). The cells were arrested in metaphase at 25°C (Figure 2B) or at 35°C (Figure 2C) by overexpressing Mps1 from the galactose inducible *GAL1* promoter, which activates the spindle checkpoint. All three mutants reduce the phosphorylation of the APC by a factor of 2 to 4 compared to wild type.

Previous studies have suggested that in mammalian tissue culture cells the protein kinase Plk is primarily responsible for phosphorylating the APC (Kotani et al., 1998). A mutant in *CDC5* , the yeast homolog of Plk, cannot activate the Hct1-dependent APC (Charles et al., 1998). To determine whether APC phosphorylation is dependent on Cdc5, we examined APC phosphorylation in a *cdc5-1* mutant, arrested in metaphase by overexpressing Mps1 at a semi-restrictive temperature of 35°C. We observed a similar reduction in APC phosphorylation as in *CDC28-VF* and *cdc28-1N* (Figure 1C), suggesting that Cdc5 contributes to APC phosphorylation in vivo.

To confirm the identities of the phosphorylated APC subunits and to determine if the APC is phosphorylated by Cdc28 in vivo, we mutated all the potential Cdc28 sites in Cdc16, Cdc23, and Cdc27. Using the weakest possible consensus phosphorylation site (serine or threonine followed by proline (S/TP)) as our criterion, we mutated six sites in Cdc16, one in Cdc23, and five in Cdc27. We refer to the resulting genes as *CDC16-6A* *CDC23-A* and *CDC27-5A*. As Figure 3A shows, most of the mutated sites fit only the minimal S/TP motif and lack a nearby basic residue found in many biochemically determined Cdk phosphorylation sites (Brown et al., 1999).

We directly assessed the ability of the mutant subunits to be phosphorylated in vivo and in vitro. In vivo, each alanine substituted subunit is resistant to phosphorylation (Figure 3B). This result cofirms our conclusion that Cdc16, Cdc27 and Cdc23 are the three major phosphorylated proteins in the APC (Figure 2) and shows that among the phsophorylation sites we mutated are the relevant in vivo sites. In addition, the phosphorylation of the different subunits is largely independent of each other. For example, the *CDC23-A* mutant eliminates the phosphorylation of Cdc23 but not that of Cdc16 and Cdc27. In vitro, Cdc23-A and Cdc27-5A are completely resistant to phosphorylation in vitro by Cdc28 (Figure 3C). Cdc16-6A is still weakly phosphorylated, though much less than the wild type protein.

The APC binds to Cks1

During the course of this work we discovered that the budding yeast APC, like the animal APC, can bind to Cks1-coupled beads (Sudakin et al., 1997). This interaction is thought to be critical for APC phosphorylation and reflects the ability of Cks1 to bring Cdc2/cyclin B complexes into proximity with the APC by interacting with both complexes simultaneously (Patra and Dunphy, 1998; Shteinberg and Hershko, 1999). Figure 4A shows that the APC from mitotically arrested yeast cells binds to Cks1-coupled beads. Comparing western blots of the material recovered from wild type and *CDC28-VF* cells reveals that less APC from *CDC28-VF* cells binds to Cks1-coupled beads. Reduced recovery of the APC does not reflect decreased binding of Cdc28-VF to Cks1 beads, since equal amounts of Cdc28-VF/Clb2 and Cdc28/Clb2 are recovered with the beads.

Figure 4A also shows that Cdc27 runs as doublet on western blots, with the upper band predominating in wild type and the lower band predominating in *CDC28-VF*. The slower mobility form of Cdc27 is a phosphorylated form because it can be converted to the faster one by treating the Cks1-bound material with lambda phosphatase (Figure 4B). We are also able to detect phosphorylation dependent mobility shifts in Cdc16 and Cdc23 (data not shown). These phosphorylation dependent shifts confirm our in vivo labeling data (Figure 2) that show the APC is phosphorylated in vivo.

To investigate the relationship between reduced Cks1 binding and reduced Cdc27 phosphorylation in *CDC28-VF* cells, we followed both through the cell cycle. Figure 4C shows that through most of the cell cycle Cdc27 is partially phosphorylated, but as cells go through mitosis and Clb2 levels peak, Cdc27 and Cdc16 phosphorylation increases (Figure 4C, arrow). The amount of phosphorylation on both subunits increases further when nocodazole treatment arrests cells in mitosis by activating the spindle checkpoint. In *CDC28-VF* cells, as Clb2 levels increase, Cdc27 phosphorylation decreases before eventually returning to its partially phosphorylated state (Figure 4C, bracket).

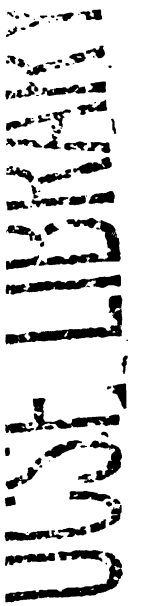
These changes in APC phosphorylation correlate with its ability to bind Cks1-coupled beads (Figure 4C, bottom). In wild type, no APC binds Cks1-coupled beads in an alpha factor arrest and its binding increases as levels of Clb2 rise. In *CDC28-VF* cells, little binding of the APC to Cks1-coupled beads is seen at any stage of the cell cycle, even though the peak levels of Clb2 are similar in wild type and *CDC28-VF* cells. These differences suggest that mitotic phosphorylation by Cdc28 is required for APC binding to Cks1-coupled beads (Sudakin et al., 1997). Although the difference in APC phosphorylation between wild type and *CDC28-VF* cells in a synchronous cell cycle is transient and subtle, it is reproducible, and it correlates with a large difference in the ability of the APC to bind Cks1-coupled beads.

We next tested whether APC phosphorylation is required for the APC to bind Cks1-coupled beads. The beads do not bind an APC containing Cdc27-5A (Figure 4D), but do bind an APC containing either Cdc16-6A and Cdc23-A. This result suggests that phosphorylation of Cdc27 is critical for Cks1 binding to the APC.

We have shown that mutations that alter the mitotic activity of Cdc28 have reduced APC phosphorylation (Figure 2B and C). Our ability to detect APC phosphorylation on western blots allowed us to examine additional mutants that affect Cdc28 activity. Mutants that reduce the mitotic activity of Cdc28 (*CDC28-VF*, *cdc28-1N*, *clb2Δ* and *cks1-38*) are hypersensitive to checkpoint arrest caused by overexpression of Mps1, whereas a mutant that primarily affects G1 activity (*cdc28-4*) , is not (Reed, 1980; Surana et al., 1991; Tang and Reed, 1993). To test if this correlation extended to the phosphorylation state of the APC, we arrested these strains in mitosis by Mps1 overexpression and immunoblotted for Cdc27 and Cdc16. This analysis correlates perfectly with our earlier findings: *cdc28-4* have normal levels of Cdc16 and Cdc27 phosphorylation whereas *clb2Δ*, *cdc28-1N* and *cks1-38* all have reduced levels and resemble *CDC28-VF* (Figure 4E).

APC phosphorylation site mutants affect mitotic but not G1 functions

We wanted to rule out the possibility that the phosphorylation site mutants had general effects on the activity of the APC as opposed to a specific effect on its mitotic, Cdc20-dependent form. Since the Hct1-dependent APC is maximally active when Cdc28 is inactive, loss of Cdc28-dependent phosphorylations should not affect Hct1-dependent APC activity in G1-arrested cells that lack active Cdc28 (Jaspersen et al., 1999; Zachariae et al., 1998). We therefore examined the activity of APC containing the alanine substituted subunits that had been isolated from G1 arrested cells. APC activity was measured in an in vitro ubiquitination assay that uses an iodinated fragment of sea urchin cyclin B as a substrate and the APC provided from anti-Cdc26 immunoprecipitates (Charles et al., 1998). Figure 5 shows that there is no difference in G1-specific APC activity between wild type cells and those carrying alanine mutations in APC subunits (*CDC16-6A* , *CDC23-A*, or *CDC27-5A*). Thus the mutations in putative Cdc28-dependent phosphorylation sites have not disrupted the ability of these subunits to associate with other APC components or produce normal levels of Hct1-dependent APC activity. In addition, cells carrying alanine-substituted APC subunits show no obvious growth defects at any temperature.



We asked if the alanine substitutions in the APC, like *CDC28-VF*, have difficulty leaving mitosis (Rudner et al., accompanying paper). Wild type and *CDC16-6A CDC23-A CDC27-5A* cells were arrested in G1 by the mating pheromone alpha factor and then released into the cell cycle. Once cells had budded, alpha factor was re-added to arrest cells that had completed the cycle. *CDC16-6A CDC23-A CDC27-5A* cells show a 20 minute delay in sister chromatid separation (Figure 6A). Clb2 and Clb3 proteolysis are delayed by more than 40 minutes. This defect is not due to slower mitotic entry, because wild type and *CDC16-6A CDC23-A CDC27-5A* cells initiate budding, degrade Sic1 and form a short mitotic spindle at the same time (Figure 6A and data not shown).

Mutating APC phosphorylation sites also causes an increased sensitivity to spindle checkpoint arrest caused by Mps1 overexpression (Hardwick et al., 1996). Serial dilutions of wild type, *CDC28-VF*, mutants in single APC subunits, double mutants and the triple mutant were spotted on plates where Mps1 is induced to high levels (Figure 6B). Both *CDC16-6A* and *CDC27-5A* are sensitive to Mps1 overexpression and combining the two mutants creates a phenotype very similar to that of *CDC28-VF*. The *CDC23-A* mutation alone has little phenotype but exacerbates the effect of both the *CDC16-6A* and *CDC27-5A* mutations. These data suggest that phosphorylation of the APC subunits contributes to the ability to overcome the spindle checkpoint and suggest that the alanine substituted APC, like *CDC28-VF*, may be defective in the Cdc20-dependent APC.

To test Cdc20-dependent APC function more directly, we examined the ability of these non-phosphorylatable APC mutants to support Pds1 degradation in vivo. Pds1 is normally unstable in anaphase with a half life of about 10 minutes (Jaspersen et al., 1998). We arrested wild type and non-phosphorylatable APC mutants in anaphase (using the *cdc15-2* mutant), induced Pds1 expression from the *GAL1* promoter by adding galactose for one hour, then shut the promoter off by adding glucose and examined the rate of Pds1 degradation. We have previously shown that in this anaphase arrest, *CDC28-VF* and *clb2Δ* stabilize Pds1 (Rudner et al., accompanying paper). We see a similar effect when the *CDC27-5A* and *CDC16-6A* mutants are combined with *cdc15-2*. The half life of Pds1 is increased to 30 minutes in anaphase-arrested *CDC27-5A*, and to more than 90 minutes in *CDC16-6A* cells (Figure 7A).

We also have examined the association of Cdc20 with the APC in the alanine substituted mutants at the *cdc15-2* block, a time when the Cdc20-dependent APC is active. This association is impaired in *CDC28-VF* (Rudner et al., accompanying paper). We arrested *cdc15-2*, *cdc15-2 CDC16-6A*, and *cdc15-2 CDC27-5A* cells in anaphase, immunoprecipitated the APC with anti-Cdc26 antibodies, and examined the amount of

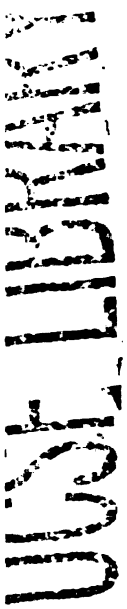
associated Cdc20. The association of Cdc20 to the APC is reduced in *CDC27-5A* cells and severely impaired in *CDC16-6A* cells (Figure 7B).

The role of Cdc5 in APC phosphorylation

Different groups debate whether Cdc2 (Cdk1) or Plk is the major APC kinase in vivo (Kotani et al., 1998; Lahav-Baratz et al., 1995; Patra and Dunphy, 1998; Shteinberg and Hershko, 1999; Shteinberg et al., 1999). Since we find reduced APC phosphorylation is reduced in a cdc5-1 mutant (Figure 2C), we tested whether purified recombinant Cdc5 could phosphorylate the APC in vitro. Like Cdc28, Cdc5 phosphorylates Cdc16, Cdc27 and Apc9 (Figure 8A), but unlike Cdc28, appears to not phosphorylate Cdc23. Cdc5 also phosphorylates proteins that have the molecular weights of several other APC subunits (Apc1, Apc2, Apc4 and 5) (Zachariae et al., 1996). We have not confirmed the identity of these proteins because there is little evidence that these proteins are major targets of phosphorylation in vivo (Figure 2). The ability of human Plk to activate the APC in vitro depends on pre-treatment with Cdc2/cyclin B complexes (Kotani et al., 1998), a result that has been interpreted to suggest that Cdc2 activates Plk's kinase activity against the APC. In our hands, however, Cdc5's ability to phosphorylate of the APC does not increase when the kinase is pre-treated with purified Cdc28/Clb2/Cks1 complexes (data not shown).

We next tested whether purified Cdc5 can phosphorylate the alanine substituted APC. The Cdk sites we mutated on Cdc16, Cdc23 and Cdc27 are also potential sites of phosphorylation by Cdc5. Substrates of the frog homolog of Cdc5, Plx1, become epitopes for the MPM-2 antibody after phosphorylation by Plx1 (Kumagai and Dunphy, 1996) and MPM-2 recognizes phosphorylation at SP or TP sites (Westendorf et al., 1994). In contrast to their effect on phosphorylation by Cdc28, the APC phosphorylation site mutants had no effect on in vitro phosphorylation of the APC by recombinant Cdc5 (Figure 8B). This observation makes it likely that the reduced in vivo APC phosphorylation seen in

cdc5-1 cells is an indirect effect of reduced Cdc5 activity rather than a direct in vivo phosphorylation of these APC subunits by Cdc5.



Discussion

We have shown that the budding yeast APC subunits, Cdc16, Cdc23 and Cdc27, are phosphorylated in vivo and in vitro. Phosphorylation in vivo depends on Cdc28 and in vitro it is catalyzed by pure Cdc28/Clb2/Cks1 complexes. Mutating potential Cdc28 phosphorylation sites in Cdc16, Cdc23, and Cdc27 abolishes their in vivo phosphorylation and compromises the mitotic but not the G1 functions of the APC. We have also shown that Cdc5 affects APC phosphorylation in vivo and can catalyze APC phosphorylation in vitro. Our analysis of APC phosphorylation site mutants in vivo and in vitro, however, argues that in vivo Cdc5 indirectly induces the phosphorylation of Cdc16, Cdc23 or Cdc27 rather than directly modifying these subunits.

The APC is phosphorylated in budding yeast

Our results agree with studies on other organisms that show mitosis-specific APC phosphorylation. Cdc16, Cdc23, Cdc27, and Apc1 are phosphorylated in frogs, Apc1, Cdc16 and Cdc27 are phosphorylated in mammalian tissue culture cells, and Cdc16 (Cut 9) is phosphorylated in fission yeast (Kotani et al., 1999; Patra and Dunphy, 1998; Peters et al., 1996; Yamada et al., 1997). Although APC phosphorylation has been shown to activate the Cdc20-dependent APC in mammalian tissue culture and clam egg extracts (Kotani et al., 1998; Shteinberg et al., 1999) and Cks1 depletions prevent mitotic exit in frog extracts (Patra and Dunphy, 1996), this is the first report to examine the in vivo function of APC phosphorylation. Although phosphorylation of Cdc16, Cdc23 and Cdc27 is not essential for viability in budding yeast, our studies suggest that it stimulates Cdc20-dependent APC activity and Cdc20 binding to the APC in vivo.

Cdc27 remains partially phosphorylated in G1 cells (Figure 4C). The presence of slower migrating Cdc27 in G1 cells could arise two ways: during the exit from mitosis, if

Cdc28-catalyzed phosphorylation declines after phosphatases have been inactivated, or in G1, by phosphorylation catalyzed by another kinase. Because Cdc27-5A runs as a single band on western blots in G1 (Figure 5) we favor the possibility that G1 phosphorylation on Cdc27 remains from the previous mitosis. This finding would suggest that the phosphatase that removes phosphorylation from the APC is only active in mitosis. Protein phosphatase 2A (PP2A) has been proposed to play such a role in clams and frogs (Lahav-Baratz et al., 1995; Vorlaufer and Peters, 1998).

In one report, Plk has been identified as the major kinase of the mammalian APC (Kotani et al., 1998), although others have argued that this role is played by Cdc2 (Lahav-Baratz et al., 1995; Patra and Dunphy, 1998; Shteinberg and Hershko, 1999; Shteinberg et al., 1999). We asked if its budding yeast homolog Cdc5 plays a similar role. In vivo, APC phosphorylation is reduced in the *cdc5-1* mutant and purified Cdc5 phosphorylates the APC in vitro (Figure 2C and 8A). Three observations argue that in living cells Cdc5 does not directly phosphorylate the APC subunits we have studied: i) phosphorylation site mutations that completely block phosphorylation of Cdc16, Cdc23 and Cdc27 in vivo, do not block in vitro phosphorylation of these subunits by Cdc5 (Figure 8B), ii) purified Cdc28/Clb2/Cks1, that lacks detectable Cdc5, efficiently phosphorylates immunoprecipitated APC (Figure 1), and iii) the same mutations that block Cdc28-catalyzed phosphorylation in vitro also block in vivo APC phosphorylation (Figure 3).

If Cdc5 does not phosphorylate Cdc16, Cdc23 and Cdc27 directly, how does it regulate the phosphorylation of these subunits? Cdc5 may be responsible for phosphorylating other APC subunits (Apc1, 2, 4 and 5 are potential substrates, Figure 8) in vivo, and the phosphorylation of these subunits may affect the phosphorylation of the Cdc28 targets Cdc16, Cdc23 and Cdc27. Alternatively, Cdc5 may modulate Cdc28/Clb/Cks1 activity or localization.

Phosphorylation stimulates Cdc20-dependent APC activity

We have shown that phosphorylation site mutants in the APC reduce activation of the Cdc20-dependent APC. The half-life of Pds1 is increased in *CDC27-5A* and *CDC16-6A* cells and this defect in Cdc20 function could be explained by the observed inability of Cdc20 to bind an APC containing Cdc16-6A. This data supports genetic experiments showing that reduced mitotic Cdc28 activity compromises the Cdc20-dependent APC (Rudner et al., accompanying paper).

If Cdc20 binding and activity depend on a phosphorylated APC, why is the triple mutant *CDC16-6A CDC23-A CDC27-5A* viable? Even in the triple mutant there is some residual Cdc20 binding to the APC (data not shown), which is presumably sufficient to drive the metaphase to anaphase transition. The residual binding of Cdc20 to the APC could depend on the phosphorylation of the other subunits. In support of this idea, we see weak phosphorylation of proteins we believe to be Apc1, Apc4, Apc5, and Apc9 in some in vivo labelings (data not shown), and a protein we believe to be Apc9 is phosphorylated in vitro by Cdc28/Clb2/Cks1 complexes (data not shown and Figure 1) (Zachariae et al., 1996). In addition, *cdc28-1N*, a mutation in Cdc28 that cannot bind Cks1 (Kaiser et al., 1999) ; and data not shown), and *cks1-38*, have reduced APC phosphorylation (Figures 1C and 4E). These two mutants are temperature sensitive for growth and arrest in mitosis (Piggott et al., 1982; Tang and Reed, 1993), suggesting that APC phosphorylation may be essential. Alternatively, it has been proposed that the primary defect in *cdc28-1N* and *cks1-38* is in proteasome function (Kaiser et al., 1999), though proteasome activity was examined in G1, not in mitosis, leaving the relevance of this finding to the exit from mitosis uncertain.

Our data suggests that activation of the APC by phosphorylation opposes its inhibition by the spindle checkpoint. Although *CDC16-6A CDC23-A CDC27-5A* is viable, its delay in mitosis (Figure 6A) becomes lethal when the spindle checkpoint is activated (Figure 6B). Both APC phosphorylation and the spindle checkpoint affect the

ability of Cdc20 to activate the APC but have no effects on the G1, Hct1-dependent activity of the APC.

Regulation of APC phosphorylation

Phosphorylation of the APC in frogs and clams in vitro depends on homologs of the small Cdk binding protein, Cks1, and in clams, Cks1 stimulates Cdc20-dependent APC activity (Patra and Dunphy, 1998; Shteinberg and Hershko, 1999; Shteinberg et al., 1999). In budding yeast, the role of Cks1 remains uncertain. Although we add purified Cks1 to our in vitro kinase reactions, Cks1 is not required for APC phosphorylation in vitro (Figure 1 and data not shown). However, APC phosphorylation in vivo clearly depends on Cks1 (Figure 4E) and phosphorylation of Cdc27 is required for APC binding to Cks1-coupled beads (Figure 4D). We do not think that the binding of the APC to Cks1-coupled beads correlates with the ability of Cdc28 to phosphorylate the APC in vivo: although an APC containing Cdc27-5A does not bind to Cks1-coupled beads, Cdc16 and Cdc23 are fully phosphorylated in a *CDC27-5A* mutant. Despite this in vivo finding, we do see reduced in vitro phosphorylation of Cdc16 and Cdc23 in an APC containing Cdc27-5A (Figure 3C).

Mutants that affect the mitotic form of Cdc28 have reduced levels of phosphorylation of the APC, whereas *cdc28-4* cells, which are defective in the G1 form of Cdc28 (Reed, 1980), show normal phosphorylation of Cdc27 and Cdc16. We were surprised to discover that APC phosphorylation in *cdc28-4* appears to be normal (Figure 4E), because this mutant has approximately 20% the amount of Cdc28 protein as wild type cells at the permissive temperature of 23°C and very little detectable Cdc28-associated kinase activity when immunoprecipitated from cell lysates (Surana et al., 1991); and data not shown). A possible explanation of the absence of mitotic defects in *cdc28-4* cells is that the specific activity of each Cdc28-4 molecule is equal to that of wild type Cdc28, although the total number of active kinases is drastically reduced. The specific activity of

individual Cdc28 molecules may be critical for APC phosphorylation because one Cdc28/Clb2/Cks1 complex may bind persistently to the APC. Once bound to the APC this single complex might be responsible for multiple phosphorylations. If the steady state phosphorylation of the APC is determined by the balance between phosphorylation by Cdc28 and dephosphorylation by protein phosphatases, and Cdc28 remains bound to the APC, a drop in specific activity of Cdc28 would reduce the phosphorylation and activity of the APC.

How do cells escape from mitosis? If activating Cdc28/Clb/Cks1 complexes activates the Cdc20-dependent APC, which in turn triggers chromosome segregation, how do cells ensure that the lag between activating Cdc28 and activating the Cdc20-dependent APC is long enough to assemble a spindle and align chromosomes on it? Although one answer is that the spindle checkpoint inhibits Cdc20 in cells with mis-aligned chromosomes, this explanation is not enough (Hwang et al., 1998; Kim et al., 1998). Inactivating the spindle checkpoint does not kill yeast cells, implying other mechanisms exist to block premature activation of the Cdc20-dependent APC. Another possible mechanism is regulating the abundance of Cdc20. High levels of *CDC20* transcripts are restricted to mitotic cells and APC-dependent proteolysis restricts the abundance of Cdc20 (Kramer et al., 1998; Prinz et al., 1998; Shirayama et al., 1998; Weinstein, 1997). None of these forms of regulation exist in early frog embryos, where Cdc20 (Fizzy) levels are constant through the cell cycle and spindle depolymerization does not induce mitotic arrest (Lorca et al., 1998; Minshull et al., 1994). In addition, overexpressing Cdc20 in budding yeast raises the level of Cdc20 mRNA and protein, but does not advance the exit from mitosis, suggesting that other mechanisms must exist to regulate Cdc20-dependent APC activity (Prinz et al., 1998). Regulating the rate of Cdc28-catalyzed APC phosphorylation provides an additional mechanism. If this phosphorylation were slow relative to spindle assembly, most cells would manage to align their chromosomes on the spindle before activating the Cdc20-dependent APC which in turn induces Pds1 destruction and anaphase.

Figure 1 The APC is phosphorylated in vitro by Cdc28

Wild type (ADR376), *CDC27-MBP* (ADR1705), *CDC16-myc6* (K6180), *CDC23-HA* (SLJ378) and *cdc5-1* (JC145) were grown overnight in YEP+2% glucose at 23°C to log phase, arrested in G1 by alpha factor (1μg/ml) for 3 hours, harvested, lysed and the APC immunoprecipitated with anti-Cdc26 antibody. The immunoprecipitates were treated with purified Cdc28-His_6, Clb2-MBP, Cks1 and [γ-^{32}P]-ATP, washed to remove phosphorylated Clb2-MBP, and run on a polyacrylamide gel which was subjected to autoradiography (top) or western blotting (bottom). *cdc5-1* cells were shifted to 37°C for an additional one hour of alpha factor treatment. As controls, cell lysate was mock precipitated in the absence of anti-Cdc26 antibody (no anti-Cdc26) or was precipitated in the presence of anti-Cdc26 antibody, but no Cdc28, Clb2 or Cks1 was added to kinase reaction (no kinase). The western blot shows that similar amounts of APC were precipitated with the anti-Cdc26 antibody.

Figure 1

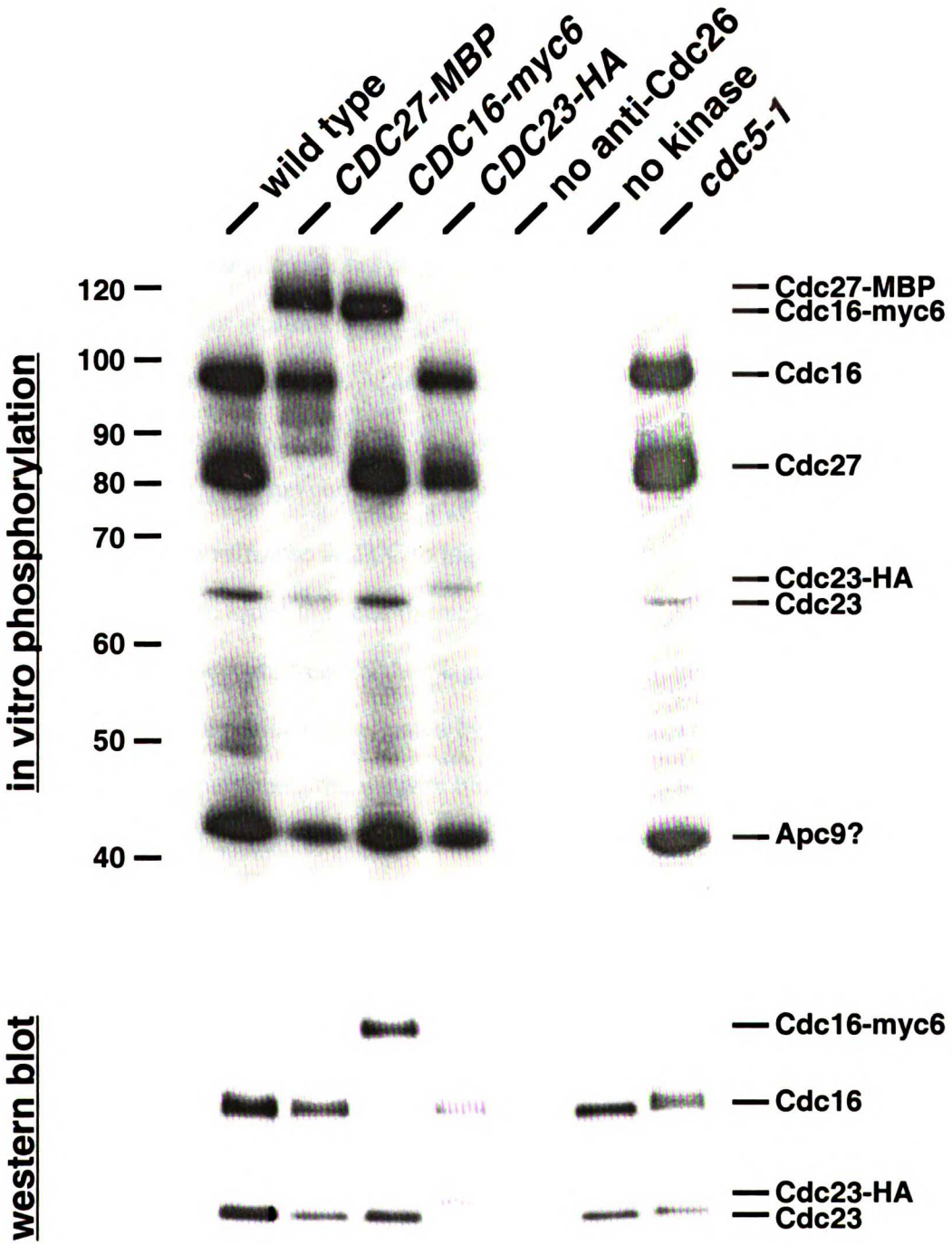

wild type
CDC27-MBP
CDC16-myc6
CDC23-HA
no anti-Cdc26
no kinase
cdc5-1
in vitro phosphorylation
120
100
90
80
70
60
50
40
Cdc27-MBP
Cdc16-myc6
Cdc16
Cdc27
Cdc23-HA
Cdc23
Apc9?
western blot
Cdc16-myc6
Cdc16
Cdc23-HA
Cdc23

Figure 2 The APC is phosphorylated in vivo

(A) APC phosphorylation is greatest in mitosis. Wild type (ADR376) and *cdc26Δ* (LH307) were grown overnight in YEP+2% glucose at 23°C to log phase and then arrested in G1 with alpha factor (1μg/ml), in S-phase with hydroxyurea (200mM) or in mitosis with nocodazole (10μg/ml) for 3 hours Cells were then transferred to phosphate free-CSM+2% glucose containing $^{32}PO_4$, and alpha factor, HU or nocodazole as indicated. After 1 hour cells were harvested, lysed and the APC immunoprecipitated with anti-Cdc26 antibody. Immunoprecipitates were run on a polyacrylamide gel which was subjected to either autoradiography (top) or western blotting (bottom).

(B&C) *CDC28-VF*, *clb2Δ*, *cdc28-1N*, and *cdc5-1* have reduced APC phosphorylation in vivo. All srains contain *pGAL-MPS1*. wild type (KH153), *CDC28-VF* (KH181), *clb2Δ* (ADR1606), *cdc28-1N* (ADR1899), *cdc5-1* (JC165) and *cdc26Δ* (ADR2023) were grown overnight in YEP+2% raffinose at 23°C to log phase and then transferred to YEP+2% galactose for 4 hours to arrest the cells in mitosis by Mps1 overexpression. Cells were then transferred to phosphate free-CSM+2% galactose containing $^{32}PO_4$ and treated as described in (A). In (B) cells were arrested by Mps1 overexpression at 23°C, whereas in (C) at 35°C. In all experiments the western blots shown below the autoradiographs illustrate that the same amount of APC was immunoprecipitated in all strains (except for *cdc26Δ* strains, where no APC was precipitated).

Figure 2

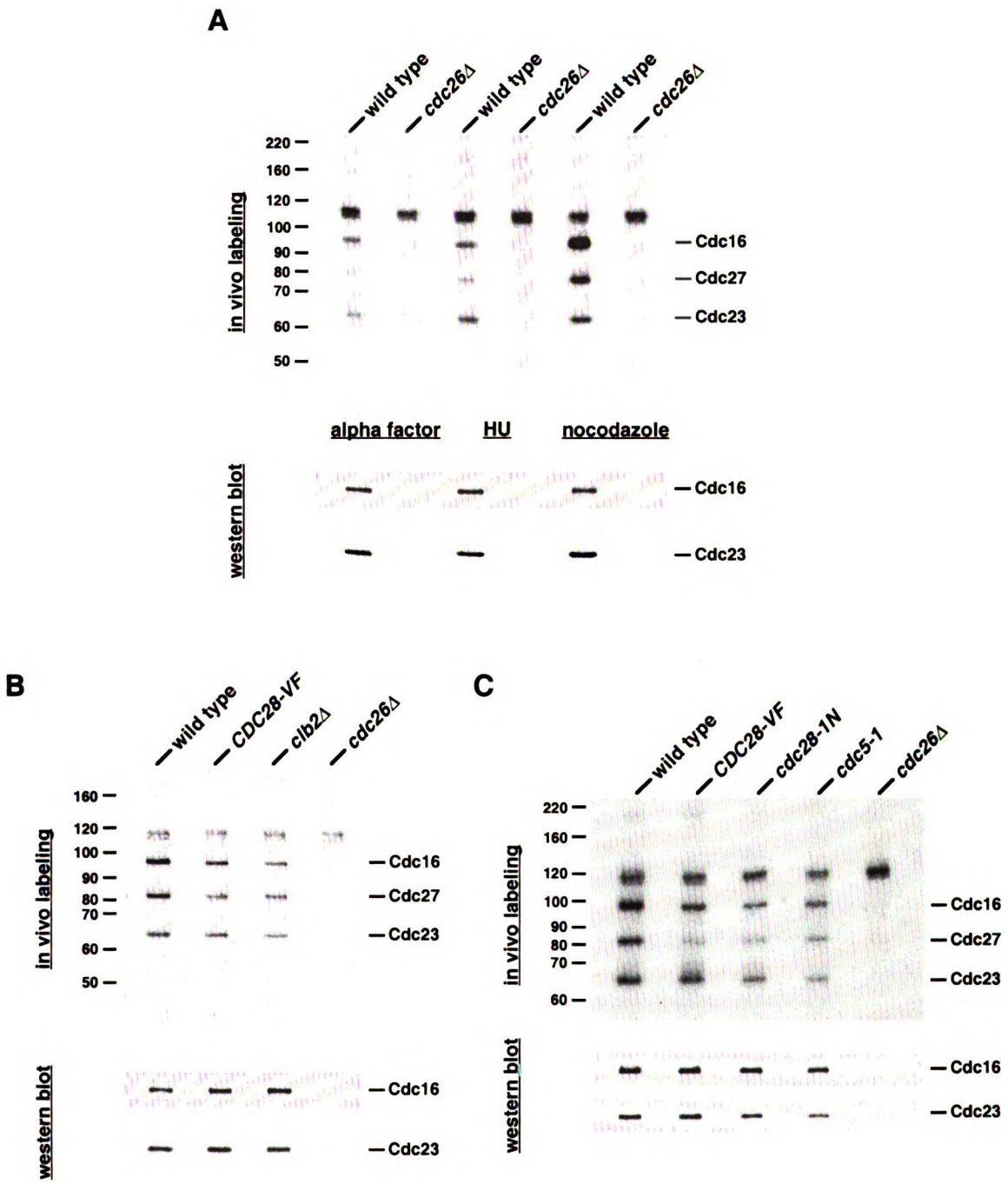

A
wild type
cdc26Δ
wild type
cdc26Δ
wild type
cdc26Δ
220
160
120
100
90
80
70
60
50
in vivo labeling
Cdc16
Cdc27
Cdc23
alpha factor
HU
nocodazole
western blot
Cdc16
Cdc23
B
wild type
CDC28-VF
clb2Δ
cdc26Δ
160
120
100
90
80
70
60
50
in vivo labeling
Cdc16
Cdc27
Cdc23
western blot
Cdc16
Cdc23
C
wild type
CDC28-VF
cdc28-1N
cdc5-1
cdc26Δ
220
160
120
100
90
80
70
60
in vivo labeling
Cdc16
Cdc27
Cdc23
western blot
Cdc16
Cdc23

Figure 3 The APC is phosphorylated on potential Cdc28 phosphorylation sites

(A) All serine/proline (SP) and threonine/proline (TP) sites on Cdc16, Cdc23 and Cdc27 were mutated to alanine/proline (AP).

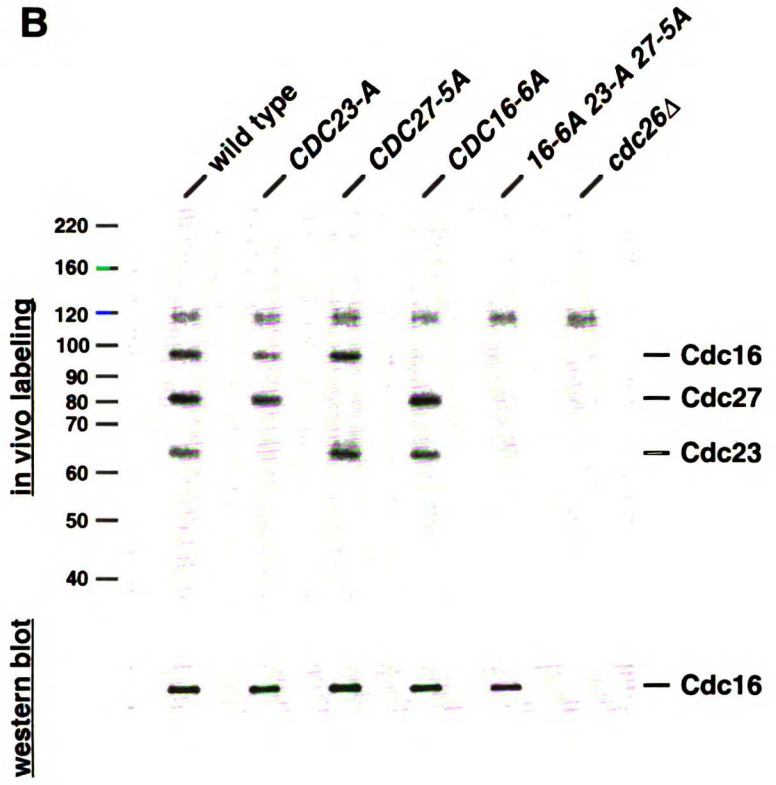
(B) Phosphorylation site mutants are resistant to phosphorylation in vivo. All strains contain *pGAL-MPS1*. wild type (KH153), *CDC16-6A* (ADR1975), *CDC23-A-HA* (ADR1973), *CDC27-5A-HA* (ADR1974) and *CDC16-6A CDC23-A CDC27-5A* (ADR1979) and *cdc26Δ* (ADR2023) were grown in the presence of $^{32}PO_4$ as described in Figure 1B.

(C) Phosphorylation site mutants are resistant to phosphorylation in vitro. The APC was isolated and phosphorylated in vitro as described in Figure 1 for: *cdc26Δ* (LH307), *CDC23-A* (ADR2030), wild type (ADR376), *CDC27-5A* (ADR2031), *CDC16-6A* (ADR2029) and *CDC16-6A CDC23-A CDC27-5A* (ADR2032).

Figure 3

A

CDC16		*CDC23*		*CDC27*	
PM**SP**SE	44	DE**SP**LR	59	SN**SP**PS	267
AA**SP**FV	59			TQ**TP**KN	304
AT**SP**YQ	95			LV**SP**SS	328
AN**SP**LI	103			LT**TP**PS	351
IM**TP**HQ	115			FK**TP**RN	397
LL**TP**QE	406				



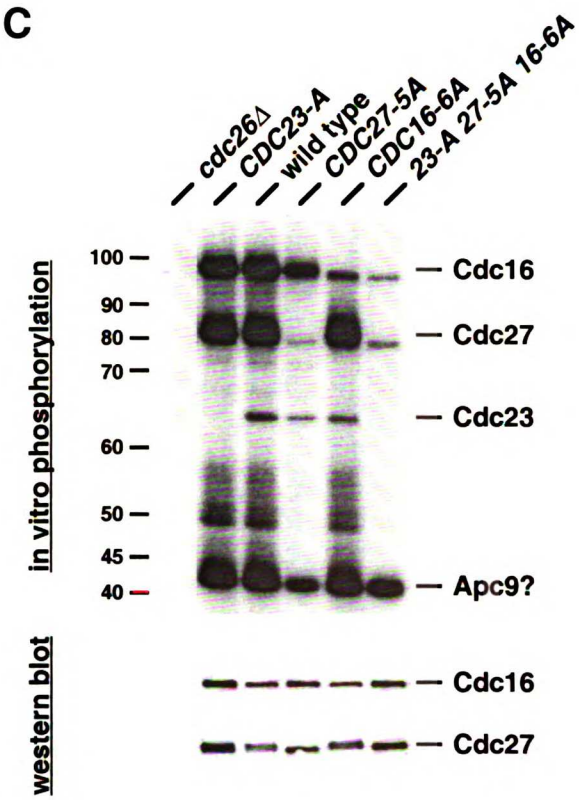


Figure 4 The APC associates with Cks1-coupled beads

(A) The APC from *CDC28-VF* associates poorly with Cks-coupled beads. Wild type (JM425) and *CDC28-VF* (JM434) were grown overnight in YEP+2% glucose at 23°C to log phase and arrested in mitosis with nocodazole (10μg/ml) for 3 hours Cells were harvested, lysed and mixed with Cks1-coupled beads. Western blots of the material bound to the Cks1-coupled beads show that the APC and Cdc28/Clb2 bind to the beads.

(B) Cdc27 phosphorylation can be seen by western blotting. Wild type APC isolated as described in (A), was treated either with lambda phosphatase, lambda phosphatase and inhibitors, or inhibitors alone.

(C) APC association to Cks1-coupled beads changes through the cell cycle. Wild type (ADR1389) and *CDC28-VF* (ADR1252) were grown overnight at 30°C in YPD to log phase, arrested in G1 with alpha factor (1μg/ml) and at t=0 (alpha factor) the cells were released from the G1 arrest. At t=75, alpha factor (1.5μg/ml) was added back to the cultures to re-arrest the cells in the next G1. A parallel sample was arrested in mitosis with nocodazole (10μg/ml). Samples were taken at the indicated times and processed for Western blots (top) or for Cks-coupled bead pulldowns (bottom). The arrow indicates that in wild type cells when Clb2 levels peak (t=75), Cdc16 and Cdc27 phosphorylation increases. The bracket indicates that in *CDC28-VF* cells when Clb2 levels are peaking, Cdc27 phosphorylation decreases.

(D) An APC containing Cdc27-5A does not bind to Cks1-coupled beads. The strains in Figure 3B and *CDC28-VF pGAL-MPS1* (KH181) were grown overnight in YEP+2% raffinose at 23°C to log phase and then transferred to YEP+2% galactose for 4 hours to arrest the cells in mitosis by Mps1 overexpression. Samples were taken and processed for western blots (cell lysate), immunoprecipitation with anti-cdc26 antibodies or Cks1-coupled bead pulldowns as described in Experimental Procedures. Equal amounts of lysates were used for the Cks1-coupled bead pulldown and the anti-Cdc26

immunoprecipitation, though a longer exposure is shown for the Cks1-coupled bead pulldown. Clb2 is shown as a loading control.

(E) Mitotic Cdc28 activity is required for APC phosphorylation. Wild type (ADR376), *CDC28-VF* (JM434), *cdc28-1N* (ADR483), *cdc28-4* (ADR842), *clb2Δ* (ADR313) and *cks1-38* (ADR1767) were grown overnight in YEP+2% glucose at 23°C to log phase and arrested in mitosis with nocodazole (10μg/ml) for 3.5 hours Samples were taken at the indicated times and processed for western blots. Clb3 is shown as a loading control.

Figure 4

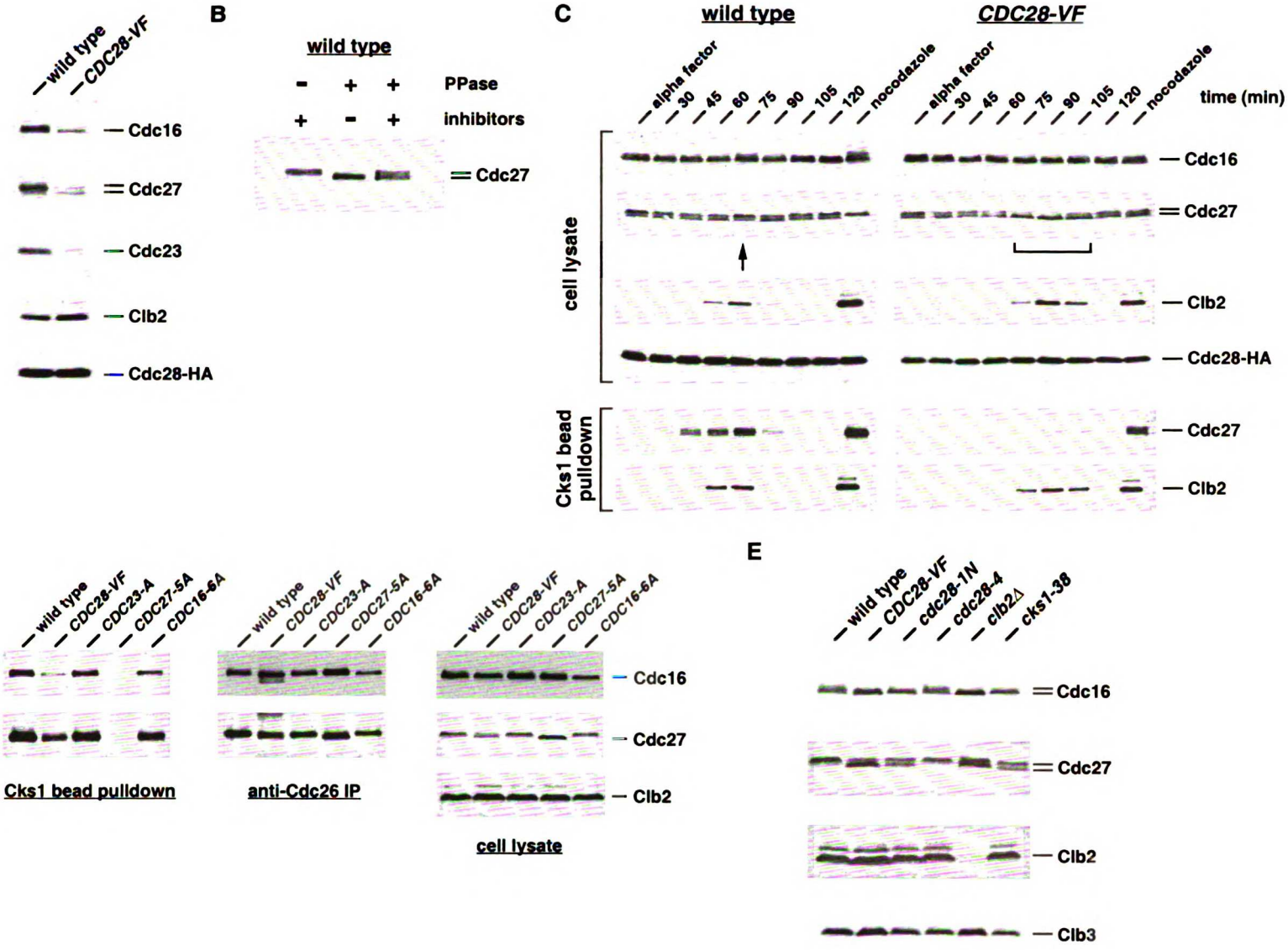

A
wild type
CDC28-VF
Cdc16
Cdc27
Cdc23
Clb2
Cdc28-HA
B
wild type
PPase
inhibitors
Cdc27
C
wild type
CDC28-VF
alpha factor
30
45
60
75
90
105
120
nocodazole
time (min)
cell lysate
Cdc16
Cdc27
Clb2
Cdc28-HA
Cks1 bead pulldown
Cdc27
Clb2
D
wild type
CDC28-VF
CDC23-A
CDC27-5A
CDC16-6A
Cdc16
Cdc27
Clb2
Cks1 bead pulldown
anti-Cdc26 IP
cell lysate
E
wild type
CDC28-VF
cdc28-1N
cdc28-4
clb2Δ
cks1-38
Cdc16
Cdc27
Clb2
Clb3

Figure 5 The alanine substituted APC has normal G1 APC activity

The strains described in Figure 3C were grown overnight at 30°C in YEP+2% glucose to log phase, arrested in G1 with alpha factor (1μg/ml) for 3 hours The cells were harvested, lysed and the APC was immunoprecipitated with anti-Cdc26 antibodies, and the in vitro ubiquitination activity of the immunoprecipitates was measured. The substrate for the in vitro ubiquitination is an iodinated N-terminal fragment of sea urchin cyclin B (CycB). Western blotting of the immunoprecipitates (bottom) shows that equal amounts of Cdc16 and Cdc27 are present in the APC isolated from each of the strains.

Figure 5

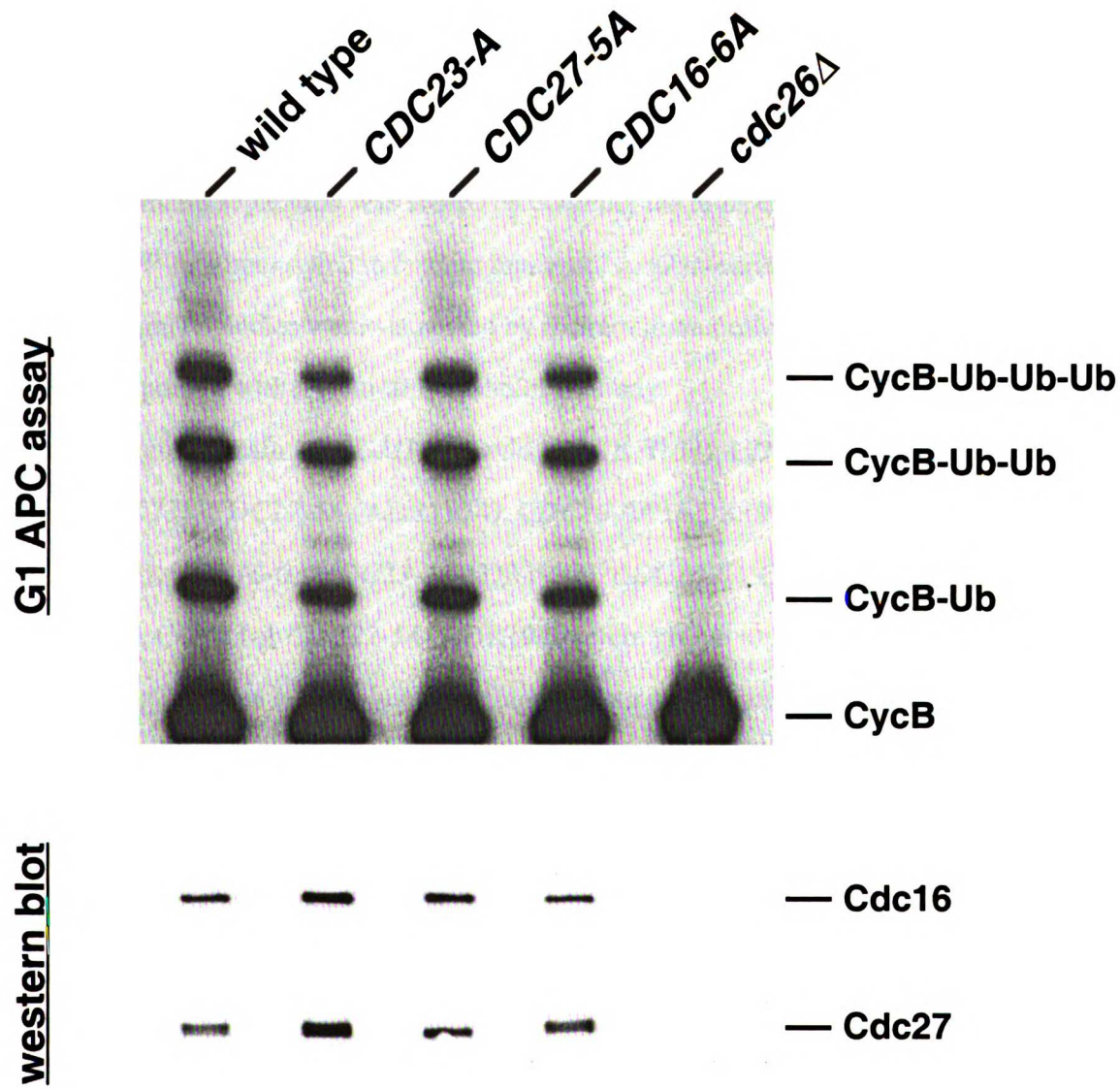
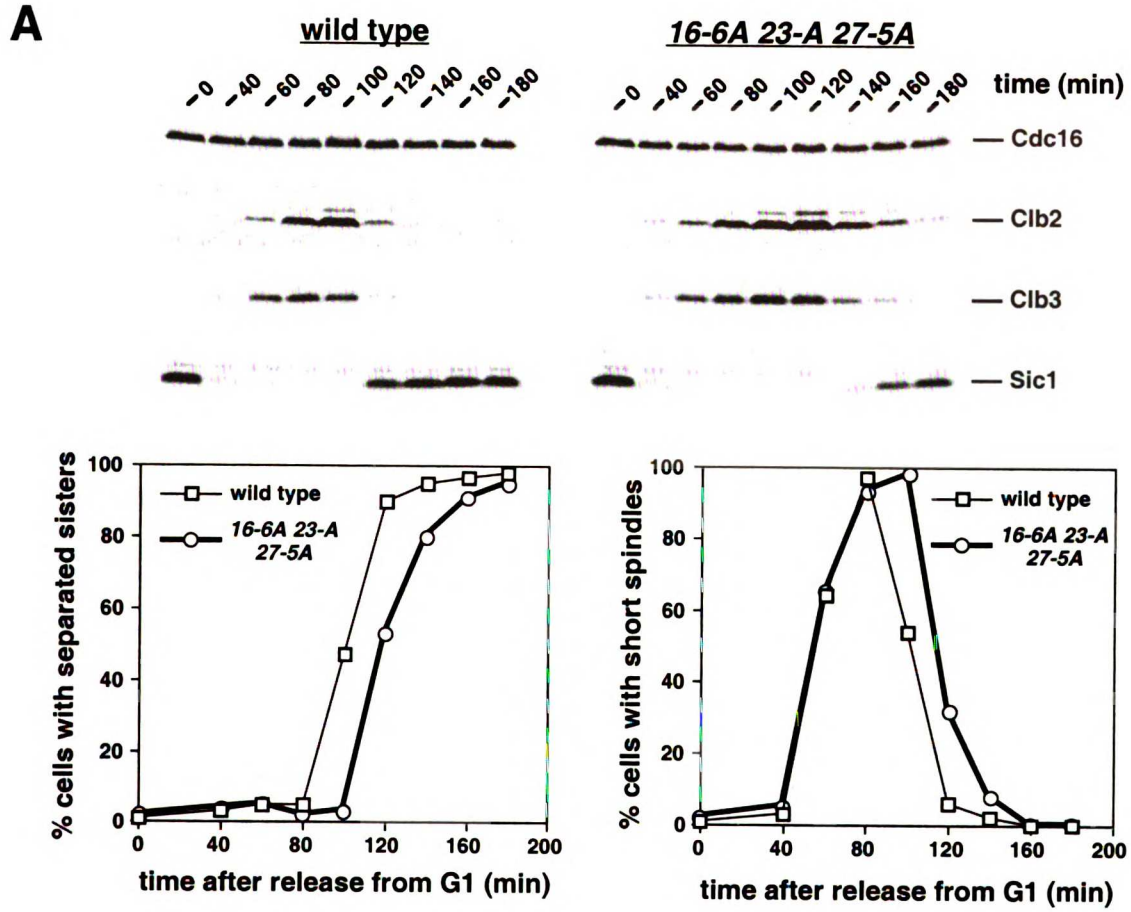

wild type
CDC23-A
CDC27-5A
CDC16-6A
cdc26Δ
G1 APC assay
CycB-Ub-Ub-Ub
CycB-Ub-Ub
CycB-Ub
CycB
western blot
Cdc16
Cdc27

Figure 6 The alanine substituted APC delays in mitosis and is sensitive to spindle checkpoint-dependent arrest

(A) Wild type (—□—, ADR2061) and *CDC16-6A CDC23-A CDC27-5A* (—○—, ADR2064) were grown overnight at 23°C in YPD to mid log phase, arrested in G1 with alpha factor (1μg/ml) and at t=0 the cells were released from the G1 arrest. At t=80, alpha factor (1.5μg/ml) was added back to the cultures to re-arrest the cells in the next G1. (top) Samples were taken at the indicated times and processed for Western blots. (bottom, left) Sister chromatid separation was scored by counting the number of fluorescent spots (one or two) of GFP-lacI bound to 256 tandem repeats of *lacO* integrated at the *LEU2* locus. (bottom, right) Spindles were visualized by indirect immunofluorescence of formaldehyde fixed cells probed with an anti-alpha tubulin antibody.

(B) All strains contain *pGAL-MPS1*. wild type (KH153), *CDC28-VF* (KH181),*CDC23-A* (ADR1973), *CDC27-5A* (ADR1974), *CDC16-6A* (ADR1975), *CDC23-A CDC27-5A* (ADR1976), *CDC16-6A CDC23-A* (ADR1977), *CDC16-6A CDC27-5A* (ADR1978) and *CDC16-6A CDC23-A CDC27-5A* (ADR1979) were grown to saturation for two days in YEP+ 2% glucose at 30°C, diluted ten-fold and four-fold serial dilutions were prepared in a multi-well dish and spotted onto YEP+2% glucose (left) or YEP+2% galactose (right). The plates were incubated at 30°C for 2 days.

Figure 6


A
wild type
16-6A 23-A 27-5A
0 40 60 80 100 120 140 160 180
time (min)
Cdc16
Clb2
Clb3
Sic1
% cells with separated sisters
wild type
16-6A 23-A 27-5A
time after release from G1 (min)
% cells with short spindles
wild type
16-6A 23-A 27-5A
time after release from G1 (min)

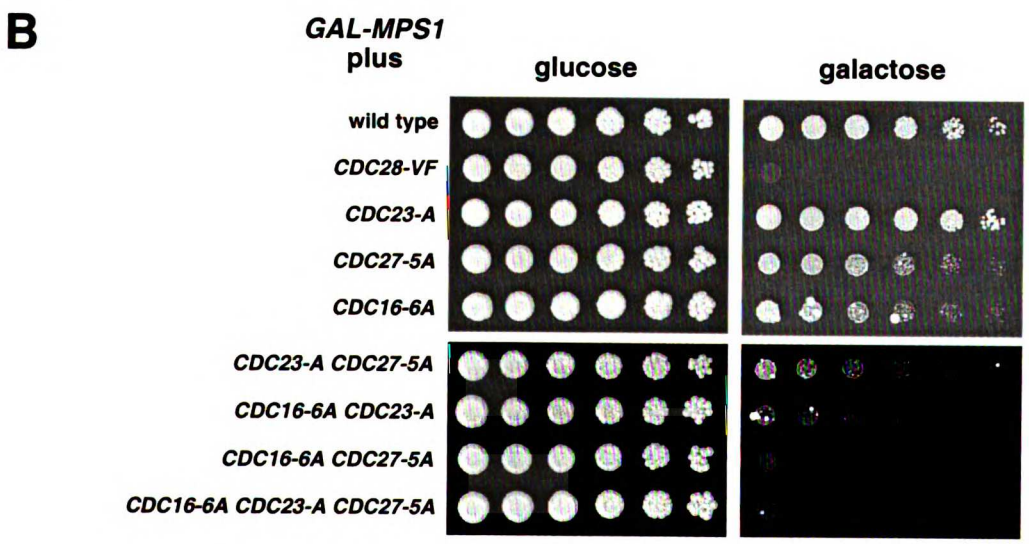
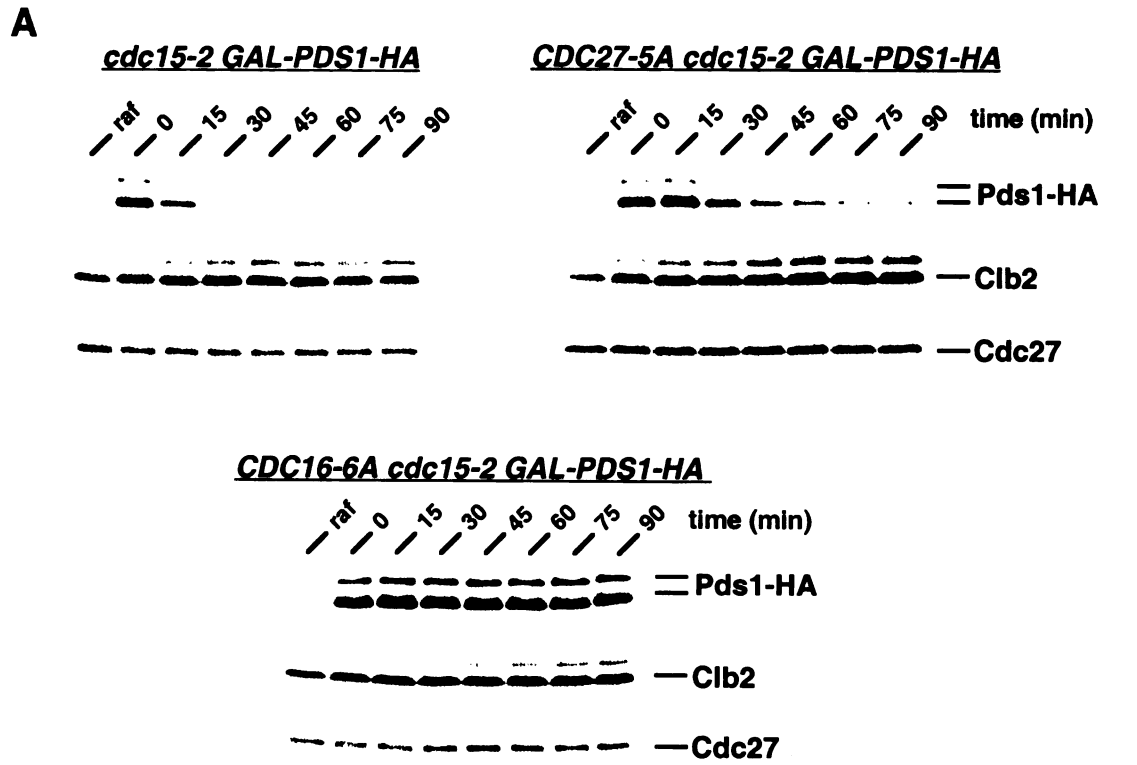

B
GAL-MPS1 plus
glucose
galactose
wild type
CDC28-VF
CDC23-A
CDC27-5A
CDC16-6A
CDC23-A CDC27-5A
CDC16-6A CDC23-A
CDC16-6A CDC27-5A
CDC16-6A CDC23-A CDC27-5A

Figure 7 The alanine substituted APC is defective in Cdc20-dependent APC function

(A) Pds1 is stabilized in anaphase in *CDC16-6A* and *CDC27-5A*. *cdc15-2 GAL-PDS1-HA* (ADR1968), *cdc15-2 CDC27-5A GAL-PDS1-HA* (ADR1999) and *cdc15-2 CDC16-6A GAL-PDS1-HA* (ADR2003) were grown overnight at 23°C in YEP+2% raffinose to log phase and shifted to 37°C to arrest the cells in anaphase (raf). When greater then 85% of the cells had reached anaphase (after 4 hours, as judged by nuclear division which was scored by DAPI staining), Pds1-HA expression was induced for 1 hour by the addition of 2% galactose, and at t=0 its expression was terminated by the addition of 2% glucose. Samples were taken at the indicated times and processed for western blots. Clb2 and Cdc27 are shown as a loading controls.

(B) Cdc20 binding to the APC is impaired in *CDC16-6A*. *cdc15-2 CDC20-myc12* (ADR1790), *cdc15-2 CDC27-5A CDC20-myc12* (ADR1987), *cdc15-2 CDC20-myc12 CDC16-6A* (ADR1990) and *cdc26Δ CDC20-myc12* (ADR2036) were grown overnight in YEP+2% glucose at 23°C to log phase and transferred into fresh YEP+2% glucose at 37°C. When greater than 85% of the cells were arrested in anaphase (4 hours, as judged by nuclear division which was scored by DAPI staining), the cells were harvested, lysed and the APC immunoprecipitated with polyclonal anti-Cdc26 antibodies. The amount of Cdc20-myc12 bound to the APC was determined by western blotting the immunoprecipitates with the 9E10 antibody. Equal amounts of Cdc23 was precipitated with the anti-Cdc26 antibodies (left) and equal amounts of cell lysate were used in the immunoprecipitation (right, cell lysate). *cdc26Δ* , which arrests in metaphase, not anaphase, accumulates high levels of Cdc20 because Cdc20 stability is regulated by the APC (Prinz et al., 1998; Shirayama et al., 1998).

Figure 7


A
cdc15-2 GAL-PDS1-HA
CDC27-5A cdc15-2 GAL-PDS1-HA
raf
0
15
30
45
60
75
90
time (min)
Pds1-HA
Clb2
Cdc27
CDC16-6A cdc15-2 GAL-PDS1-HA
raf
0
15
30
45
60
75
90
time (min)
Pds1-HA
Clb2
Cdc27

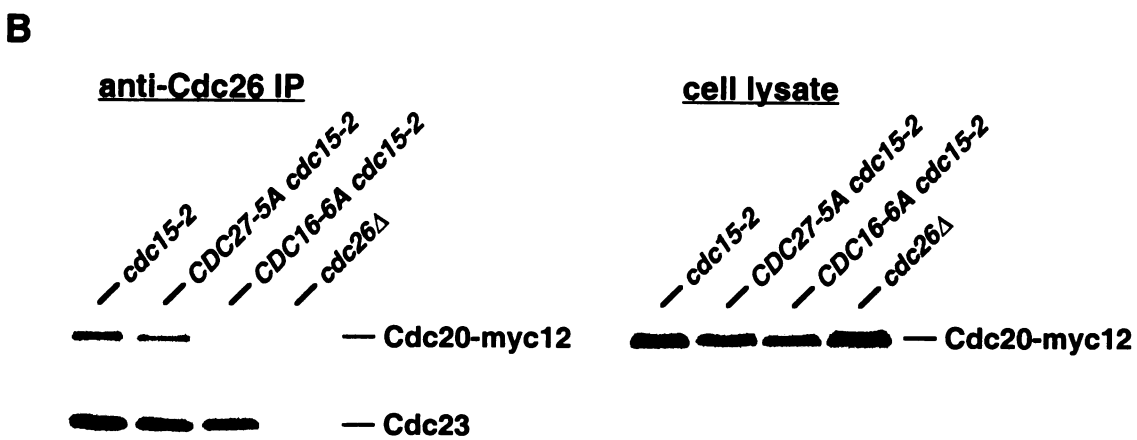
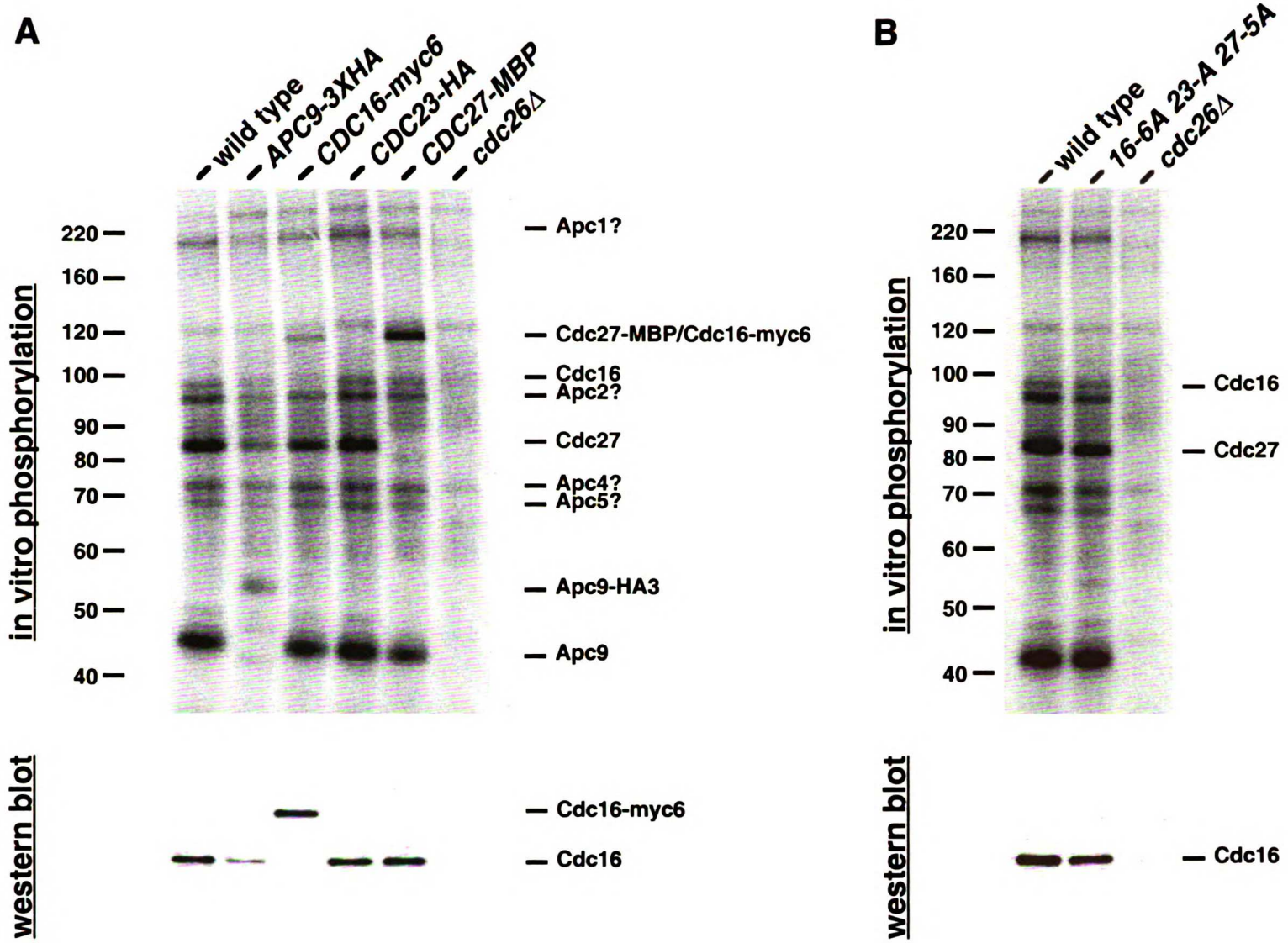

B
anti-Cdc26 IP
cell lysate
cdc15-2
CDC27-5A cdc15-2
CDC16-6A cdc15-2
cdc26Δ
Cdc20-myc12
Cdc23
cdc15-2
CDC27-5A cdc15-2
CDC16-6A cdc15-2
cdc26Δ
Cdc20-myc12

Figure 8 Cdc5 phosphorylates the APC in vitro

(A) The APC was isolated from the following strains as described in Figure 1: Wild type (ADR376), *APC9-HA3* (ADR2042), *CDC16-myc6* (K6180), *CDC23-HA* (SLJ378), *CDC27-MBP* (ADR1705), and *cdc26Δ* (LH307). The immunoprecipitates were treated with purified His_6-HA-Cdc5 and [γ-^{32}P]-ATP, washed to remove phosphorylated His_6-HA-Cdc5 and run on a polyacrylamide gel which was subjected to autoradiography (top) or western blotting (bottom). The western blot shows that similar amounts of APC were precipitated with the anti-Cdc26 antibody from all strains except *APC9-HA3*, which does not fully complement a *apc9Δ*.

(B) The APC was isolated from Wild type (ADR376), *CDC16-6A CDC23-A CDC27-5A* (ADR2032), and *cdc26Δ* (LH307), and phosphorylated by purified His_6-HA-Cdc5.

Figure 8


A
wild type
APC9-3XHA
CDC16-myc6
CDC23-HA
CDC27-MBP
cdc26Δ
in vitro phosphorylation
220
160
120
100
90
80
70
60
50
40
Apc1?
Cdc27-MBP/Cdc16-myc6
Cdc16
Apc2?
Cdc27
Apc4?
Apc5?
Apc9-HA3
Apc9
western blot
Cdc16-myc6
Cdc16
B
wild type
16-6A 23-A 27-5A
cdc26Δ
in vitro phosphorylation
220
160
120
100
90
80
70
60
50
40
Cdc16
Cdc27
western blot
Cdc16

Chapter 3

CDC55 regulates sister chromatid separation and inhibitory phosphorylation of Cdc28

Introduction

The spindle checkpoint monitors the attachment of kinetochores to the mitotic spindle. The presence of as little as one unattached kinetochore prevents activation of the Cdc20-dependent APC, and thus exit from mitosis (Rieder et al., 1995; Wells and Murray, 1996). In budding yeast the *MAD1-3*, *BUB1* and *3*, and *MPS1* genes are required for the checkpoint, and were isolated as mutants in that are unable to arrest in mitosis after depolymerization of the mitotic spindle by the microtubule inhibitor benomyl (Hoyt et al., 1991; Li and Murray, 1991; Weiss and Winey, 1996) The protein products of these genes are thought to be recruited to unattached kinetochores and there generate a signal that eventually results in Mad2 inhibition of Cdc20 (Chen et al., 1996; Kallio et al., 1998; Waters et al., 1999). Exactly how unattached kinetochores recruit this complex, how the signal is amplified and translated into a soluble inhibitor of the APC, and how the signal decays is not known, but many of the checkpoint components and their vertebrate homologues have been localized to kinetochores and can be found in a number of distinct protein complexes (Brady and Hardwick, 2000; Chen et al., 1998; Chen et al., 1996; Taylor and McKeon, 1997).

In addition to these six checkpoint mutants, two other mutants have been found that are involved in the spindle checkpoints. *bub2Δ* , initially thought to behave like *bub1* and *bub3* mutants, now is thought to function in a later checkpoint that may monitor the delivery of the daughter nucleus into the daughter bud during anaphase (Alexandru et al., 1999; Hoyt et al., 1991). Rather than target the Cdc20-dependent APC, the Bub2 checkpoint prevents the release of Cdc14 from the nucleolus and thus the inactivation of Cdc28 (Cerutti and Simanis, 2000). *cdc55Δ*, although quite similar to the *mad* mutants, is thought to function in a separate pathway from the Mad proteins (Minshull et al., 1996; Wang and Burke, 1997). The checkpoint defect of *cdc55Δ* cells was initially thought to be caused by increased inhibitory phosphorylation on Cdc28. *CDC28-VF*, which prevents

this phosphorylation, suppresses *cdc55Δ* and inhibitory phosphorylation on Cdc28 in *cdc55Δ* cells is elevated. In addition, *CDC55* encodes a B subunit of protein phosphatase 2A (PP2A) which is known to regulate the kinase and phosphatase that regulate inhibitory phosphorylation of Cdk1 in other organisms (Coleman and Dunphy, 1994; Healy et al., 1991; Kinoshita et al., 1993; Lee et al., 1994; Mueller et al., 1995). However, the extensive characterization of *CDC28-VF* described in Chapters 1 and 2 has led to the conclusion that *cdc55Δ*, like *CDC28-VF*, has at least two phenotypes, one involved in regulating inhibitory phosphorylation on Cdc28 and the other affecting the activity of the Cdc20-dependent APC. Because this story is incomplete and because our misconceptions about *cdc55Δ* were so deep, I've presented the data historically, tracking my changing ideas about *cdc55Δ*.

Results

cdc55Δ is a spindle checkpoint mutant

Jeremy Minshull isolated *cdc55-215* in a screen for novel checkpoint mutants. The characterization of this mutant is described in detail in Minshull et al. 1996, but below I will summarize this data and present the experiments that I performed for this paper. *cdc55-215* behaves identically to *cdc55Δ*, and in all experiments below, the deletion is used rather than the allele. *cdc55Δ*, like the other mad mutants, die rapidly when grown in benomyl or nocodazole, microtubule poisons that activate the spindle checkpoint. Also like *mad* mutants, an S-phase delay caused by treatment with hydroxyurea (HU), which inhibits DNA synthesis, rescues the benomyl sensitivity of *cdc55Δ*. The death of *cdc55Δ* cells is associated with a re-entry into the cell cycle as evidenced by rebudding when grown in nocodazole. Recent data has also shown that like *mad* mutants, *cdc55* mutant cells do not delay passage through mitosis due to the presence of short linear chromosomes (S. Davis, B. Stern and A.W. Murray, personal communication)

In order to better understand what happens to *cdc55Δ* cells as they prematurely exit mitosis in the presence of nocodazole, Jeremy Minshull, Aaron Straight and I examined key markers of mitotic progression: sister separation, Cdc28-associated kinase activity, and Clb protein levels. Cells were arrested in G1 with the mating pheromone alpha factor, and released from this arrest into media containing nocodazole, which, like benomyl, causes spindle depolymerization and activation of the checkpoint. In order to look at only one cycle, alpha factor was re-added to the cultures after cells had budded, in order to re-arrest the cells that had exited mitosis in G1. Figure 1 examines Clb2 and Clb3 protein stability, and the data examining Clb2-associated kinase activity and sister separation can be found in Figure 2, and was performed by Jeremy Minshull and Aaron Straight. Wild type cells treated with nocodazole activate the spindle checkpoint and arrest with their sister

chromatids together, Clb2-associated kinase activity high and Clb2 and Clb3 proteins stable. A *mad2Δ* mutant, on the other hand separate their sister chromatids, inactivates Cdc28/Clb2 complexes and degrades Clb proteins. *cdc55Δ* cells, like *mad2Δ* cells separate their sisters and inactivate Cdc28/Clb2 complexes, but Clb proteins remain quite stable, though at a somewhat lower level than in wild type. These events occur slower than in *mad2Δ* mutants because *cdc55Δ* cells grow significantly slower than *mad* cells (data not shown). *cdc55Δ* and *mad2Δ* cells die during this experiment, while viability of wild type stays high (data not shown).

We were surprised to find that Clb2-associated kinase activity falls in *cdc55Δ* mutants despite little apparent Clb2 or Clb3 proteolysis. We therefore tested whether the inhibition of Cdc28 activity was due to some other method of inhibition. One way in which Cdc28 can be inhibited is by inhibitory phosphorylation on residues threonine 18 and tyrosine 19. The *CDC28-VF* mutant (described in detail in Chapter 1) mutates these sites of phosphorylation and is resistant to phosphorylation (Figure 3, Chapter 1). *cdc55Δ CDC28-VF* cells suppress both the fall in Clb2-associated kinase activity and the premature sister separation, suggesting that excessive inhibitory phosphorylation on Cdc28 might cause the *cdc55Δ* phenotype (Figure 1 and 2). Although we see good suppression of these two traits, *cdc55Δ CDC28-VF* cells still die during this experiment. This suppression is specific for *cdc55Δ*, because *mad* mutants are not suppressed by *CDC28-VF* (data not shown).

A second way in which Cdc28-associated activity could be inhibited in *cdc55Δ* cells is by the binding of Sic1, a CDK inhibitor in budding yeast (Mendenhall, 1993). *cdc55Δ sic1Δ* cells, however, behave like *cdc55Δ* cells (Jeremy Minshull, unpublished data), making it unlikely that Sic1 is involved in the spindle checkpoint.

I performed epistasis analysis between *mad2Δ* and *cdc55Δ* cells in order to distinguish whether *MAD2* and *CDC55* operate in the same checkpoint pathway, or in independent pathways. Using the difference in Clb2 degradation as a distinguishing

phenotype, I compared wild type, *mad2Δ*, *cdc55Δ* and *mad2Δ cdc55Δ* in a timecourse identical to the one described above (Figure 3). In the double mutant, *cdc55Δ mad2Δ*, Clb2 was only partially degraded, a phenotype intermediate between the *mad2Δ* and *cdc55Δ* phenotypes. Although not conclusive, this data is most consistent with the idea that these two genes operate in separate checkpoint pathways, and the Clb2 degradation phenotype reflects a combination of the two defects.

cdc55Δ cells have high levels of inhibitory phosphorylation on Cdc28

Because some aspects of the *cdc55Δ* checkpoint phenotype are suppressed by *CDC28-VF*, I wondered if the *cdc55Δ* defect was due to increased levels of inhibitory phosphorylation on Cdc28. Threonine 18 is not normally phosphorylated in vivo, so I focused on detecting the phosphorylation of tyrosine 19. An epitope tagged Cdc28 was immunoprecipitated from wild type, *cdc55Δ*, *CDC28-VF* and *cdc55Δ CDC28-VF* cells, and then probed by western blot with a phospho-tyrosine specific antibody (Figure 4). The four strains were either grown asynchronously, arrested in G1 by alpha factor, or treated with nocodazole (which will arrest three of the strains in mitosis, but not *cdc55Δ*). No phospho-tyrosine is detected on Cdc28 in G1 in any of the strains, but asynchronously grown or nocodazole treated *cdc55Δ* cells have reproducibly higher levels of tyrosine phosphorylated Cdc28 than wild type cells (Figure 4A). The strains containing *CDC28-VF* contain no tyrosine phosphorylated Cdc28. *cdc55Δ* cells do not arrest in nocodazole, which could explain why Cdc28 is hyper-phosphorylated compared to wild type during nocodazole treatment. However, I also see increases in tyrosine phosphorylated Cdc28 in *cdc55Δ* cells which pass through a synchronous cell cycle (see Chapter 4, Figure 1) and in cells arrested by overexpression of a non-degradable Clb2 (*CLB2-Δ176*) (Figure 4B). I do not know if there is also a change in threonine 18 phosphorylation in *cdc55Δ* cells.

Swe1 (the budding yeast Wee1) is the protein kinase that phosphorylates Cdc28 on tyrosine 19, and Mih1 (the budding yeast Cdc25) is the phosphatase that removes the phosphorylation (Booher et al., 1993; Russell et al., 1989). Inhibitory phosphorylation on Cdc28 is altered in *swe1Δ* and *mih1Δ* cells (see Chapter 1, Figure3). Changes in phosphorylation of tyrosine 19 on Cdc28 in *cdc55Δ* cells could result from activation of the Swe1 kinase, or inhibition of the Mih1 phosphatase, or some combination of the two. Homologues of these two proteins are regulated by phosphorylation in other organisms (Coleman and Dunphy, 1994), so I investigated if the phosphorylation of Swe1 is altered in *cdc55Δ* cells. Lysates of asynchronous cells were probed with an anti-Swe1 antibody (Figure 5A). In wild type cells, Swe1 runs as a heterogeneous mixture of bands. These different forms of Swe1 are phosphorylated isoforms because lambda phosphatase treatment of immunoprecipitates of an epitope tagged Swe1 collapse the forms to slower migrating forms (Figure 5B). *cdc55Δ* cells contain Swe1 predominantly in a hyperphosphorylated form (Figure 5A). I also looked at Swe1 phosphorylation in *mih1Δ* cells, and Swe1 is slightly shifted to its hyperphosphorylated forms. This data suggests that the hyperphosphorylated form of Swe1 is more active, though this data does not rule out that Mih1 activity is also affected. Because I have detected increased inhibitory phosphorylation in *hct1Δ* cells (see Appendix 1, Figure 8), I also investigated if Swe1 phosphorylation is changed in *hct1Δ* cells. The banding pattern of Swe1 is similar in wild type and *hct1Δ* cells (Figure 5A).

APC independent sister chromatid separation

At the time that the above experiments were performed, the Clb proteins were the only well characterized substrates of the APC. I knew sister separation depended on APC function, so I thought that perhaps sister separation in the *cdc55Δ* mutant was triggered simply by the drop in Clb2-associated kinase activity in *cdc55Δ* . In support of an APC-

independent trigger for sister separation, I observed that *cdc55Δ* can weakly suppress the APC mutant, *cdc23-1*, at semi-permissive temperatures for *cdc23-1* function (Figure 6A). This suppression is similar to that seen in *cdc23-1 mad* double mutants (see Appendix 4, Figure 2), but unlike *cdc23-1 mad* doubles, *cdc55Δ* strongly suppresses the lethality of *cdc23-1* in a rapid death assay performed at 37° (Figure 6B). *cdc23-1* also suppresses the nocodazole sensitivity of *cdc55Δ* in a rapid death assay (Figure 6B). Others have also published that *cdc55Δ* suppresses the temperature sensitivity of *cdc20-1*, a mutant in an activator of the APC (Tavormina and Burke, 1998).

I therefore wondered if sister separation could be triggered by simply inactivating Cdc28. Overexpression of Sic1, a CDK inhibitor, causes a rapid inhibition of Cdc28/Clb-associated kinase activity (Amon, 1997). Rather than use the wild type copy of Sic1, I used a version in which multiple phosphorylation sites have been mutated to alanine (referred to as Sic1-mut), rendering the protein resistant to SCF-mediated degradation. Wild type and *cdc23-1* cells were arrested in mitosis at 26°C or 33°C in raffinose and nocodazole, and then high levels of Sic1-mut were driven from the inducible *GAL1* promoter by addition of galactose. Sic1-mut overexpression inhibits Clb2-associated kinase one hour after induction in both wild type and *cdc23-1* cells (Figure 7A and 7B). Clb2 protein is degraded in wild type cells, but remains stable in *cdc23-1* cells indicating that the APC is largely inactive even at 26°C in *cdc23-1* (Figure 7A). Sister separation was monitored using fluorescent in situ hybridization with a cosmid probe that hybridizes to the arm of chromosome III. Sister separation occurs in greater than 50% of wild type cells during this experiment (Figure 7C). However, despite the rapid inhibition of Cdc28 activity in *cdc23-1* cells, no sister separation occurs (Figure 7C). Although sister separation cannot be triggered independently of the APC, this experiment shows that Clb2-associated kinase activity inhibits the activation of the APC, and that rapid inhibition of kinase activity can override the spindle checkpoint. Similar work has been published (Amon, 1997) and numerous studies have identified Hct1 and Sic1 as the two likely targets

of Cdc28 that are activated when Cdc28-associated activity is inhibited (Jaspersen et al., 1999; Zachariae et al., 1998).

Although this result suggested that sister separation in *cdc55Δ* must depend on the APC, we wondered if the method by which Cdc28-associated activity is inhibited has an affect on the outcome of the experiment. I therefore tested whether inhibition of Cdc28-associated activity by high levels of Swe1, the kinase responsible for phosphorylating the inhibitory tyrosine on Cdc28, might trigger sister separation. Overexpression of Swe1 from the *GAL1* promoter does not trigger sister separation in a *cdc23-1* mutant arrested at 35°C (data not shown), nor does it trigger sister separation or Clb proteolysis when expressed in wild type cells arrested by nocodazole (data not found and see Chapter 4, Figure 4). These experiments were also performed in an *mih1Δ* background to ensure high levels of inhibition and again no sister separation was ever observed (see Chapter 4, Figure 6). I considered overexpressing both Sic1 and Swe1 in a *cdc23-1 mih1Δ* cell (and would have torqued the system further by including *cdc55Δ*, but *cdc55Δ mih1Δ* cells are inviable) but other data (see Figure 8) made me realize that sister separation in *cdc55Δ* cells was APC dependent.

Pds1 is degraded in *cdc55Δ* cells

Once it became clear that the anaphase inhibitor Pds1 (the budding yeast homologue of fission yeast *cut2*) is the key regulator of sister separation and is a target of the APC (Cohen-Fix et al., 1996; Yamamoto et al., 1996a; Yamamoto et al., 1996b), I investigated whether Pds1 is degraded when sister separation occurs in *cdc55Δ* cells grown in nocodazole. wild type, *cdc55Δ*, *mad2Δ* and *cdc55Δ CDC28-VF* cells containing an epitope tagged Pds1 were arrested in G1 with alpha factor, and then released into fresh media containing nocodazole to activate the spindle checkpoint. Alpha factor was re-added to arrest cells that had exited mitosis in the next G1. Wild type cells arrest in mitosis with

high levels of Pds1 and Clb2, and no Sic1 (Figure 8). *mad2Δ* cells do not arrest, degrade Clb2 and Pds1, and reaccumulate Sic1 as they re-arrest in alpha factor. *cdc55Δ* cells, as shown above (Figure 1) stabilize Clb2, but do not stabilize Pds1 which is degraded at the same time that sister separation occurs in similar experiments. In addition, Sic1 levels re-accumulate, confirming that *cdc55Δ* cells exit mitosis and re-arrest with alpha factor. The suppression of *cdc55Δ* by *CDC28-VF* is also confirmed in this experiment. Pds1 levels stay high in *cdc55Δ CDC28-VF*, and only start to fall slightly at the end of the time course, as Sic1 levels begin to rise (Figure 8).

Given my current understanding of APC regulation, this experiment suggests that Cdc20 is inappropriately activated in *cdc55Δ* cells causing Pds1 degradation, but for some reason, Hct1 cannot be activated. Eventually due to accumulation of Sic1, or small amounts of Cdc20-dependent Clb proteolysis, *cdc55Δ* cells exit mitosis, though with little Clb2 degradation. If this interpretation is correct, what causes the inappropriate Cdc20 activation in *cdc55Δ* cells?

Cdc16 is hyperphosphorylated in *cdc55Δ* cells.

Cdc20 is inhibited by the Mad checkpoint, and is activated indirectly by the phosphorylation of the APC (see Chapter 2). The data above suggests that *cdc55Δ* acts independently of the Mad pathway, so I investigated whether *cdc55Δ* cells have changes in the amount of APC phosphorylation. APC phosphorylation is only high in mitosis, so I examined the in vivo phosphorylation of the APC in *cdc15-2*, a mutant that arrests in anaphase with active Cdc20-dependent APC (Jaspersen et al., 1998), *cdc15-2 cdc55Δ* and *mad1Δ cdc15-2* cells. The phosphorylaion of Cdc16, Cdc23 and Cdc27 was identical in *cdc15-2* and *cdc15-2 mad1Δ* (Figure 9A), but the phosphorylation of Cdc16 was five-fold higher and the phosphorylation of Cdc23 and Cdc27 was two-fold higher in *cdc55Δ cdc15-2* cells (Figure 9A). This difference is particularly interesting because mutation of the

phosphorylation sites in Cdc16 causes a dramatic loss of Cdc20 binding to the APC (see Chapter 2, Figure 7).

To investigate whether the difference in Cdc16 phosphorylation is relevant to the *cdc55Δ* phenotype, I combined *cdc55Δ* and *CDC16-6A*, which replaces the endogenous *CDC16* with a version that is non-phosphorylatable (see Chapter 2, Figure 3), to see if *CDC16-6A* suppresses the checkpoint defect of *cdc55Δ*. *cdc55Δ CDC16-6A* cells are as sensitive to checkpoint activation, both when tested on plates containing benomyl, and when tested in a rapid death assay using nocodazole (Figure 9B and 9C). This result questions the relevance of the increased phosphorylation of Cdc16 in *cdc55Δ* cells, but it is difficult to know if the increase in phosphorylation of Cdc27 and Cdc23 is important for the *cdc55Δ* phenotype, or if although *cdc55Δ CDC16-6A* cells are sensitive to benomyl and nocodazole, they are checkpoint proficient.

Discussion

Why is *cdc55Δ* checkpoint defective?

The analysis described above leaves little doubt that *cdc55Δ* is spindle checkpoint defective. In addition, a recent selection for checkpoint mutants has found multiple new alleles of *CDC55* (Sam Davis and Bodo Stern, unpublished data). Although *cdc55Δ* has many traits in common with *mad* mutants, its stabilization of Clb2 during mitotic exit and its suppression by *CDC28-VF*, makes it stand out as different.

It still remains unclear what causes the checkpoint defect of *cdc55Δ*. Inhibitory phosphorylation on Cdc28 is clearly raised in *cdc55Δ* cells (Figure 4), and the suppression of *cdc55Δ* by *CDC28-VF* supports the conclusion that this defect in Cdc28 activity is responsible for the checkpoint defect (Figure 1, 2 and 8). But the analysis of *CDC28-VF* in Chapter 1 raises the possibility that the suppression of *cdc55Δ* by *CDC28-VF* is due to *CDC28-VF*'s defect in APC activation. The obvious experiment, which has only been performed in a cursory way (Jeremy Minshull and Andrew Murray, personal communication), is to test if *swe1Δ* or *swe1Δ CDC28-V* suppress the checkpoint defect of *cdc55Δ*. If suppression of inappropriate sister separation is observed, than inhibitory phosphorylation of Cdc28 is important, if not, than there is a second defect in *cdc55Δ* cells responsible for the spindle checkpoint defect. It is possible that inhibitory phosphorylation contributes to the *cdc55Δ* phenotype, but that other defects are required for the full checkpoint defective phenotype.

The suppression of *cdc23-1* and *cdc20-1* by *cdc55Δ*, and the changes in APC phosphorylation in *cdc55Δ* make it likely that *cdc55Δ* hyperactivates the Cdc20-dependent APC (Figure 6 and 9). If this is true, then the suppression by *CDC28-VF* becomes even more suspect because in Chapters 1 and 2 I have shown that *CDC28-VF* is defective in activating and phosphorylating the Cdc20-dependent APC. It seems likely that the

suppression by *CDC28-VF* simply reflects its defect in APC activation either being stronger than *cdc55Δ's* hyperactivation of the APC, or Cdc28 and Cdc55 regulate the same subunits of the APC. Phosphorylation of the APC subunits Cdc16, Cdc23 and Cdc27 affect Cdc20 binding and activation of the APC, and so might be the common target of Cdc28 and Cdc55. This model is also supported by the fact that *CDC55* encodes a B subunit of PP2A (Healy et al., 1991). PP2A has been shown to inhibit the APC in vitro and an okadaic acid sensitive phosphatase co-purifies with the clam APC (Lahav-Baratz et al., 1995; Shteinberg et al., 1999). In addition to APC phosphorylation, it is also possible that Cdc20 phosphorylation, or Pds1 phosphorylation might be affected by Cdc28 and Cdc55 (see Appendix 3).

If the Cdc20-dependent APC is activated inappropriately in *cdc55Δ* cells, how come it doesn't cause activation of the Hct1-dependent APC and degradation of Clb2? This may be because only partial activation of the APC occurs and cells get stuck at a point in the cell cycle with low levels Cdc20-dependent APC activity, but with insufficient proteolysis of Clb proteins to activate Hct1. The *cdc55Δ mad2Δ* double mutant described above argues against this idea because removal of the Mad pathway should further activate Cdc20 and cause *cdc55Δ mad2Δ* cells to behave as *mad2Δ* cells. Instead, I find that *cdc55Δ mad2Δ* cells have an intermediate phenotype between *cdc55Δ* and *mad2Δ* (Figure 3).

Alternatively, *CDC55* may also regulate Hct1 activation or Cdc14 release from the nucleolus (Shou et al., 1999; Visintin et al., 1999). One way to distinguish these possibilities would be to examine the phenotype of a *cdc55Δ bub2Δ* double mutant. *bub2Δ* cells, like *cdc55Δ* cells, are able to suppress *cdc20-1* mutants, and hyperactivate the Hct1-dependent APC (Shirayama et al., 1999; Tavormina and Burke, 1998) (and see Appendix 1). *bub2Δ cdc55Δ* cells might now show complete degradation of Clb2 when grown in nocodazole.

A third possibility is that the increased Cdc20 activity in *cdc55Δ* mutants blocks Hct1 activation of the APC. This could occur either because Hct1 doesn't bind a highly

phosphorylated APC well, or because Hct1 and Cdc20 compete for binding to the APC. This last possibility seems less likely because Hct1 and Cdc20 are thought to be sub-stochiometric activators, but there is currently no data about how many APC complexes are competent to bind Hct1 or Cdc20.

CDC55 regulates inhibitory phosphorylation of Cdc28

CDC55 also plays a role regulating inhibitory phosphorylation on Cdc28. The increase of phosphorylation is responsible for its hyperpolarized growth morphology. This is typical for mutants that are unable to accumulate high enough levels of active Cdc28/Clb complexes, and are therefore unable to switch from polarized to isotropic growth (Lew and Reed, 1993).

Why do *cdc55Δ* cells increase inhibitory phosphorylation on Cdc28? Swe1 might become more active, or simply stay active at a time when it is usually degraded. Swe1 is normally present only during S-phase and early mitosis, after which it is targeted for degradation by SCF mediated degradation (Chapter 4) (Sia et al., 1998). Alternatively, Mih1 activity might be decreased in *cdc55Δ* cells, or remain inactive at times when it is normally active. Mih1 protein is present throughout the cell cycle, but becomes dephosphorylated at the same time that Swe1 is expressed (chapter 4, and lew ref). Although both Swe1 and Mih1 may be regulated by PP2A as has been shown in other systems (Clarke et al., 1993; Mueller et al., 1995), Figure 5 shows that Swe1 is hyperphosphorylated in *cdc55Δ*, and is a likely target of PP2A in budding yeast, and that phosphorylation activates Swe1. However, the opposite has been shown to be the case for the fission yeast and frog Wee1 proteins.

If PP2A dephosphorylates Swe1 directly, then *cdc55Δ* cells should have decreased PP2A activity. However, deletion of the catalytic subunits of PP2A, *pph21Δ pph22Δ*, suppresses the hyperpolarization of *cdc55Δ*. These inconsistencies may be because Swe1

is not the target of *CDC55*, but that its phosphorylation changes in response to lower activity of Mih1. A final possibility is that deletion of *CDC55* increases the phosphatase activity associated with Rts1, a B'-subunit of PP2A in budding yeast, and that increased Rts1 activity is responsible for some or all of the *cdc55Δ* phenotypes (Shu et al., 1997). This seems possible because overexpression of Rts1 causes hyperpolarization similar to *cdc55Δ*. Some of this confusion may be settled with the biochemical characterization of the recently discovered Z-complex, that contains Zds1, Cdc55 and other components of PP2A (Doug Kellogg, personal communication).

Figure 1 Clb proteins are stable in *cdc55Δ* cells treated with nocodazole

Wild type (ADR21), *mad2Δ* (ADR55), *cdc55Δ* (ADR496) and *cdc55Δ CDC28-VF* (ADR764) were grown overnight at 23°C in YPD to mid log phase, arrested in G1 with alpha factor (10μg/ml) for 2.5 hours and at t=0 the cells were released from the G1 arrest. At t=90, alpha factor (15μg/ml) was added back to the cultures to prevent re-accumulation of Clb proteins in cells that had exited from mitosis. Samples were taken at the indicated times, lysed and immunoblotted for Clb2, Clb3 and Tub1 proteins. Tub1 is shown as a loading control.

Figure 1

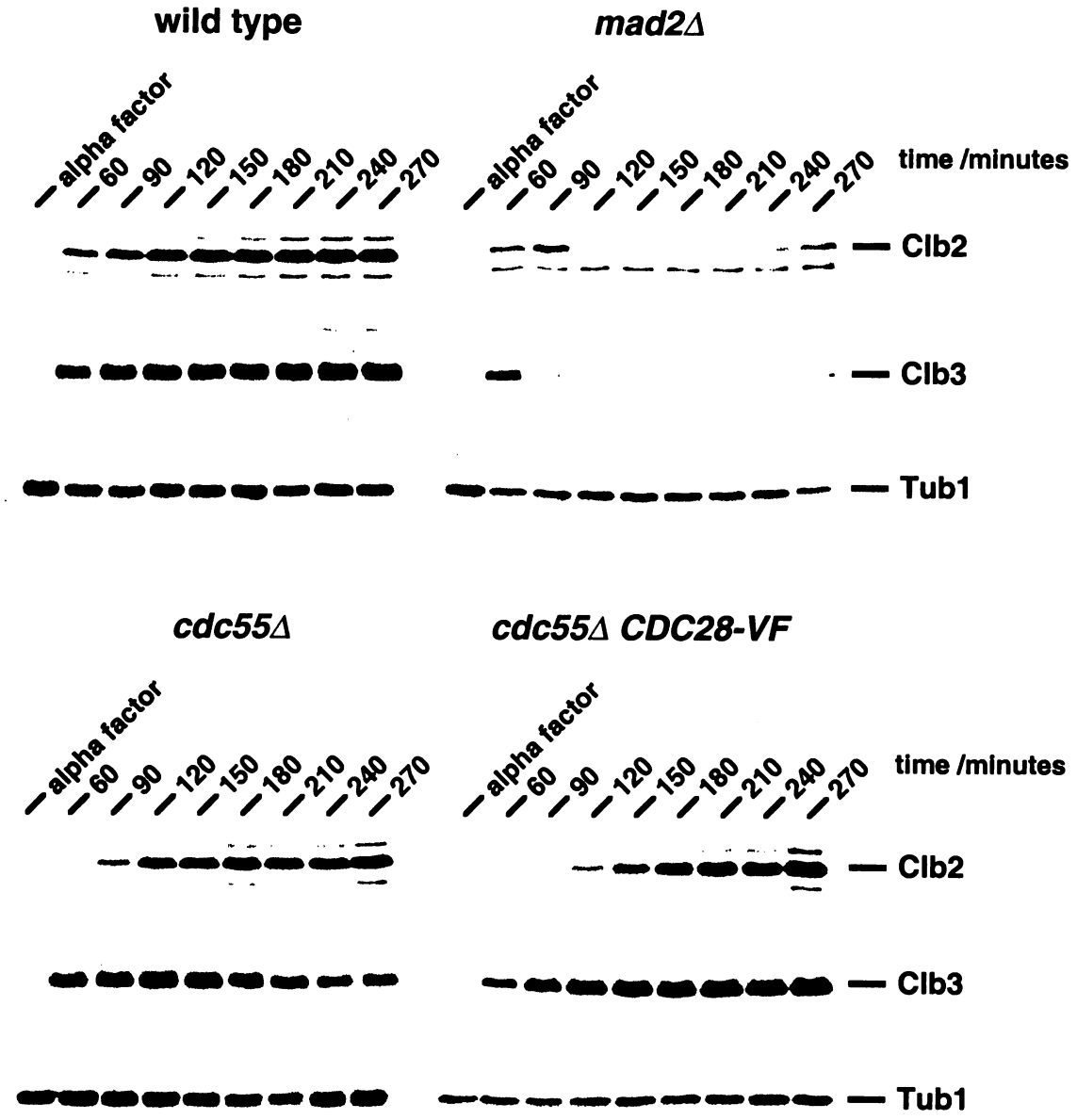

wild type
mad2Δ
alpha factor
60
90
120
150
180
210
240
270
time /minutes
Clb2
Clb3
Tub1
cdc55Δ
cdc55Δ CDC28-VF

Figure 2 Sister chromatids separate and Clb2-associated kinase activity falls in *cdc55Δ* cells

Wild type (JM478), *mad2Δ* (JM479), *cdc55Δ* (JM480) and *cdc55Δ CDC28-VF* (JM477) were grown overnight at 23°C in YPD to mid log phase, arrested in G1 with alpha factor (10μg/ml) for 2.5 hours and at t=0 the cells were released from the G1 arrest. For the last 30 minutes of the alpha factor arrest, cells were transferred to CSM-his+2% dextrose containing 10mM 3-aminotriazole and alpha factor (10μg/ml) to induce expression of *pHIS3-GFP-lacI*. At t=90, alpha factor (15μg/ml) was added back to the cultures to prevent re-accumulation of Clb proteins in cells that had exited from mitosis. Duplicate samples were taken at 30 minute intervals. One set was lysed, Clb2 immunoprecipitated from the samples and immunoprecipitates tested for their ability to phosphorylate histone H1. The kinase reactions were run on a 15% polyacrylamide gel and radiolabeled phosphate incorporation was measured using a Phosphorimager. The other set was washed, fixed and sister chromatid separation was scored by counting the number of fluorescent spots (one or two) in the nucleus of GFP-lacI bound to 256 tandem repeats of *lacO* integrated at the *TRP1* locus.

Figure 2

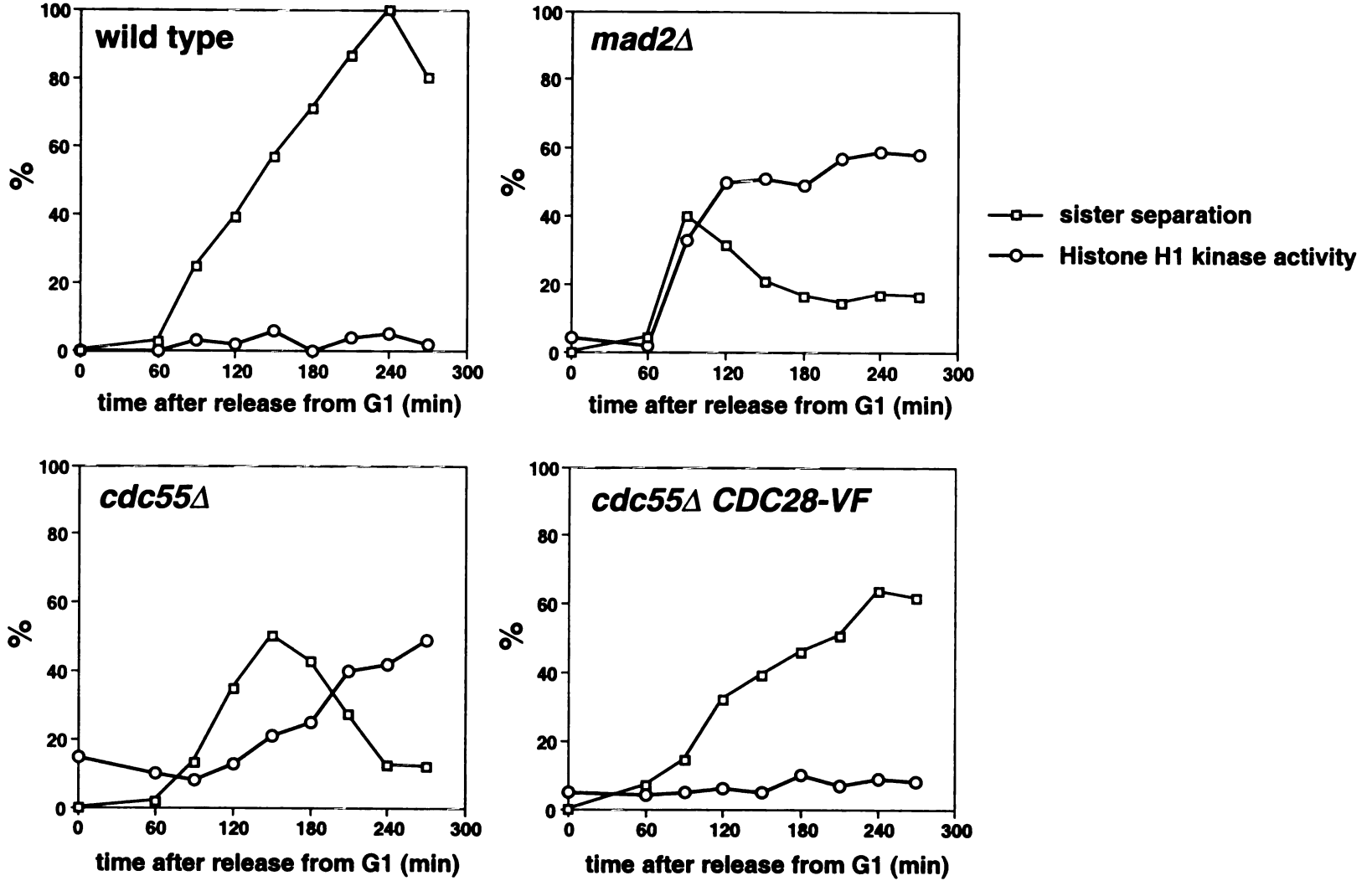

wild type
mad2Δ
cdc55Δ
cdc55Δ CDC28-VF
%
time after release from G1 (min)
0
20
40
60
80
100
0
60
120
180
240
300
sister separation
Histone H1 kinase activity

Figure 3 Clb2 is stable in *cdc55Δ mad2Δ* cells treated with nocodazole

Wild type (ADR21), *mad2Δ* (ADR55), *cdc55Δ* (ADR496) and *cdc55Δ mad2Δ* (ADR618) were grown overnight at 23°C in YPD to mid log phase, arrested in G1 with alpha factor (10μg/ml) for 2.5 hours and at t=0 the cells were released from the G1 arrest. At t=90, alpha factor (15μg/ml) was added back to the cultures to prevent re-accumulation of Clb proteins in cells that had exited from mitosis. Samples were taken at the indicated times, lysed and immunoblotted for Clb2.

Figure 3

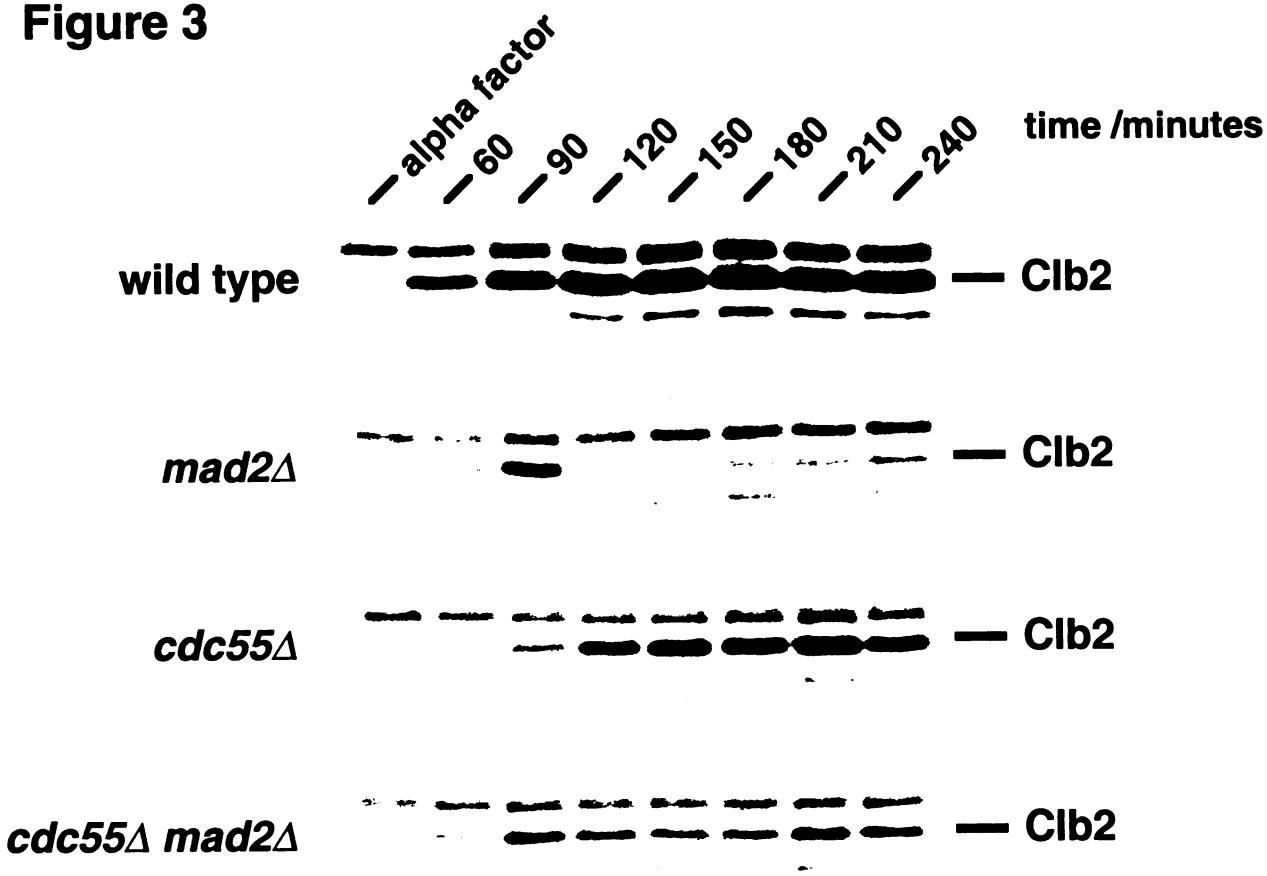
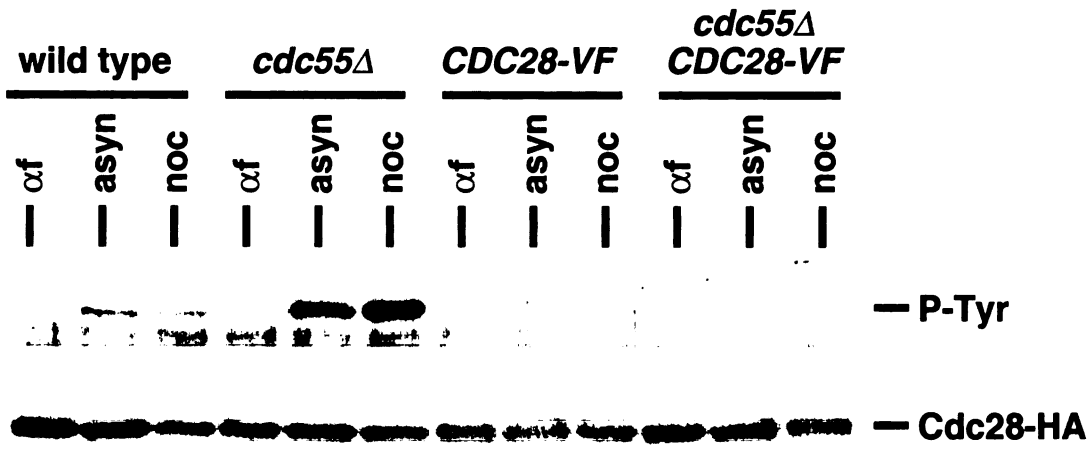

alpha factor
60
90
120
150
180
210
240
time /minutes
wild type
Clb2
mad2Δ
Clb2
cdc55Δ
Clb2
cdc55Δ mad2Δ
Clb2

Figure 4 Inhibitory phosphorylation on Cdc28 is increased in *cdc55Δ*

(A) Inhibitory phosphorylation in asynchronous and nocodazole treated cells. *CDC28-HA* (JM425), *CDC28-VF-HA* (JM434), *cdc55Δ CDC28-HA* (JM445) and *cdc55Δ CDC28-VF-HA* (ADR764) were grown overnight at 23°C in YPD to mid log phase. The cultures were split, and cells were arrested in G1 with alpha factor (10μg/ml), treated with nocodazole (10μg/ml), or grown asynchronously for 3 hours. The cells were harvested, lysed and Cdc28-HA was immunoprecipitated with the 12CA5 antibody. The immunoprecipitates were run out on a polyacrylamide gel and probed either with a phosphotyrosine antibody (top) or with 12CA5 (bottom) as a control for equal loading. (B) Inhibitory phosphorylation in S-phase arrested and anaphase arrested cells. (left) *CDC28-HA* (JM425) and *cdc55Δ CDC28-HA* (JM445) were grown overnight at 23°C in YPD to mid log phase. The cultures were split, and cells were arrested in S-phase with hydroxyurea (0.2M) or grown asynchronously for 3 hours. (right) *CDC28-HA pGAL-CLB2Δ176* (ADR777) and *cdc55Δ CDC28-HA pGAL-CLB2-Δ176* (ADR771) were grown overnight at 23°C in YEP+2% raffinose to mid log phase. The cultures were spilt and cells were transferred to YEP+2% galactose to induce expression of *pGAL-CLB2-Δ176* or fresh YEP+2% raffinose for 4 hours. The cells were then processed as described in part (A).

Figure 4

A


wild type
cdc55Δ
CDC28-VF
cdc55Δ CDC28-VF
αf
asyn
noc
P-Tyr
Cdc28-HA

B

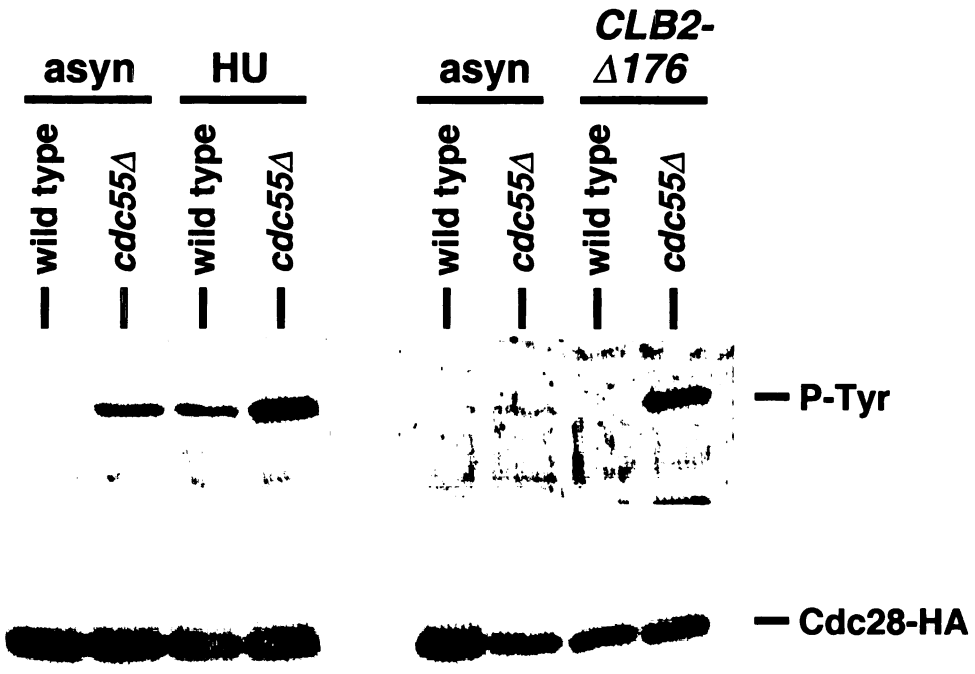

asyn
HU
asyn
CLB2-Δ176
wild type
cdc55Δ
P-Tyr
Cdc28-HA

Figure 5 Swe1 is hyperphosphorylated in *cdc55Δ* cells

(A) Swe1 runs at a slower mobility in *cdc55Δ*. Wild type (ADR21), *CDC28-VF* (JM434), *swe1Δ* (ADR601), *mih1Δ* (ADR877), *cdc55Δ::HIS3* (ADR496), *cdc55Δ::LEU2* (ADR472), *hct1Δ::HIS3* (ADR1340) and *hct1Δ::LEU2* (ADR1322) were grown overnight at 23°C in YPD to mid log phase, cells were harvested, lysed and immunoblotted for Swe1.

(B) Swe1 is phosphorylated. *pGAL-SWE1-HA* (ADR606) and *cdc55Δ pGAL-SWE1-HA* (ADR608) were grown overnight at 23°C in YEP+2% raffinose to mid log phase. The cells were transferred to YEP+2% galactose to induce *pGAL-SWE1-HA*, harvested, lysed and Swe1-HA was immunoprecipitaed with the 12CA5 antibody. Immunoprecipitates were then treated with or without lambda phosphatase.

Figure 5

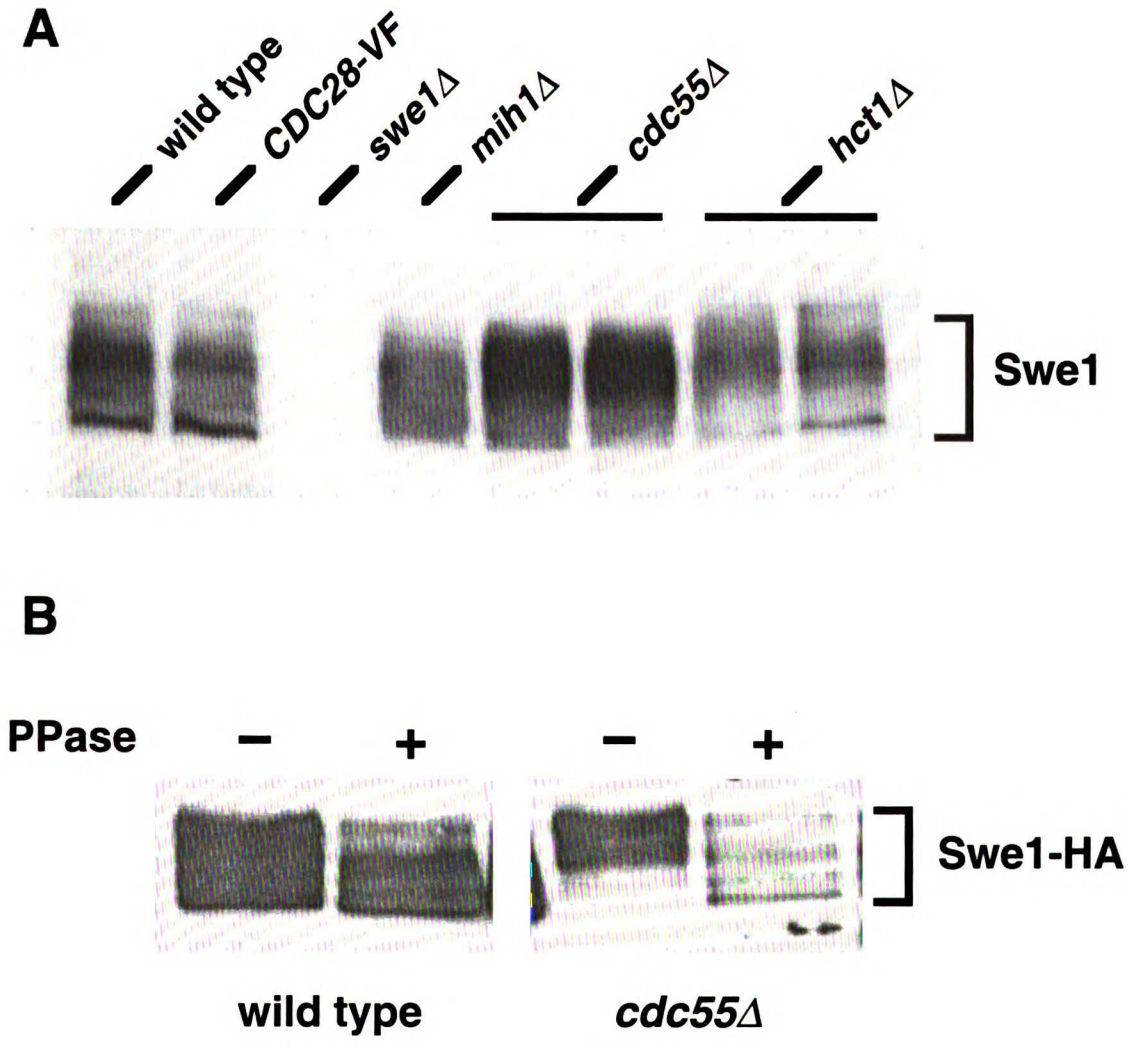
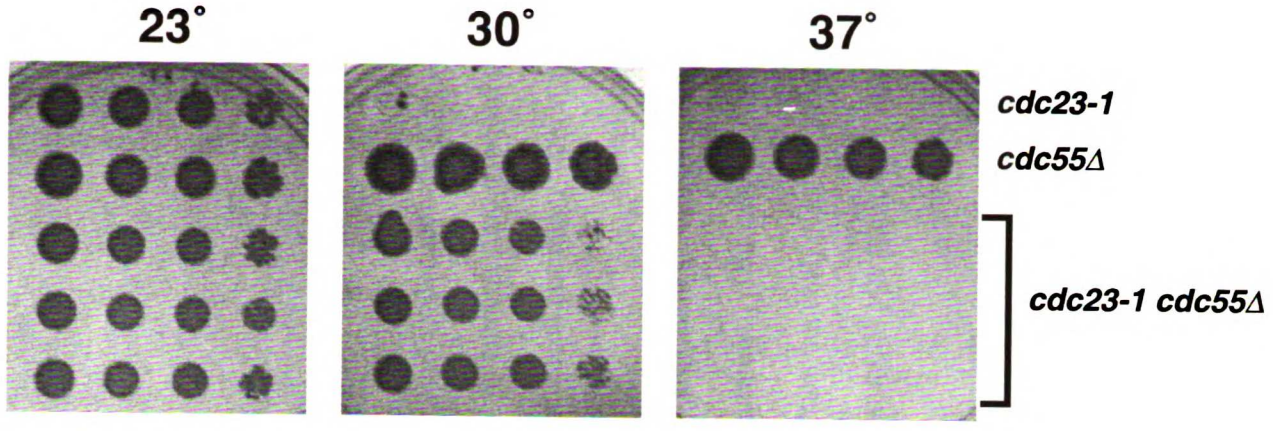

A
wild type
CDC28-VF
swe1Δ
mih1Δ
cdc55Δ
hct1Δ
Swe1
B
PPase
–
+
–
+
Swe1-HA
wild type
cdc55Δ

Figure 6 *cdc55Δ* suppresses *cdc23-1*

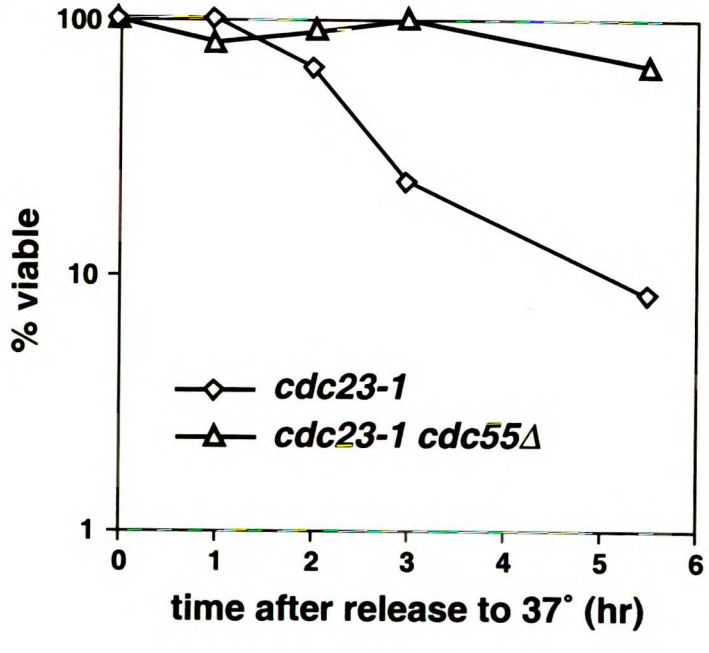
(A) *cdc55Δ cdc23-1* grows at 30°C. *cdc23-1* (ADR719), *cdc55Δ* (ADR496) and *cdc55Δ cdc23-1* (ADR725-7) were grown to saturation for two days in YEP+ 2% glucose at 23°C, diluted ten-fold and four-fold serial dilutions were prepared in a multi-well dish and spotted onto YEP+2% glucose plates. The plates were incubated at 23°C, 30°C and 37°C for 2-3 days.

(B) *cdc55Δ* suppresses *cdc23-1* and *cdc23-1* suppresses *cdc55Δ*. *cdc23-1* (—◇—, ADR719), *cdc55Δ cdc23-1* (—△—, ADR726), wild type (—□—, ADR21) and *cdc55Δ* (—○—, ADR496) were grown overnight at 23°C in YPD to mid log phase, and the cultures were transferred into fresh YPD at 37°C (left) or YPD at 23°C containing 10μg/ml nocodazole (right). Samples were harvested at the indicated times, diluted and plated onto YPD plates. The number of colonies at each time was compared with the number immediately before the shift to 37°C or nocodazole addition to determine the % viable cells.

Figure 6 **A**


23°
30°
37°
cdc23-1
cdc55Δ
cdc23-1 cdc55Δ

B


100
10
1
% viable
cdc23-1
cdc23-1 cdc55Δ
0
1
2
3
4
5
6
time after release to 37° (hr)

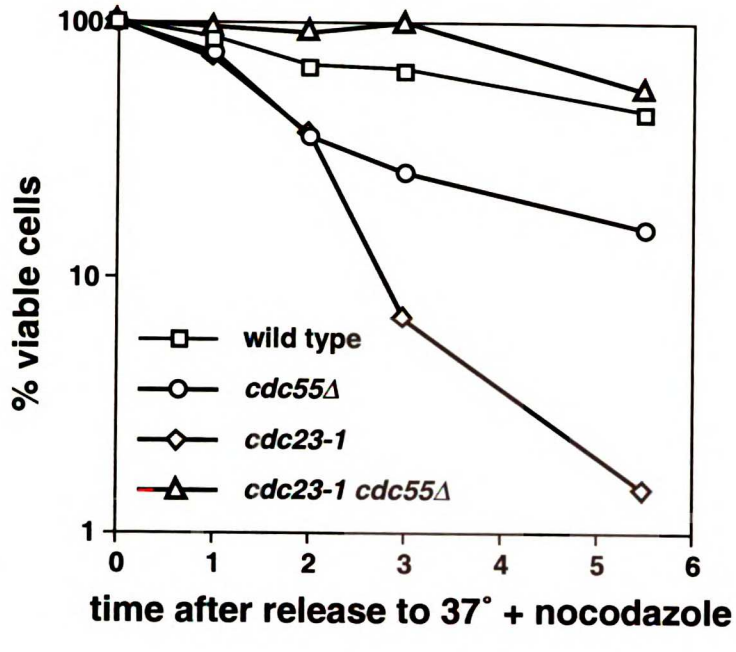

100
10
1
% viable cells
wild type
cdc55Δ
cdc23-1
cdc23-1 cdc55Δ
0
1
2
3
4
5
6
time after release to 37° + nocodazole

Figure 7 APC activity is required for sister chromatid separation

pGAL-SIC1-mut (—□—, ADR813) and *cdc23-1 pGAL-SIC1-mut* (—○—, ADR628) were grown overnight at 23°C in YEP+2% raffinose to mid log phase and arrested in mitosis with nocodazole (10μg/ml) at 23°C for 4 hours. During the last hour of the nocodazole arrest the cultures were split and shifted to 26°C (left) or 35°C (right). At t=0, 2% galactose and 10μg/ml alpha factor was added to the cultures. Cells were harvested at one hour intervals.

(A) Cells were lysed and either immunoblotted for Clb2 protein (top) or Clb2 was immunoprecipitated from the samples and immunoprecipitates tested for their ability to phosphorylate histone H1 (bottom).

(B) The phosphate incorporation into histone H1 in (A) was measured using a Phosphorimager.

(C) Cells were fixed and processed for fluorescent in situ hybridization (FISH) using a cosmid probe to chromosome III. Sister chromatid separation was scored by counting the number of fluorescent spots (one or two) in each nucleus.

Figure 7

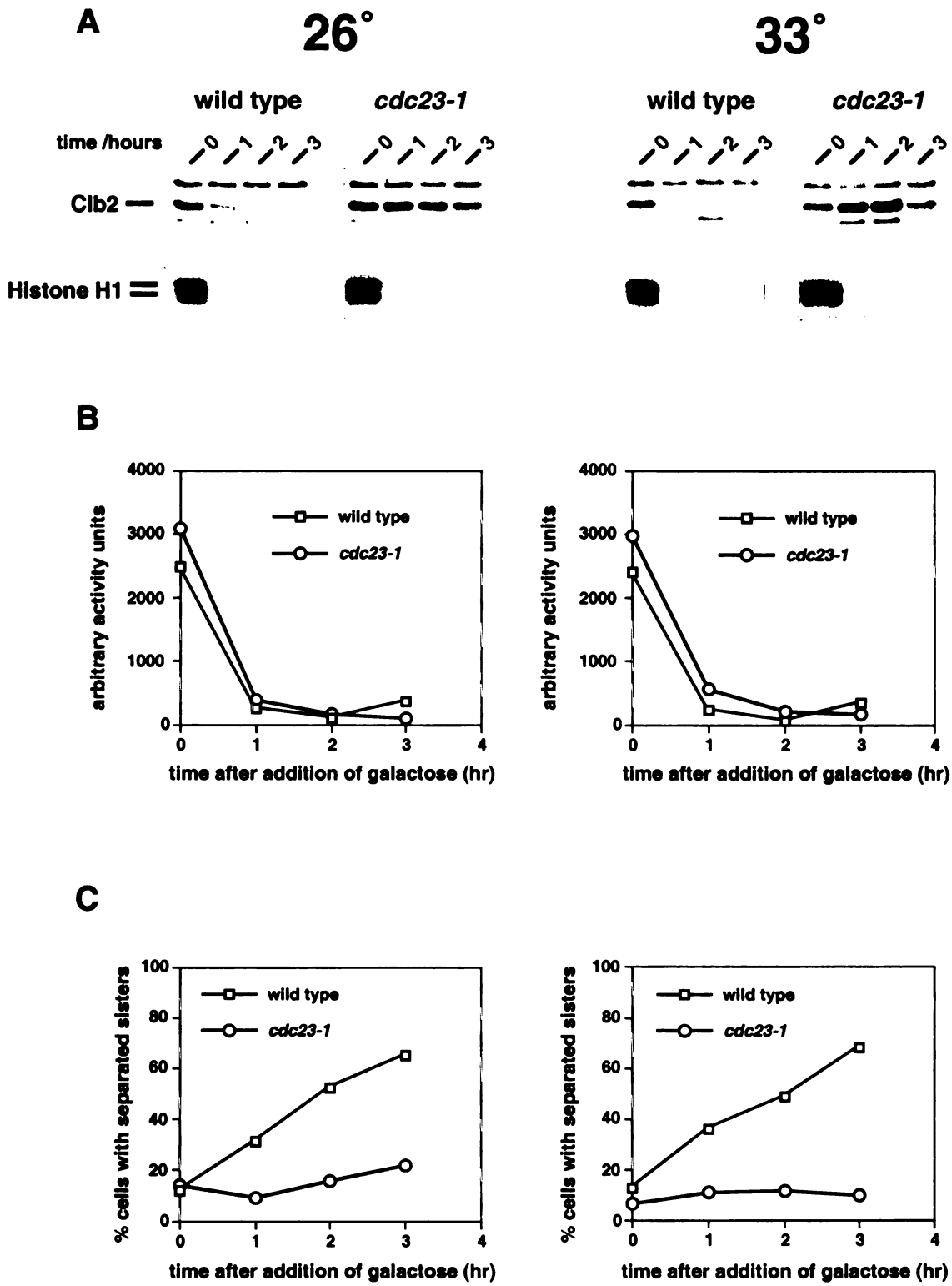

A
26°
33°
wild type
cdc23-1
wild type
cdc23-1
time /hours
0 1 2 3
0 1 2 3
0 1 2 3
0 1 2 3
Clb2
Histone H1
B
arbitrary activity units
4000
3000
2000
1000
0
wild type
cdc23-1
0 1 2 3 4
time after addition of galactose (hr)
arbitrary activity units
4000
3000
2000
1000
0
wild type
cdc23-1
0 1 2 3 4
time after addition of galactose (hr)
C
% cells with separated sisters
100
80
60
40
20
0
wild type
cdc23-1
0 1 2 3 4
time after addition of galactose (hr)
% cells with separated sisters
100
80
60
40
20
0
wild type
cdc23-1
0 1 2 3 4
time after addition of galactose (hr)

Figure 8 Pds1 is degraded when *cdc55Δ* cells exit mitosis inappropriately

All strains contain *PDS1-HA*. Wild type (ADR1002), *mad2Δ* (ADR998), *cdc55Δ* (ADR994) and *cdc55Δ CDC28-VF* (ADR1097) were grown overnight at 23°C in YPD to mid log phase, arrested in G1 with alpha factor (10μg/ml) for 2.5 hours and at t=0 the cells were released from the G1 arrest. At t=90, alpha factor (15μg/ml) was added back to the cultures to prevent re-accumulation of Clb proteins in cells that had exited from mitosis. Samples were taken at the indicated times, lysed and immunoblotted for Clb2, Sic1 and Pds1-HA.

Figure 8

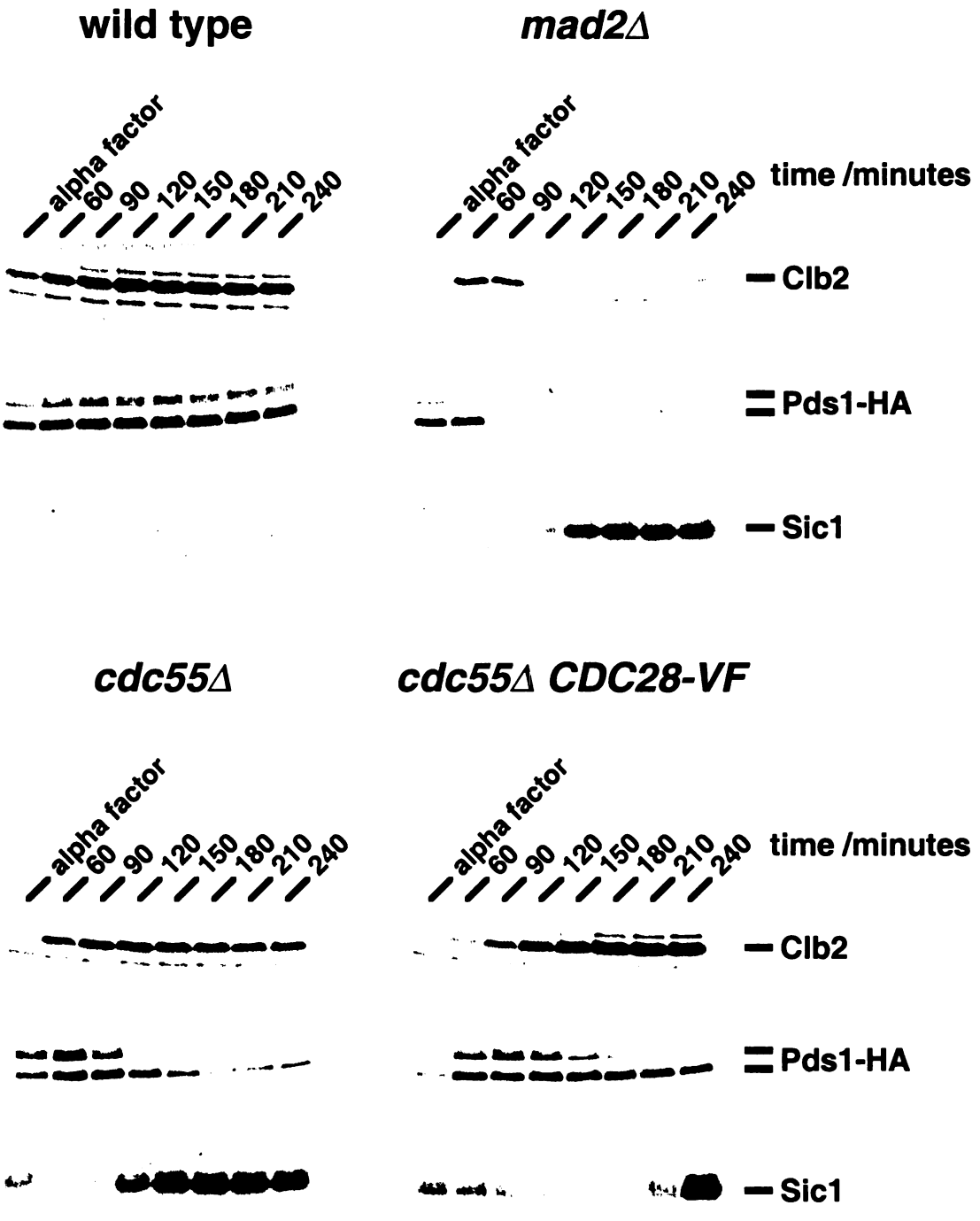

wild type
mad2Δ
alpha factor
60
90
120
150
180
210
240
time /minutes
Clb2
Pds1-HA
Sic1
cdc55Δ
cdc55Δ CDC28-VF

Figure 9 The APC is hyperphosphorylated in *cdc55Δ*

(A) *cdc55Δ cdc15-2* (ADR829), *mad1Δ cdc15-2* (ADR76), *cdc15-2* (K1993) and *cdc26Δ* (LH307) were grown overnight in YEP+2% glucose at 23°C to log phase and then arrested in anaphase (or metaphase for *cdc26Δ*) by shifting the cultures to 37°C for 4 hours. Cells were then transferred to phosphate free-CSM+2% glucose containing $^{32}PO_4$, at 37°C. After 1 hour cells were harvested, lysed and the APC immunoprecipitated with anti-Cdc26 antibody. Immunoprecipitates were run on a polyacrylamide gel which was subjected to either autoradiography (top) or western blotting (bottom).

(B) *CDC16-6A* does not suppress *cdc55Δ*. Wild type (—□—, ADR21), *CDC16-6A* (—○—, ADR2029), *cdc55Δ* (—△—, ADR496) and *cdc55Δ CDC16-6A* (—◆—, ADR2071) were grown to saturation for two days in YEP+ 2% glucose at 30°C, diluted ten-fold and four-fold serial dilutions were prepared in a multi-well dish and spotted onto YEP+2% glucose plates containing or not containing 7.5μg/ml benomyl. The plates were incubated at 23°C for 2-3 days.

(C) The same strains as in (B) were grown overnight at 23°C in YPD to mid log phase, and the cultures were transferred into fresh YPD containing 10μg/ml nocodazole. Samples were harvested at the indicated times, diluted and plated onto YPD plates. The number of colonies at each time was compared with the number immediately before nocodazole addition to determine the % viable cells.

Figure 9

A

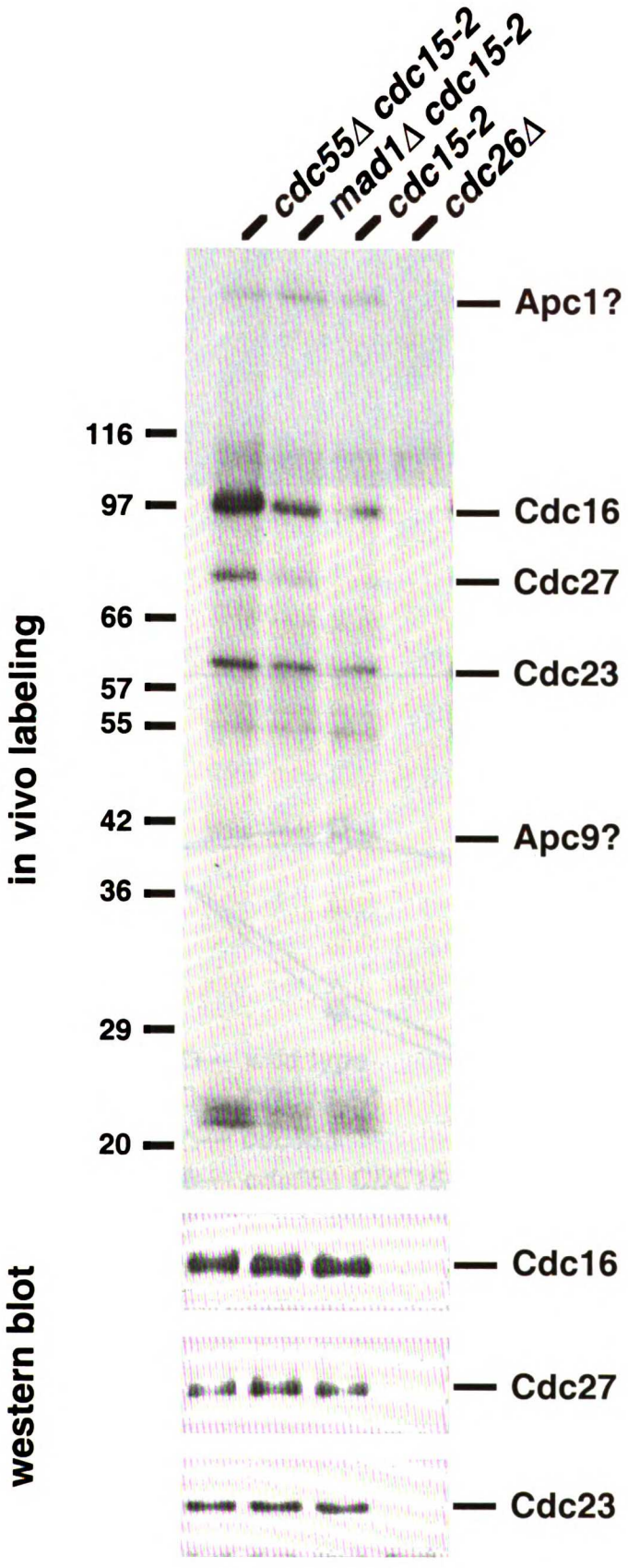
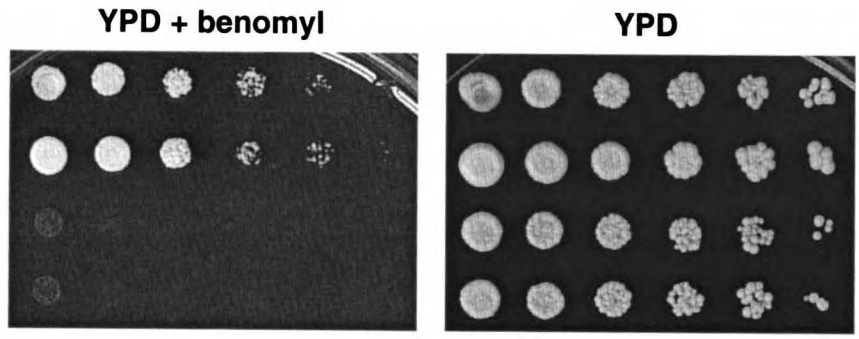
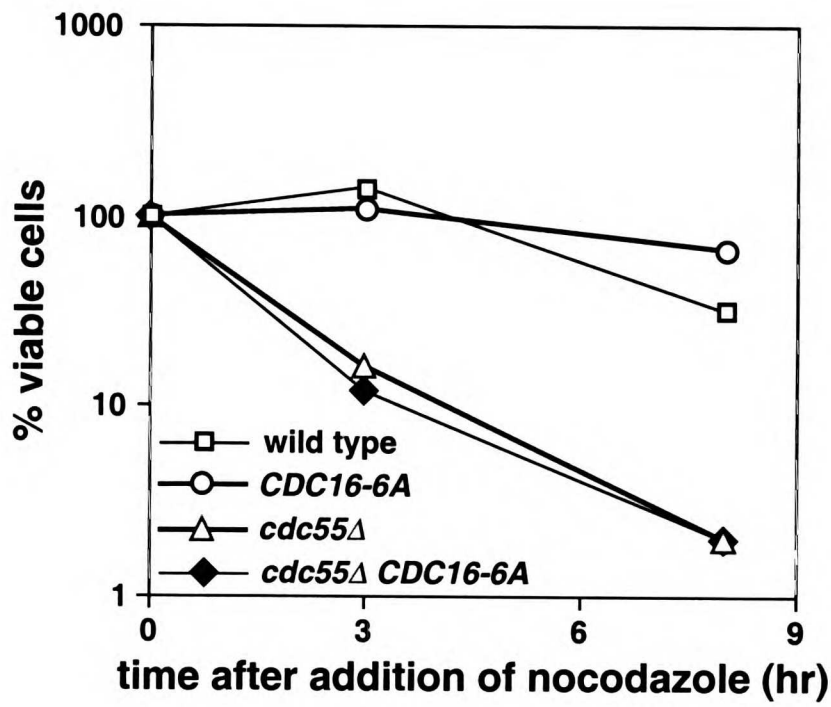

cdc55Δ cdc15-2
mad1Δ cdc15-2
cdc15-2
cdc26Δ
in vivo labeling
116
97
66
57
55
42
36
29
20
Apc1?
Cdc16
Cdc27
Cdc23
Apc9?
western blot
Cdc16
Cdc27
Cdc23

Figure 9

B


YPD + benomyl
YPD
wild type
CDC16-6A
cdc55Δ
cdc55Δ CDC16-6A

C


1000
100
10
1
% viable cells
wild type
CDC16-6A
cdc55Δ
cdc55Δ CDC16-6A
0
3
6
9
time after addition of nocodazole (hr)

Chapter 4

Inhibitory phosphorylation of Cdc28 blocks APC activation

Introduction

Inhibitory phosphorylation on Cdc2 is required for the DNA damage and replication checkpoints in most organisms. Phosphorylation of Cdc2 by the Wee1 and Mik1 kinases prevents its full activation and blocks cells before entry into mitosis (Lundgren et al., 1991). Dephosphorylation of Cdc2 by Cdc25 rapidly activates Cdc2 and drives cells into mitosis (Coleman and Dunphy, 1994). Regulation of Cdc2 in fission yeast by *wee1* and *cdc25* is also responsible for ensuring that cells enter mitosis at the appropriate cell size (Nurse, 1975). Although homologues of wee1 (Swe1) and cdc25 (Mih1) exist in budding yeast, inhibitory phosphorylation of Cdc28 is not required for the DNA damage checkpoint (Amon et al., 1992a; Booher et al., 1993; Russell et al., 1989; Sorger and Murray, 1992).

Swe1 and inhibitory phosphorylation of Cdc28 are required for the morphogenesis checkpoint, which prevents passage through mitosis if cells haven't budded, or in response to osmotic shock or actin cytoskeleton depolymerization (Lew and Reed, 1995). This checkpoint appears to delay both entry into mitosis and exit from mitosis, though it is unclear how Swe1 slows both these events.

Inhibitory phosphorylation also influences the switch from polarized to isotropic growth. Polarized growth occurs when cells first bud, and Cdc28 is complexed primarily with Cln cyclins, and Cdc28/Clb kinase activity is low. When Cdc28/Clb activity increases, growth becomes isotropic (Lew and Reed, 1993). Mutations that arrest cells with high Cln-associated kinase activity (*cdc34*) (Dahmann et al., 1995) or have decreased mitotic Cdc28 activity (*cdc28-1N, cks1-ts, clb1-4Δ*) are hyperpolarized (Fitch et al., 1992; Surana et al., 1991; Tang and Reed, 1993). In addition, mutants that have increased inhibitory phosphorylation, like *cdc55Δ* and *elm1*, are also hyperpolarized (Wang and Burke, 1997). Consistent with this finding, *swe1Δ* cells are partially defective in pseudo-hyphal growth, an alternate pattern of growth that involves extended periods of polarized growth after budding (Ahn et al., 1999).

Swe1 is also required for the pachytene checkpoint during meiosis. This checkpoint senses whether or not homologous chromosomes have completed meiotic recombination (Leu and Roeder, 1999). Components of the DNA damage checkpoint are required for arrest at the pachytene checkpoint, so it is possible that this checkpoint is quite similar to the DNA damage checkpoint in fission yeast and other organisms (Lydall et al., 1996).

The work presented in Chapter 4 and in Appendix 1 rested on the assumption that inhibitory phosphorylation of Cdc28 promoted mitotic exit. Although I no longer believe that this is true, I became interested in whether inhibitory phosphorylation of Cdc28 plays any role during mitosis. Below I show that inhibitory phosphorylation peaks during mitosis, and that modifications on Swe1 and Mih1 also occur during mitosis. High levels of inhibitory phosphorylation during mitosis prevents activation of the Cdc20-dependent APC, suggesting that rather than promoting mitotic exit, inhibitory phosphorylation blocks mitotic exit.

Results

Inhibitory phosphorylation on Cdc28 peaks in mitosis

Previous work has showed that inhibitory phosphorylation on Cdc28 is high during an S-phase arrest caused by hydroxyurea (HU), and very low in a G1 or mitotic arrest caused by the mating pheromone, alpha factor, or the microtubule poison, nocodazole, respectively (Amon et al., 1992a; Sorger and Murray, 1992). This data is supported by examining inhibitory phosphorylation in arrests caused by *cdc* mutants. G1 mutants (*cdc28-4*) and mitotic mutants (*cdc23-1*) arrest with undetectable inhibitory phosphorylation on Cdc28, while S phase or DNA damage induced arrests (*cdc9* and *cdc13*) have high levels of inhibitory phosphorylation. To obtain a higher resolution picture of how inhibitory phosphorylation on Cdc28 changes during the cell cycle, I examined the amount of phopsho-tyrosine present on immunoprecipitates of Cdc28 that was isolated from cells traversing the cell cycle. Wild type and *cdc55Δ* cells were arrested in G1 with alpha factor and then released into fresh medium. Alpha factor was re-added after cells had budded to re-arrest cells in the subsequent G1. Inhibitory phosphorylation in wild type cells peak at the same time as Clb2 levels and Clb2-associated kinase activity (t=80 minutes) (Figure 1). Although the quality of data is poor, the peak of tyrosine phosphorylated Cdc28 is clear, and the background is due only to keratin polymerized into the polyacrylamide gel (data not shown). *cdc55Δ* cells reach higher maximal levels of tyrosine phosphorylated Cdc28, but similar to wild type, these levels peak with Clb2 and Clb2-associated kinase activity (t=100 minutes). Similar results have been seen in fission yeast, though little emphasis was placed on the coincidence of high inhibitory phosphorylation and Cdc13 protein (the major B-type cyclin in fission yeast) (Hayles and Nurse, 1995). Although high levels of inhibitory phosphorylation and high levels of Clb2-associated kinase activity may seem paradoxical, I do not know the percentage of Cdc28

molecules that are tyrosine phosphorylated. In addition, studies in fission yeast have suggested that tyrosine phosphorylated Cdc2 retains substantial activity (Stern and Nurse, 1996).

I then investigated whether Swe1 and Mih1, the kinase and phosphatase responsible for regulating tyrosine phosphorylation on Cdc28, might show a similar profile of expression or modification as tyrosine phosphorylation of Cdc28. A similar timecourse as described above was performed in a wild type strain that contained an epitope tagged Mih1. Swe1 protein was also monitored in this strain using a rabbit polyclonal anti-Swe1 antibody. Mih1 protein is present throughout the cell cycle, but as Clb2 levels rise and peak, Mih1 runs with a faster mobility. Because Cdc25, the Mih1 homologue in other organisms, is highly modified by phosphorylation, we believe that these mobility changes are due to phosphorylation(Coleman and Dunphy, 1994). Swe1 protein is not present in alpha factor arrested cells, and is first seen at the same time that Clb2 is present, and as Clb2 accumulates, Swe1 runs at a slower mobility. As Clb2 peaks, Swe1 rapidly disappears. The highly modified slower migrating form is due to phosphorylation (see Chapter 4, Figure 5B)

Recently it has been shown that Swe1 transcription and stability are regulated. Transcription occurs only during S-phase and early mitosis, and Swe1 degradation is mediated by the SCF. Like Sic1, the CDK inhibitor, Swe1 is destabilized by high levels of Cdc28-associated kinase activity (Sia et al., 1998; Verma et al., 1997). This is consistent with the rapid disappearance of Swe1 after Clb2 levels peak. Comparing the profiles in Figures 1 and 2 suggests that hyper-phosphorylated Swe1 is more active and de-phosphorylated Mih1 is less active. However, Swe1 protein disappears before tyrosine phosphorylation peaks, so it is also possible that hyperphosphorylated Swe1 is inactive, but Mih1 is inactivated before Swe1, so net tyrosine phosphorylation of Cdc28 rises until Mih1 becomes phosphorylated and re-activated. Some phosphorylations on fission yeast and frog Cdc25 are activating, and others inhibitory, so no conclusions can be made until a

more detailed biochemical analysis is performed (Kumagai and Dunphy, 1996; Kumagai et al., 1998).

As shown in Chapter 1, *mih1Δ* cells have levels of inhibitory phosphorylation on Cdc28 higher than seen in wild type cells, and no phosphorylation is detected in *swe1Δ* cells. Both *mih1Δ* and *swe1Δ* cells are healthy, but I wondered if there might be any consequence of the increased levels of inhibitory phosphorylation on Cdc28 in *mih1Δ* cells. A similar time course was performed as described above, and a small, but reproducible, 10-15 minute delay in the accumulation of Clb2 is seen in *mih1Δ* cells (figure 3). There is a greater than 15 minute delay in the fall of Clb2, suggesting that these cells may also delay mitotic exit. This small delay is abolished in a *swe1Δ mih1Δ* cell suggesting that this delay is due solely to the increased amount of inhibitory phosphorylation on Cdc28. wild type and *swe1Δ* cells traverse the cell cycle with similar kinetics as *swe1Δ mih1Δ* (Figure 3 and data not shown). The delay in accumulation of Clb2 is most likely due to a delay in the activation of *CLB2* transcription which is positively regulated by Cdc28-associated activity (Amon et al., 1993).

A second phenotype of mih1Δ is its synthetic lethality in combination with *cdc55Δ* and *hct1Δ*. Both synthetic lethalities are rescued by *swe1Δ* (data not shown), supporting the idea that all *mih1Δ* phenotypes are caused by increased inhibitory phosphorylation on Cdc28.

Overexpression of Swe1 and Sic1 have different effects

Rapid inhibition of Cdc28 during a spindle checkpoint arrest activates APC-dependent proteolysis of Clb proteins, triggers sister chromatid separation and the exit from mitosis (amon and Chapter 3, Figure 7). I therefore wondered whether high levels of Swe1 might have the same effect (described in Chapter 3). wild type cells were arrested in mitosis by nocodazole which activates the spindle checkpoint, and high levels of Sic1-mut

(a non-degradable form of Sic1), or high levels of Swe1 were expressed from the inducible *GAL1* promoter by addition of galactose. Cells which express neither Sic1 or Swe1 remain arrested with high levels of Clb2 and Clb2-associated kinase activity (Figure 3A and B), and unseparated sister chromatids (data not found). Overexpression of Sic1-mut causes a rapid fall in Clb2-associated kinase activity and Clb2 protein, and triggers sister separation (Figure 3A and 3B, and Chapter 3, Figure 7). Overexpression of Swe1 causes a more modest fall in Clb2-associated kinase activity (to 70% of the starting level), but does not trigger Clb2 degradation or sister separation (Figure 3A and 3B, and data not found). This experiment suggests that the drop in Clb2-associated kinase activity is insufficient to activate Hct1 and Sic1.

Overexpression of Swe1 blocks APC activation

Overexpression of Swe1 is unable to override the spindle checkpoint as Sic1 can, but are there any consequences of high levels of inhibitory phosphorylation during mitosis? In order to test this, an experiment similar to the one described above was performed, but after one hour of Swe1 expression, the cells were released from the nocodazole arrest and allowed to proceed into G1. In cells which do not overexpress Swe1, Clb2 and Clb3 protein, and Clb2-associated kinase activity falls between 120 and 150 minutes after the nocodazole is washed out (figure 4A and 4B). In cells that overexpress Swe1, Clb2-associated kinase activity falls immediately after induction of Swe1, and then falls a small amount more after the nocodazole has been washed out, but remains between 10 and 20% of starting level. Surprisingly, Clb2 and Clb3 protein remain completely stable throughout this time course (Figure 4A). We don't believe that high levels of Swe1 would interfere with release from nocodazole arrest, especially because the spindle checkpoint is thought to only function during periods of high Cdc28 activity (Hardwick and Murray, 1995). We therefore believe that high levels of inhibitory phosphorylation can prevent APC activation.

This presumably effects the Cdc20-dependent APC, and not the Hct1-dependent APC, which if anything should be activated when Cdc28 is inhibited by phosphorylation.

Cdc28 phosphorylates and activates the Cdc20-dependent APC (see Chapter 2), so we wondered if high levels of inhibitory phosphorylation on Cdc28 causes dephosphorylation of the APC. The above experiment was repeated, this time in a strain that is *mih1Δ* and overexpresses Swe1. In addition to examining Clb levels, I examined the phosphorylation state of Cdc27, one of the phosphorylated APC subunits. In cells that don't overexpress Swe1, Cdc27 becomes dephosphorylated slowly as Clb proteins are degraded (Figure 5, t=165 minutes). In cells that express high levels of Swe1, Cdc27 rapidly becomes dephosphorylated after induction of Swe1, and remains dephosphorylated throughout the time course. Given the results presented in Chapter 2, this dephosphorylation may contribute to the inability of these cells to activate the Cdc20-dependent APC.

One possible explanation for the stability of Clb proteins in the above experiment is that tyrosine phosphorylated Cdc28/Clb complexes cannot be recognized by the APC. We don't believe this to be the case because co-overexpression of Swe1 and Sic1-mut in the above experiment causes rapid degradation of Clb proteins in a manner similar to overexpression of Sic1-mut alone (fig 6A). In addition, co-overexpression of Swe1 and Clb2 in alpha factor arrested cells, does not lead to accumulation of Clb2 protein (fig 6B). Both these experiments have flaws -- accumulation of Sic1 might happen sooner than Swe1 in figure 6A, and Swe1 doesn't express highly in alpha factor arrested cells, but together, they argue against a model whereby tyrosine phosphorylation itself protects Clb proteins from degradation.

Discussion

The data presented above are preliminary, but suggest that high levels of inhibitory phosphorylation might delay exit from mitosis by preventing phosphorylation of the APC. In most organisms high levels of inhibitory phosphorylation block mitotic entry, not mitotic exit. This is true in budding yeast as well. When Swe1 is expressed to high levels in G1 cells that are then allowed to traverse in the cell cycle, they show delays in the accumulation of Clb2 and Clb2-associated activity, and can arrest at a point before spindle pole body separation and spindle assembly, an arrest similar to what is seen in other organisms (Lim et al., 1996; Lin and Arndt, 1995). Therefore inhibitory phosphorylation on Cdc28 can delay mitotic exit and mitotic entry in budding yeast.

In vivo relevance?

Although mitotic entry (Lim et al., 1996; Lin and Arndt, 1995) and mitotic exit (this study) can be prevented by manipulation of Swe1, is there any in vivo relevance to these findings? The morphogenesis checkpoint is the one checkpoint in mitotically growing cells that depends on Swe1 (Lew and Reed, 1995). Although Swe1 is the target of this checkpoint, the downstream targets that halt cell cycle progression in arrested cells have not been clearly identified (McMillan et al., 1998). Typically, it is assumed that inhibition of Cdc28 is the explanation, and that this checkpoint functions like the DNA damage and replication checkpoints in fission yeast. But activation of the morphogenesis checkpoint, although it delays the accumulation of Clb protein and associated kinase activity, eventually arrests cells in mitosis and delay anaphase. This physiology could be explained if both mitotic entry and mitotic exit were prevented by high levels of inhibitory phosphorylation. This proposal also explains why *CDC28-F* cells are not as checkpoint defective as *swe1Δ* cells (McMillan et al., 1999). *CDC28-F*, in addition to being resistant to inhibitory

phosphorylation, are somewhat defective in phosphorylating and activating the Cdc20-dependent APC. Thus, its checkpoint defect is partially canceled out by its APC activation defect.

In most organisms inhibitory phosphorylation on Cdc2 is required for the DNA damage and replication checkpoints (Rhind et al., 1997; Rhind and Russell, 1998). These checkpoints arrest cells before mitotic entry. This phosphorylation is not required in budding yeast, and although this lack of conservation is surprising, an explanation has been that because S-phase and mitosis occur simultaneously in budding yeast, inhibition of Cdc28 would inhibit both processes. In budding yeast the primary target of the DNA damage checkpoint appears to be the anaphase inhibitor Pds1, and the checkpoint prevents sister chromatid separation and Clb proteolysis (Cohen-Fix and Koshalnd, 1997; Sanchez et al., 1999) The data presented above shows that inhibitory phosphorylation can prevent APC activation, so it now seems plausible to suggest that this phosphorylation might contribute to DNA damage checkpoint arrest.

This idea is reinforced by the following observations: 1. Recently it has been shown that the budding yeast Chk1 homologue plays a small role in promoting checkpoint arrest (Sanchez et al., 1999). Chk1 is required for DNA damage checkpoint arrest and phosphorylates and inhibits Cdc25 in fission yeast, frogs and mammalian cells, but in budding yeast it is proposed to phosphorylate Pds1, not Mih1 (Furnari et al., 1999; Furnari et al., 1997; Kumagai et al., 1998). Sasnchez et al. did not consider the possibility that teh budding yeast Chk1 might phosphorylate Mih1 and have the same function in budding yeast as in other organisms. 2. Although Swe1 is not required for DNA damage checkpoint arrest, inhibitory phosphorylation is induced to high levels in cells containing DNA damage (Amon et al., 1992a; Sorger and Murray, 1992). These cells bud normally, so there is no reason to believe that the morphogenesis checkpoint has been activated. 3. Mutations in CDC5 promote arrest by the DNA damage checkpoint (Sanchez et al., 1999). In frog extracts the Cdc5 homologue, Plx1, is the major activating kinase of Cdc25, and

therefore is a potential target of the DNA damage checkpoint (Kumagai and Dunphy, 1996). In budding yeast, Cdc5 is primarily involved in activating the Hct1-depndent APC at the end of mitotis, but may also activate the Cdc20-dependent APC (Charles et al., 1998; Toczyski et al., 1997). No one has investigated whether Cdc5 may activate Mih1 as well.

Cdc2 and Plx1 both regulate Cdc25 and the APC

The results of this chapter, Chapter 2 and numerous studies in other organisms suggest that Cdc28 and Cdc5 may both activate Mih1 (Cdc25) and the APC. The activation of both Cdc25 and the APC has been proposed to require the small Cdk binding protein Cks1 (Suc1) (Patra et al., 1999). In budding yeast, Cdc5 is also able to bind Cks1 beads and this binding is independent of APC binding to Cks1 beads (see Appendix 2) and is independent of Cdc28 binding to Cks1 beads (data not shown). The dual regulation of Cdc25 by these two kinases has been shown to be essential for the rapid exponential activation of the Cdc25 in an in vitro oocyte maturation assay (Karaiskou et al., 1999). The activation proceeds in three steps. Linear Cks1-independent phosphorylation of Cdc25 by Cdc2 slowly activates Cdc25, the small amount of phosphorylation then allows further activation of Cdc25 by Plx1, and this phosphorylation then allows exponential Cks1-dependent phosphorylation by Cdc2. Such a scheme may be used to activate the Cdc20-dependent APC, although the different kinases may target different sub-units of the APC, rather than different phosphorylation sites on the same sub-unit (see Chapter 2). Activation of Cdc25 triggers entry into mitosis and activation of the APC triggers mitotic exit. The data presented in this chapter also ensures dependency between these two events. The APC cannot be activated until Cdc28 becomes dephosphorylated, thus ensuring that mitotic entry precede mitotic exit.

Figure 1 Inhibitory phosphorylation on Cdc28 peaks in mitosis

CDC28-HA (JM425) and *cdc55Δ CDC28-HA* (JM445) were grown overnight at 30°C in YPD to mid log phase, arrested in G1 with alpha factor (10μg/ml) and at t=0 the cells were released from the G1 arrest. At t=60, alpha factor (15μg/ml) was added back to the cultures to re-arrest the cells in the next G1. Samples were taken at the indicated times, lysed and immunoblotted for Clb2, or Ccc28-HA and Clb2 were immunoprecipitated. Cdc28-HA was immunoprecipitated with the 12CA5 antibody and the immunoprecipitates were run out on a polyacrylamide gel and probed either with a phosphotyrosine antibody or with 12CA5 as a control for equal loading. Clb2 was immunoprecipitated with a Clb2 antibody and the immunoprecipitates tested for their ability to phosphorylate histone H1.

Figure 1

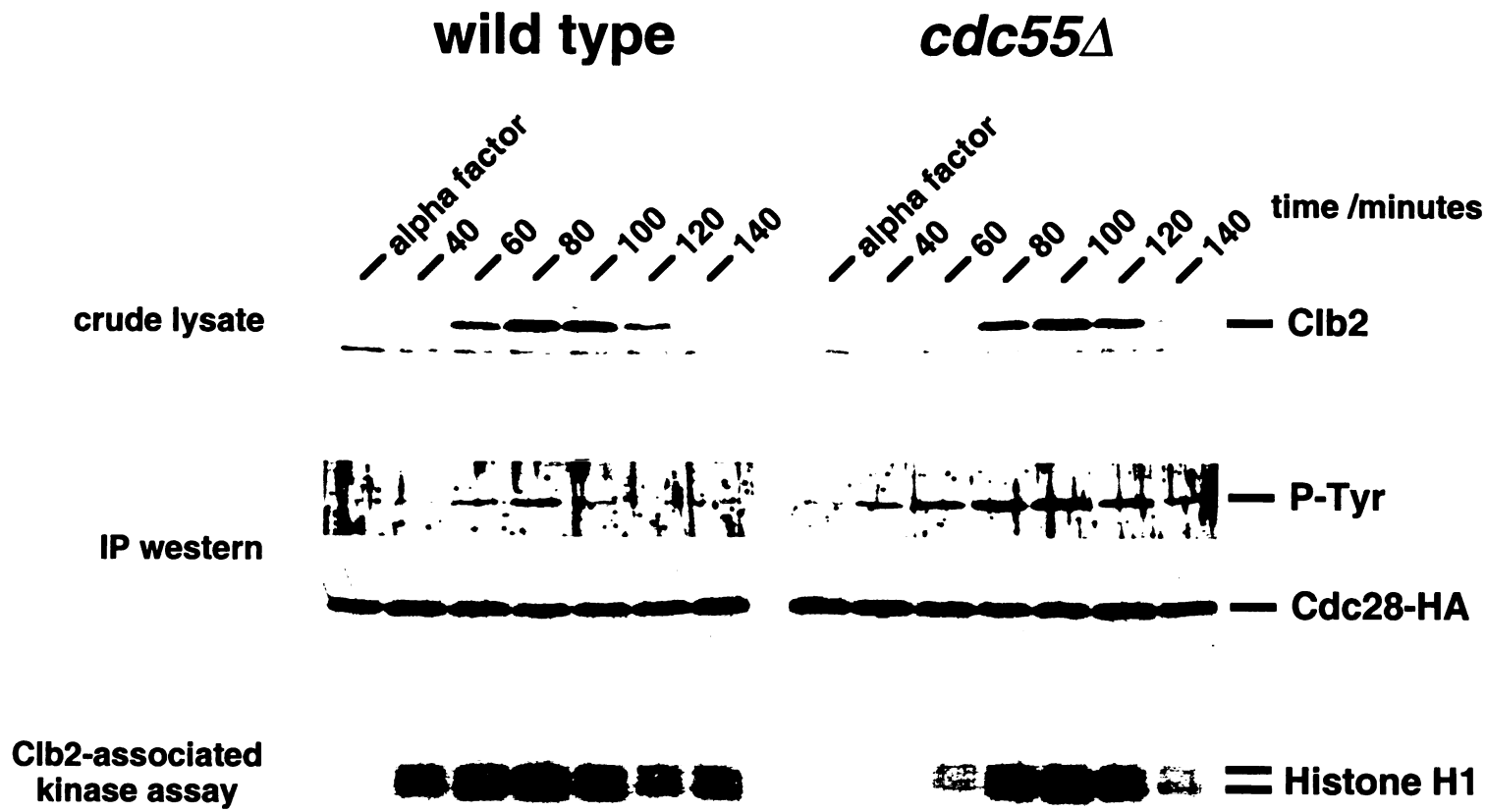

wild type
cdc55Δ
alpha factor
40
60
80
100
120
140
time /minutes
crude lysate
Clb2
IP western
P-Tyr
Cdc28-HA
Clb2-associated kinase assay
Histone H1

Figure 2 Swe1 and Mih1 protein levels and phosphorylation state change during mitosis

MIH1-myc12 (ADR1471) were grown overnight at 30°C in YPD to mid log phase, arrested in G1 with alpha factor (10μg/ml) and at t=0 the cells were released from the G1 arrest. At t=60, alpha factor (15μg/ml) was added back to the cultures to re-arrest the cells in the next G1. A portion of the overnight cultures was arrested in mitosis with nocodazole (10μg/ml) for 2.5 hours. Samples were taken at the indicated times, lysed and immunoblotted for Swe1, Mih-myc12, Clb2, Clb3 and Sic1.

Figure 2

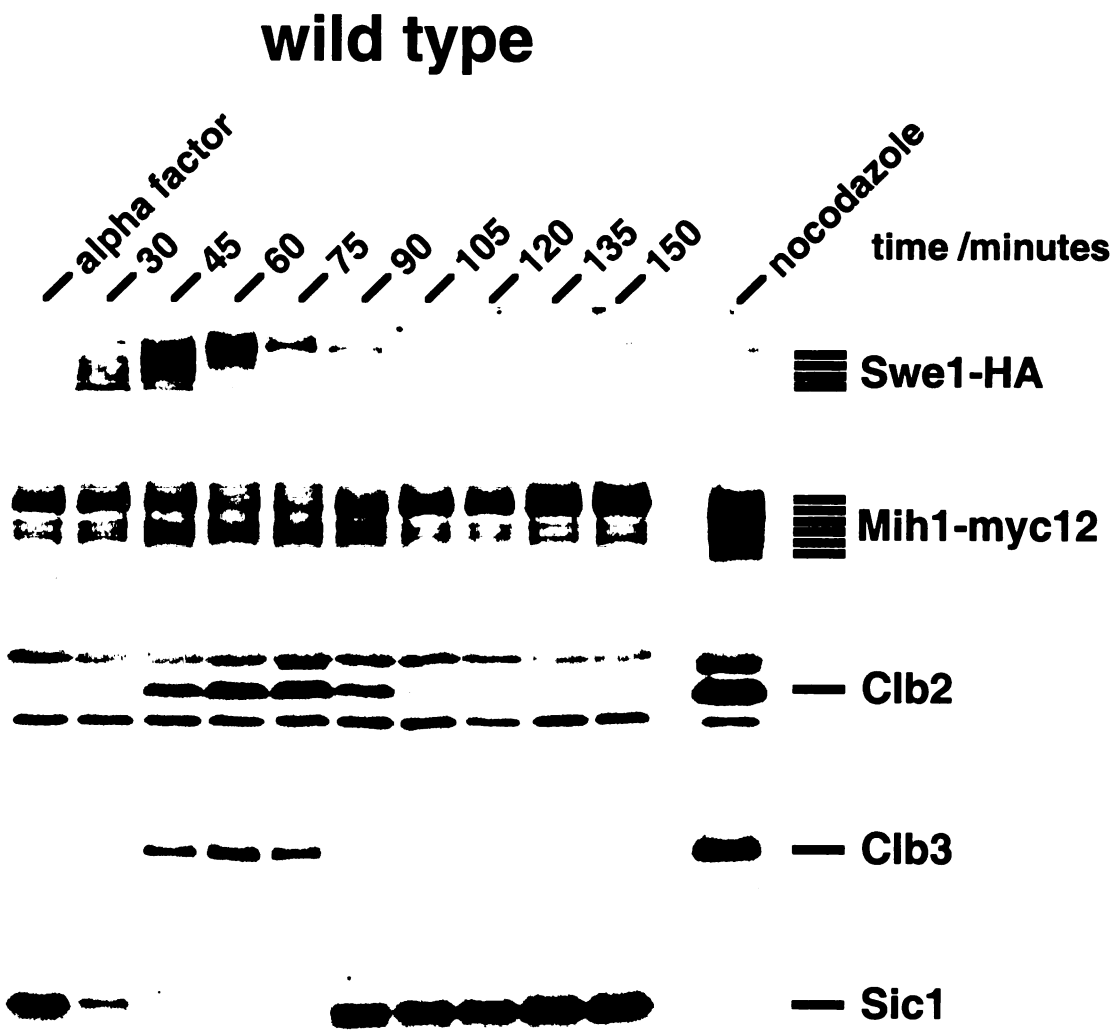

wild type
alpha factor
30
45
60
75
90
105
120
135
150
nocodazole
time /minutes
Swe1-HA
Mih1-myc12
Clb2
Clb3
Sic1

Figure 3 *mih1Δ* delays entry into mitosis

Wild type (ADR376), *mih1Δ* (ADR1367) and *mih1Δ swe1Δ* (ADR1369) were grown overnight at 23°C in YPD to mid log phase, arrested in G1 with alpha factor (1μg/ml) and at t=0 the cells were released from the G1 arrest. At t=90, alpha factor (1.5μg/ml) was added back to the cultures to re-arrest the cells in the next G1. Samples were taken at the indicated times, lysed and immunoblotted for Clb2.

Figure 3

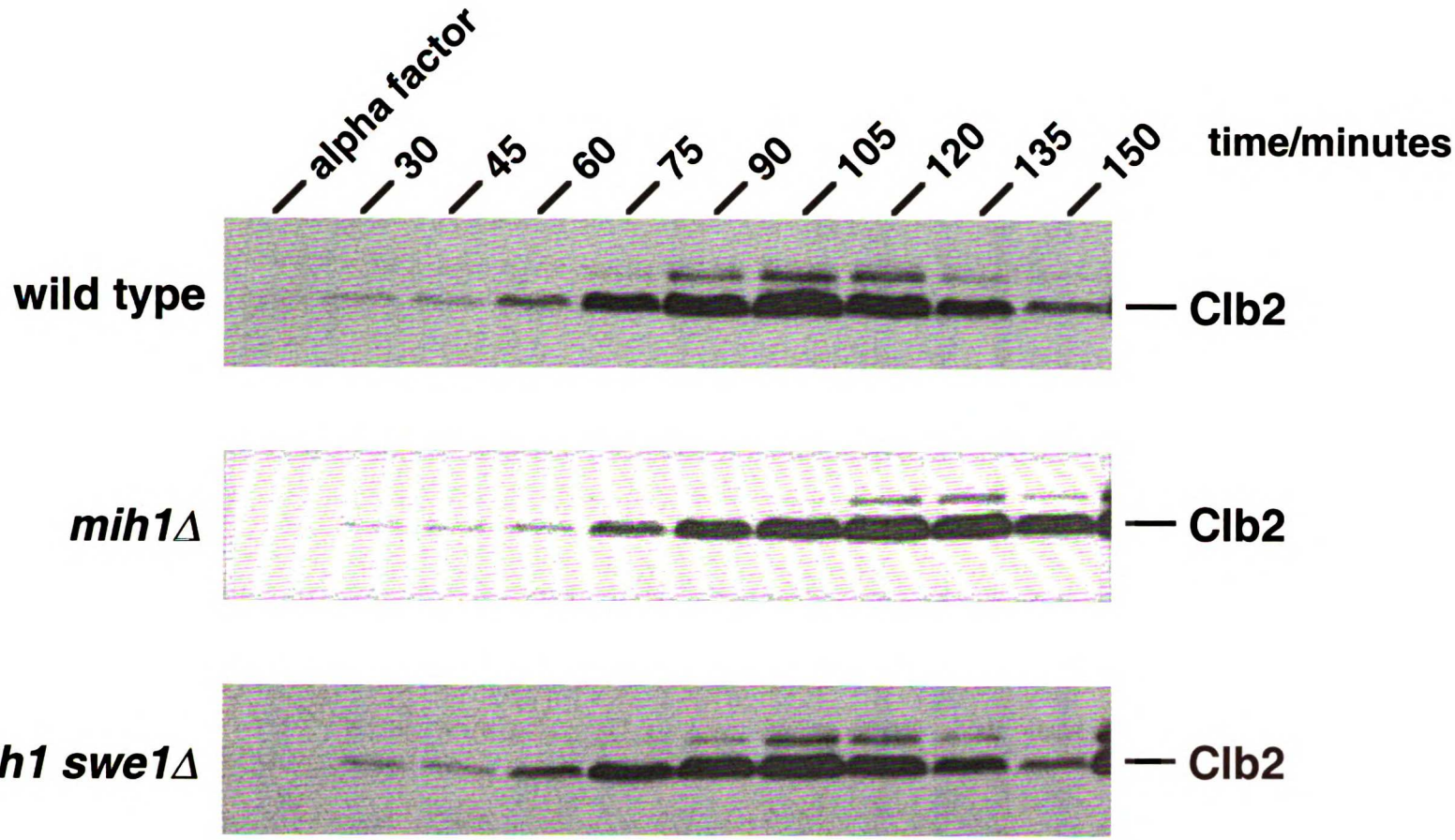

alpha factor
30
45
60
75
90
105
120
135
150
time/minutes
wild type
Clb2
mih1Δ
Clb2
mih1 swe1Δ
Clb2

Figure 4 Overexpression of Swe1 inhibits Cdc28 activity, but Clb2 remains stable

Wild type (—□—, ADR376), *pGAL-SWE1-HA* (—○—ADR797) and *pGAL-SIC1-mut* (—△—, ADR813) were grown overnight at 23°C in YEP+2% raffinose to mid log phase and arrested in mitosis with nocodazole (10μg/ml) at 23°C for 4 hours. Expression of *pGAL-SWE1* and *pGAL-SIC1-mut* was induced by addition of 2% galactose, and alpha factor (1μg/ml) was added at the same time to arrest cells that exited mitosis in the next G1. Duplicate samples were harvested at the indicated times. (A) One set was lysed and immunoblotted for Clb2. (B) The other set was lysed and Clb2 was immunoprecipitated with a Clb2 antibody and the immunoprecipitates tested for their ability to phosphorylate histone H1. The kinase reactions were run on a 15% polyacrylamide gel and radiolabeled phosphate incorporation was measured using a Phosphorimager, and graphed.

Figure 4

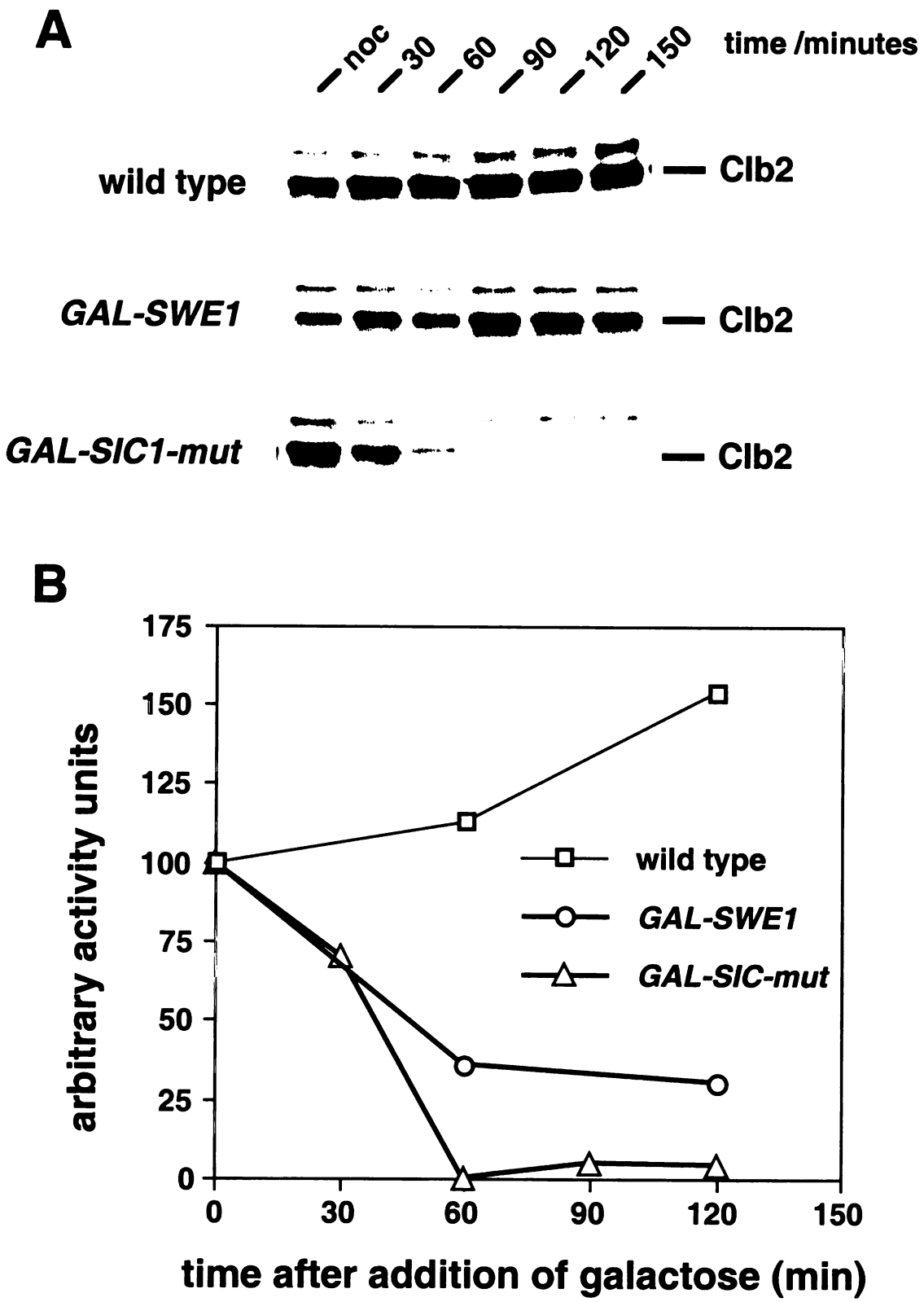
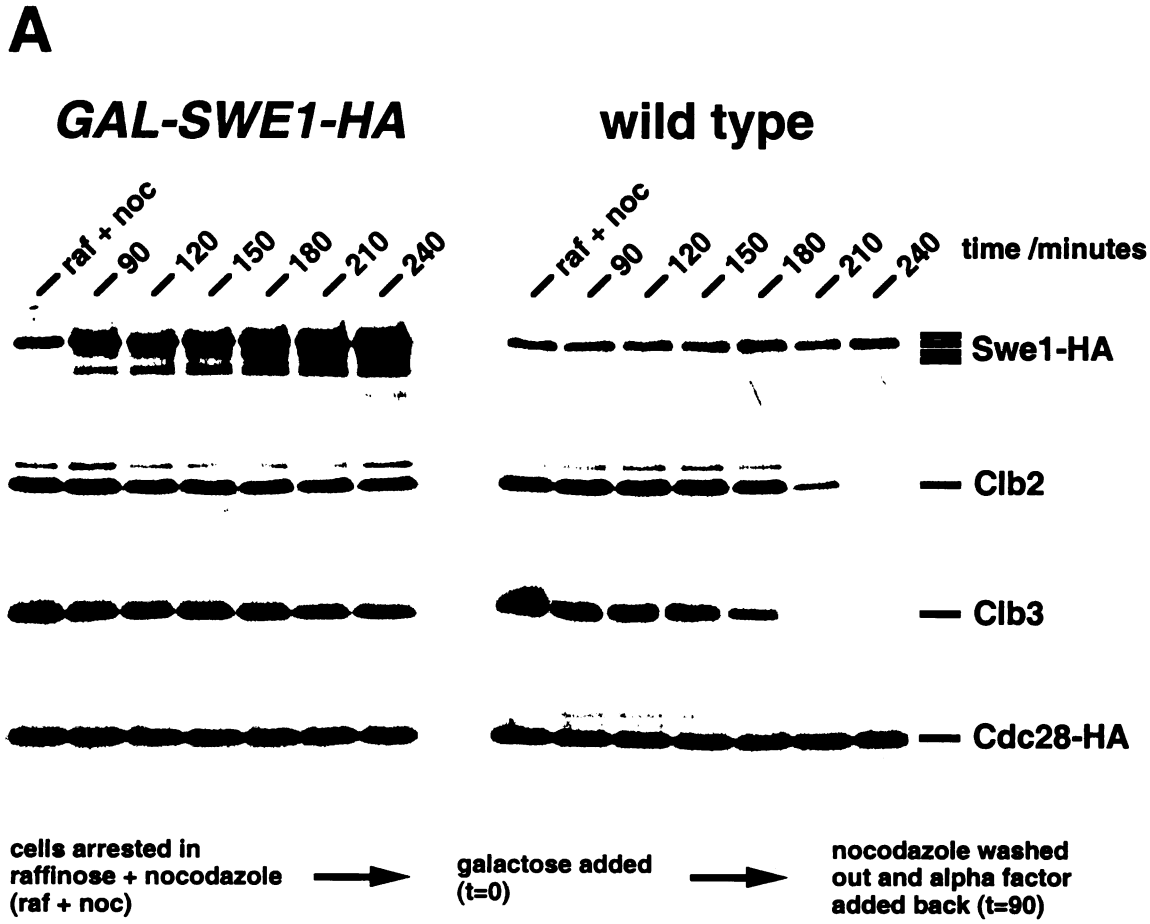

A
noc
30
60
90
120
150
time /minutes
wild type
Clb2
GAL-SWE1
Clb2
GAL-SIC1-mut
Clb2
B
175
150
125
100
75
50
25
0
arbitrary activity units
wild type
GAL-SWE1
GAL-SIC-mut
0
30
60
90
120
150
time after addition of galactose (min)

Figure 5 Overexpression of Swe1 blocks APC activation after release from spindle checkpoint arrest

Wild type (—□—, ADR376), *pGAL-SWE1-HA* (—○—ADR797) were grown overnight at 23°C in YEP+2% raffinose to mid log phase and arrested in mitosis with nocodazole (10μg/ml) at 23°C for 4 hours. Expression of *pGAL-SWE1* was induced by addition of 2% galactose (t=0). After 90 minutes of growth in galactose, the cells were transferred to fresh YEP+2% galactose containing 1μg/ml alpha factor, but without nocodazole. Duplicate samples of cells were harvested at the indicated times. (A) One set was lysed and immunoblotted for Swe1-HA, Clb2, Clb3 and Cdc28-HA. (B) The other set was lysed and Clb2 was immunoprecipitated with a Clb2 antibody and the immunoprecipitates tested for their ability to phosphorylate histone H1. The kinase reactions were run on a 15% polyacrylamide gel and radiolabeled phosphate incorporation was measured using a Phosphorimager, and graphed.

Figure 5


A
GAL-SWE1-HA
wild type
raf + noc
90
120
150
180
210
240
time /minutes
Swe1-HA
Clb2
Clb3
Cdc28-HA
cells arrested in raffinose + nocodazole (raf + noc)
galactose added (t=0)
nocodazole washed out and alpha factor added back (t=90)

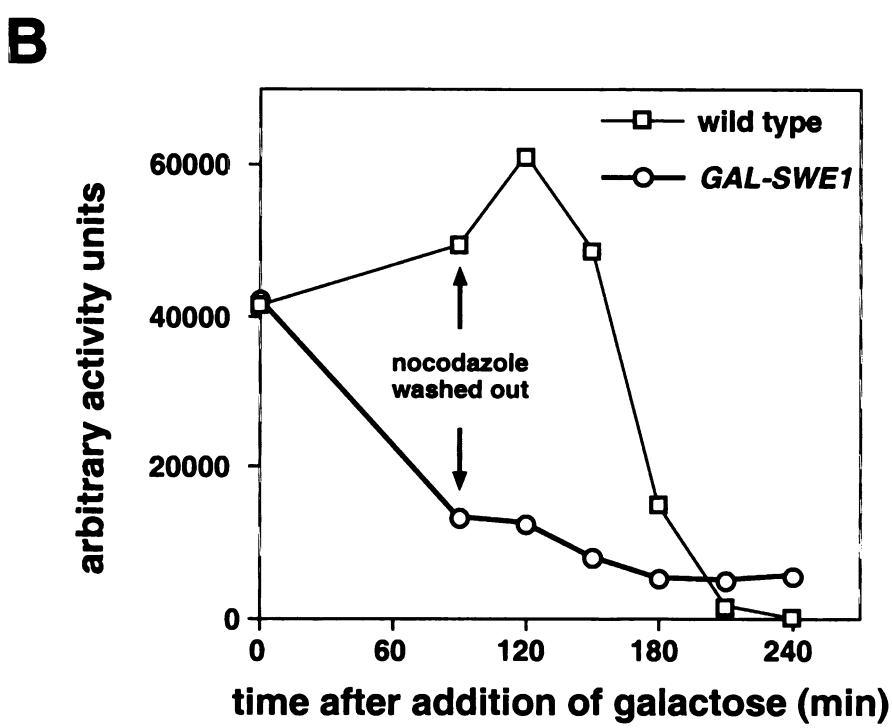

B
wild type
GAL-SWE1
arbitrary activity units
60000
40000
20000
0
nocodazole washed out
0
60
120
180
240
time after addition of galactose (min)

Figure 6 Overexpression of Swe1 causes dephosphorylation of Cdc27

Wild type (JM425) and *pGAL-SWE1-HA mih1Δ CDC28-HA* (ADR1373) were grown overnight at 23°C in YEP+2% raffinose to mid log phase and arrested in mitosis with nocodazole (10μg/ml) at 23°C for 4 hours. Expression of *pGAL-SWE1* was induced by addition of 2% galactose (t=0). After 60 minutes of growth in galactose, the cells were transferred to fresh YEP+2% galactose containing 10μg/ml alpha factor, but without nocodazole. Samples of cells were harvested at the indicated times, lysed and immunoblotted for Cdc27, Clb2, Clb3, Sic1, Swe1-HA, Cdc28-P-Tyr and Cdc28-HA.

Figure 6

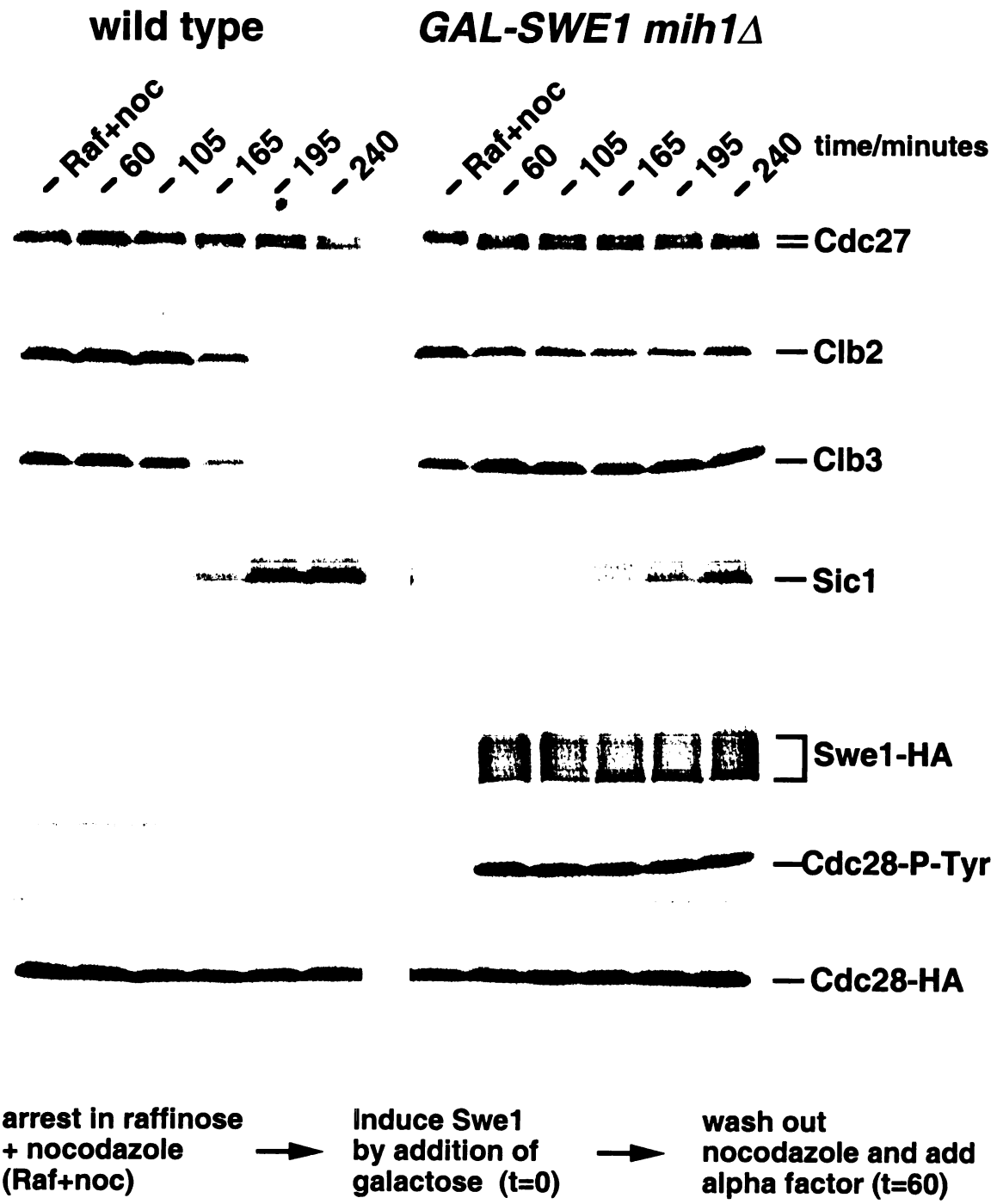
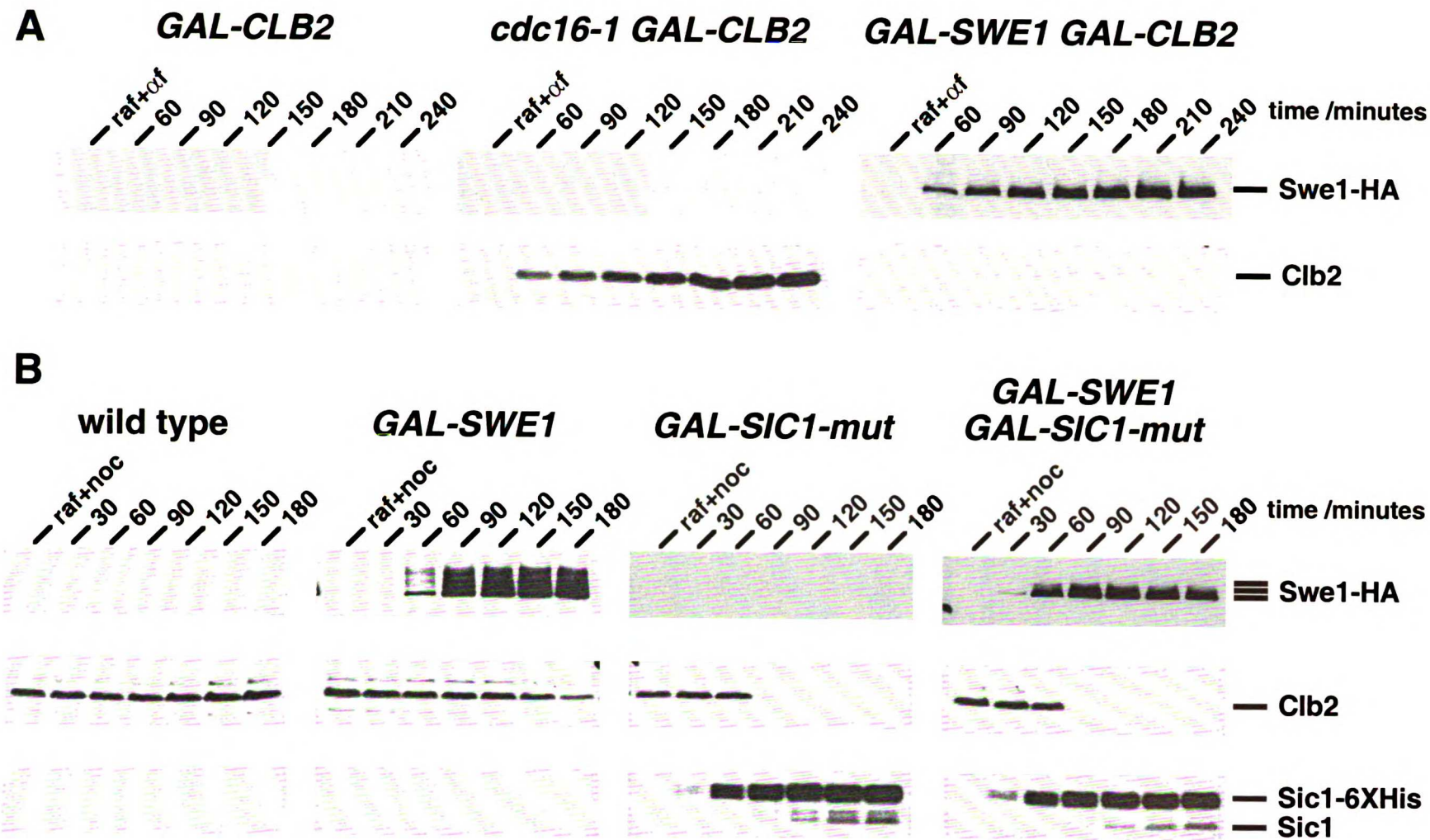

wild type
GAL-SWE1 mih1Δ
Raf+noc
60
105
165
195
240
time/minutes
Cdc27
Clb2
Clb3
Sic1
Swe1-HA
Cdc28-P-Tyr
Cdc28-HA
arrest in raffinose + nocodazole (Raf+noc)
Induce Swe1 by addition of galactose (t=0)
wash out nocodazole and add alpha factor (t=60)

Figure 7 Inhibitory phosphorylation of Cdc28 does not protect Clb2 from destruction

(A) Clb2 does not accumulate when Swe1 and Clb2 are overexpressed in G1. All strain contain *pGAL-CLB2*. Wild type (ADR1544), *cdc16-1*(ADR1542) and *pGAL-SWE1-HA mih1Δ* (ADR1547) were grown overnight at 23°C in YEP+2% raffinose to mid log phase and arrested in G1 with alpha factor (1μg/ml) at 23°C for 3 hours. Expression of *pGAL-SWE1* and *pGAL-CLB2* was induced by addition of 2% galactose (t=0). Samples of cells were harvested at the indicated times, lysed and immunoblotted for Clb2, Swe1-HA

(B) Clb2 is degraded when Swe1 and Sic1-mut are co-overexpressed. Wild type (ADR809), *pGAL-SWE1-HA* (ADR797), *pGAL-SIC1-mut* (ADR813) and *pGAL-SWE1-HA pGAL-SIC1-mut* (ADR796) were grown overnight at 23°C in YEP+2% raffinose to mid log phase and arrested in mitosis with nocodazole (10μg/ml) at 23°C for 4 hours. Expression of *pGAL-SWE1* and *pGAL-SIC1-mut* was induced by addition of 2% galactose, and alpha factor (1μg/ml) was added at the same time to arrest cells that exited mitosis in the next G1. Samples were harvested at the indicated times, lysed and immunoblotted for Swe1-HA, Clb2 and Sic1.

Figure 7


A
GAL-CLB2
cdc16-1 GAL-CLB2
GAL-SWE1 GAL-CLB2
raf+αf
60
90
120
150
180
210
240
time /minutes
Swe1-HA
Clb2
B
wild type
GAL-SWE1
GAL-SIC1-mut
GAL-SWE1
GAL-SIC1-mut
raf+noc
30
60
90
120
150
180
time /minutes
Swe1-HA
Clb2
Sic1-6XHis
Sic1

Appendix 1

Supplemental data for Chapter 1

Introduction

I began working on *CDC28-VF* because it had been shown to suppress some of the checkpoint defects of *cdc55Δ* and I believed that inhibitory phosphorylation of Cdc28 was an alternative pathway out of mitosis (see Chapter 3). When Kevin hardwick discovered that overexpression of Mps1 killed *CDC28-VF*, and I noticed that *CDC28-VF* delayed exiting mitosis after release from a nocodazole arrest, we believed that inhibitory phosphorylation of Cdc28 might be important for adaptation to spindle checkpoint arrest.

Many signaling pathways adapt, eventually reducing the output of the pathway in response to a prolonged stimulus (Detwiler and Gray-Keller, 1996; Parkinson, 1993; Reed, 1991; Torre et al., 1995) . For a cell cycle checkpoint, adaptation allows cells to gamble that division in the face of a persistent defect will produce at least some viable progeny. Plant and animal cells adapt to persistent spindle defects(Andreassen and Margolis, 1994; Kung et al., 1990b; Minn et al., 1996; Rieder and Palazzo, 1992). The most dramatic example is seen in plant and animal cells treated with colcemid, a microtubule depolymerizing drug. After a prolonged mitotic arrest, cyclin B and Cdc2 activity fall, sister chromatids separate, and the cells return to interphase without undergoing cytokinesis, thus doubling their ploidy(Kung et al., 1990a; Rieder and Palazzo, 1992) This increase in ploidy is tolerated in some cell types, but in others, it triggers apoptosis.

I therefore wondered if inhibitory phosphorylation on Cdc28 was induced after long periods of checkpoint arrest and caused an APC-independent exit from mitosis. I was particularly interested in this idea because the *cdc5-ad* mutant in budding yeast had recently been characterized as being defective in adaptation to DNA damage arrest (Toczyski et al., 1997). Chapter 1 describes how I realized that the defect in *CDC28-VF* is not due a lack of inhibitory phosphorylation on Cdc28 and Chapter 3 details how this information has

affected what I think causes the checkpoint defect in *cdc55Δ* cells, but for years I studied *CDC28-VF* believing it was defective in adaptation.

This appendix is split into three parts. Part 1 is largely supplemental data for Chapter 1, and the experiments support the idea that *CDC28-VF* is defective in adaptation, but are easily reinterpreted in light of the data presented in Chapter 1. Part 2 describes some of my efforts to show that inhibitory phosphorylation is induced during "adaptation" to checkpoint arrest. And part 3 details the many experiments I did trying to figure out what was wrong with *CDC28-VF* cells once I realized it was not a lack of inhibitory phosphorylation. These experiments produced mainly negative results, but are experiments I don't want anyone to repeat, and I hope illustrate how difficult it was to arrive at the conclusions of Chapter 1.

Results

Part 1

CDC28-VF is sensitive to checkpoint arrest

Figure 2 in Chapter 2 shows that *CDC28-VF* is sensitive to overexpression of the Mps1 checkpoint kinase. To be sure that this wasn't a checkpoint-independent interaction of Mps1 and *CDC28-VF*, I examined the sensitivity to many treatments that activate the spindle checkpoint.

CDC28-VF, like *mad2Δ* mutants, dies on plates containing the microtubule depolymerizing drug benomyl (Figure 1A) (Li and Murray, 1991). When cell division on benomyl plates is examined microscopically in microcolony assays, *CDC28-VF* does not behave like *mad* mutants. *mad* mutants divide at a rate similar to untreated wild type cells, while *CDC28-VF* cells arrest in mitosis for long periods of time, slowly going through a small number of divisions before dying (Figure 1B). Only 3% of *CDC28-VF* cells form colonies on benomyl plates. Wild type cells divide slowly at first, but then eventually their growth rate speeds up, and 74% of wild type cells eventually form colonies on benomyl plates (Figure 1B). I believed that this growth, like the eventual growth of wild type cells that express high levels of Mps1, was adaptation to checkpoint arrest, but it was difficult to rule out the possible effects of drug metabolism and active pumping of the drug out of the yeast cells.

Exiting mitosis in the presence of spindle defects is potentially risky, so I wondered if *CDC28-VF*, which arrested for longer periods of time on benomyl plates, might have better viability than wild type cells during short term treatment in nocodazole. The rationale for this experiment came from the finding that *cdc5-ad* cells, which are defective in adaptation to DNA damage, have increased viability relative to wild type cells after DNA

damage (Toczyski et al., 1997). This increased viability presumably occurs because *cdc5-ad* arrests at the checkpoint for longer periods of time, and therefore has more time to complete DNA repair.

Between six and twelve hours of growth in liquid media containing nocodazole, wild type cells lose viability (Figure 1C). This corresponds to the same time that wild type cells begin rebudding (figure 1A). *CDC28-VF* cells lose viability at the same rate as wild type cells in this experiment (Figure 1C). In addition, *CDC28-VF* cells lose viability precipitously between 10 and 12 hours after induction of high levels of Mps1 (data not shown).

Mutations in genes that encode microtubule motors, tubulin subunits, microtubuule regulators, and components of the spindle pole body, all activate the spindle checkpoint. I tested whether *CDC28-VF* has any synthetic interactions with these mutations. *CIN8* encodes a microtubule motor, and *cin8Δ* deletions are temperature sensitive in some backgrounds, but not in the W303 background (Hoyt et al., 1992; Roof et al., 1992). However, the *cin8Δ CDC28-VF* double mutant becomes temperature sensitive (Figure 2A), and examination of this double mutant grown at 37°C reveals a tight mitotic arrest (Table 1). This type of synthetic interaction is also seen in double mutants of *CDC28-VF* and *kip1Δ* (another microtubule motor), *cin1Δ* (a gene important for microtubule stability(Hoyt et al., 1990; Stearns et al., 1990)), and *mps2-1* cells (a SPB component (Winey et al., 1991)) (Table 1 and Figure 2B). *mps2-1* cells have been shown to adapt to their checkpoint arrest and diploidize, and I wondered if *CDC28-VF* might block this adaptation. I never was able to obtain convincing evidence that *mps2-1* adaptation occurred in W303 cells (data not shown).

Short linear centromeric mini-chromosomes activate the spindle checkpoint (Wells and Murray, 1996) These chromosomes are maintained in wild type cells but cause a transient *MAD* dependent arrest in mitosis. Eventually the arrest is overcome, and the cells exit mitosis and form viable progeny. This arrest may be caused by improper attachment of the mini-chromosome to the

spindle. The loss rate of the mini-chromosome doesn't improve in cells that delay compared to cells that don't delay, so it appears that these cells resume division without repairing the defect. This is the best example of adaptation to spindle checkpoint arrest in budding yeast.

We have studied the effect of *CDC28-VF* on strains that carry short linear centromeric mini-chromosomes. We performed these experiments without making any assumptions about the ability of *CDC28-VF* strains containing mini-chromosomes to proliferate. A haploid strain that was either *MAD2* or *mad2Δ* was mated to a lawn of cells that were *mad2Δ* and harbored the mini-chromosome. The matings were performed on media that only support the growth of diploids containing the mini-chromosome. There were three variables in these experiments: the *CDC28* alleles of the diploid (*CDC28/CDC28* or *CDC28-VF/CDC28-VF*), the status of the spindle checkpoint (intact in *MAD2/mad2Δ* diploids, defective in *mad2Δ/mad2Δ* diploids) and the nature of the mini-chromosome: a short linear centromeric mini-chromosome (pVL106-L), the equivalent circular centromeric mini-chromosome (pVL106-C), or the equivalent short linear acentric mini-chromosome (pVL111-L) (Figure 3A). *CDC28-VF/CDC28-VF* diploids whose spindle checkpoint is intact grow very poorly when the short linear centromeric mini-chromosome is present, and show a weaker delay in the presence of a circular mini-chromosome (Figure 3B and 3C). The short linear centromeric mini-chromosome also causes a mild growth inhibition in *CDC28/CDC28* diploids (Figure 3B), consistent with its ability to cause mitotic delays in a substantial fraction of cell divisions (Wells and Murray, 1996) The ability of centromeric mini-chromosomes to inhibit growth of *CDC28-VF/CDC28-VF* diploids is abrogated completely by inactivating the spindle checkpoint (Figure 3B).

Figure 3C also shows that an acentric linear mini-chromosome has no effect on the growth of any of the diploids, confirming the requirement for a centromere to activate the spindle checkpoint (Pangilinan and Spencer, 1996; Wells and Murray, 1996). The growth defects of *CDC28-VF/CDC28-VF* diploids that carry mini-chromosomes are not due to defects in mating, since the absolute frequency of diploids formed in the matings is independent of the *CDC28* alleles and the nature of the mini-chromosome (data not shown).

If the same matings are done, and buds of the zygote are followed microscopically in a microcolony assay, long mitotic delays and eventually lethality are observed in *CDC28-VF* cells (Figure 3D). This phenomenon has been used by others to perform a selection for novel checkpoint mutants that allow viability of *CDC28-VF* cells containing short linear chromosomes (Sam Davis and Bodo Stern, unpublished data)

Although *CDC28-VF* cells might be defective in "adaptation" to checkpoint arrest in all the examples above, a simpler explanation is that it has defects in APC activation, and checkpoint arrest makes this defect lethal. It is important to note that a strong adaptation mutant would be expected to arrest permanently with continued checkpoint activation. Although *CDC28-VF* does show long delays after checkpoint activation, it does eventually divide a number of times before dying.

CDC28-VF is semi-dominant

In chapter 2, I presented evidence that *CDC28-VF* is a semi-dominant mutant. Below I show some additional data for this semi-dominance. *CDC28-VF/CDC28* heterozygotes are somewhat sensitive to overexpression of Mps1 (fig 4A), and co-overexpression of Cdc28-VF and Mps1 is toxic to wild type cells (Figure 4B). The phenotype of *CDC28-VF* is not due to haplo-insufficiency because *CDC28/cdc28Δ* (*28/Δ*) cells overexpressing Mps1 behave like *CDC28/CDC28* (*28/28*) cells overexpressing Mps1, and *CDC28-VF/cdc28Δ* (*28-VF/Δ*) behave like *CDC28VF/CDC28-VF* (*28-VF/28-VF*) (Fig 4C). Both *CDC28/cdc28Δ* and *CDC28-VF/Δ* contain half as much Cdc28 protein as *CDC28/CDC28* and *CDC28-VF/CDC28-VF*, showing that there is no dosage compensation for Cdc28 (fig 4D).

CDC28-VF delays exit from a prolonged mitotic arrest

Wild type cells expressing high levels of Mps1 arrest in mitosis due to activation of the spindle checkpoint, but then eventually exit mitosis and resume growing (Hardwick et al., 1996). Initially I thought this resumption of growth was adaptation to the checkpoint arrest, but it may simply reflect recovery from the checkpoint arrest. *CDC28-VF* expressing high levels of Mps1 arrest in mitosis for longer periods of time than wild type cells, and eventually die. Although *CDC28-VF* cells divide a number of times before dying, I wondered if they were unable to complete all the events that occur during the exit from mitosis. Wild type and *CDC28-VF* cells were arrested in G1 by the mating pheromone alpha factor, and then Mps1 was induced to high levels from the inducible *GAL1* promoter by the addition of galactose, and cells were released into the cell cycle. After all the cells had budded (t=3 hours), alpha factor was readded to the cultures so that when cells exited the Mps1 induced arrest, they would re-arrest in the next G1. Both wild type and *CDC28-VF* cells arrest in mitosis for many hours. At 6 hours, Clb2 and Clb3 levels and their associated kinase activity peak in wild type and slowly fall through the rest of the time course (Figure 5A). The peak of Clb2, Clb3 and their associated kianse activities is delayed in *CDC28-VF* cells by 2-4 hours, and then their levels fall during the rest of the time course (Figure 5B).

Sister chromatid separation is also delayed in *CDC28-VF* cells during this experiment (data not shown). Sister separation precedes the fall in Clb levels and associated activities by a number of hours, and both wild type and *CDC28-VF* cells spend a prolonged period of time arrested in anaphase. This is reminiscent of the anaphase arrest I observe in *cdc23-1* cells overexpressing *CDC28-VF* (Chapter 1, Figure 5) and may be because cells overexpressing Mps1 accumulate enough Cdc20-dependent APC activity to trigger sister separation, but not enough to activate the Hct1-dependent APC. In

conclusion, *CDC28-VF* cells overexpressing Mps1 do not arrest permanently in mitosis, and their lethality correlates with exit from the arrest, not from an inability to exit mitosis.

I also examined whether Sic1, a CDK inhibitor that is critical for inhibiting S-phase, may contribute to the ability of cells to adapt or recover from Mps1 induced arrest (Dahmann et al., 1995). *sic1Δ* cells show only minor delays in the exit from mitosis relative to wild type cells (Figure 5C), but *sic1Δ CDC28-VF*cells permanently arrest with high Clb levels and sustained Clb-associated kinase activity (Figure 5D). Formerly I thought this experiment showed that Sic1 and inhibitory phosphorylation work together during adaptation. Given the data in Chapter 1, another explanation is that because Sic1 assists the APC in normal mitotic exit, *sic1Δ CDC28-VF* cells are simply more sensitive to checkpoint arrest.

APC mutants are sensitive to checkpoint activation

CDC28-VF is defective in activating the Cdc20-dependnet APC (Chapters 1 and 2), and I wondered if APC mutants at permissive temperatures shared any phenotypes with *CDC28-VF*. *cdc16-1* mutants are as sensitive to Mps1 overexpression as *CDC28-VF*, and like *CDC28-VF* this sensitivity is suppressed by deletion of *MAD3* (Figure 6). *cdc23-1* mutants are also very sensitive to Mps1 overexpression, and are only partially suppressed by deletion of the *MAD3* (data not shown). The suppression by *mad3Δ* is slightly complicated by the fact that *mad* mutants can partially suppress APC mutants (see Appendix 4).

I have also tested whether mutants in the activators of the APC, *cdc20-1* and *hct1Δ* are sensitive to high levels of Mps1. *cdc20-1* is very sensitive to Mps1 overexpression (data not shown), but *hct1Δ* shows no more sensitivity than wild type cells (data not found). However, *hct1Δ mad3Δ* cells are as sensitive as *hct1Δ* cells, which contrasts to the phenotype of *mad3Δ* or *CDC28-VF mad3Δ* cells which grow as if the checkpoint has not

been activated. This results suggests that if *CDC28-VF* were affecting Hct1 activity directly, one might expect *CDC28-VF mad3Δ* cells to look more like *hct1Δ* cells, and not like *mad3Δ* cells. In addition, it argues that overexpression of Mps1 might be activating the Bub2 checkpoint as well as the Mad checkpoint (Fesquet et al., 1999).

mad2Δ bub2Δ mutants do not suppress *CDC28-VF* phenotypes

Overexpression of Cdc28-VF in *cdc23-1* cells causes permanent mitotic arrest (Chapter 1, Figure 5). I believe this arrest is due to defects activating the Cdc20-dependent APC, but I wanted to confirm that neither the Mad nor the Bub2 checkpoints were being activated in this experiment. I therefore performed an experiment identical to the one described in Chapter 1, Figure 5 using *cdc23-1 GAL-CDC28-VF* and *cdc23-1 GAL-CDC28* cells that are *mad2Δ* and *bub2Δ*. As seen previously, *cdc23-1 GAL-CDC28* cells enter mitosis, exit mitosis and then re-arrest in the next G1 (Figure 7). These strains were not *bar1Δ*, so the re-arrest is only temporary. Deleting *MAD2* and *BUB2* in this strain does not significantly change its behavior, but peak Clb2 levels are lower, Sic1 expression is higher, and re-arrest in G1 is tighter. Overexpression of *cdc23-1 GAL-CDC28-VF* arrest with high levels of Clb2, and low levels of Sic1. Deletion of *MAD2*, or *MAD2* and *BUB2* in this strain leads to slightly lower levels of Clb2 in the arrest, but Clb2 levels do not fall during the timecourse. This experiment argues that *GAL-CDC28-VF* does not activate a known checkpoint. The phenotypes of *bub2Δ* in this experiment support the idea that Bub2 functions in every cycle to inhibit Hct1 and Sic1.

Part 2

Inhibitory phosphorylation on Cdc28 does not change as cells exit mitosis

Because I thought that inhibitory phosphorylation on Cdc28 contributed to mitotic exit I attempted to detect changes in this phosphorylation as cells exited mitotis. Wild type cells were arrested in G1 by alpha factor, Mps1 was induced to high levels from the inducible *GAL1* promoter by the addition of galactose and then cells were released into the cell cycle. After cells had budded alpha factor was re-added to the culture to re-arrest cells in the next G1. I then immunoprecipitated Cdc28 from extracts made from cells harvested throughout the time course, and blotted the immunoprecipitates with a anti-phosphotyrosine antibody. Tyrosine phosphorylation on Cdc28 peaked at 2 hours, which is when these cells are undergoing S-phase (Figure 8). The level of phosphorylation on Cdc28 then dropped, and remained low through the rest of the timecourse. Both nocodazole and alpha factor arrested cells contain little tyrosine phosphorylated Cdc28, so if there were a transient increase in tyrosine phosphorylated Cdc28, I hoped to see it. No such increase was ever observed.

Many variations of this experiment were performed. In order to remove APC-dependent effects on the exit from mitosis I analyzed levels of inhibitory phosphorylation on Cdc28 in *cdc16-1* and *cdc23-1* mutant cells exiting a transient nocodazole or Mps1 induced arrest. These experiments were also performed in *swe1Δ* cells to examine if any Swe1-independent phosphorylation on Cdc28 might be occurring. I neither detected a change in phosphorylation of Cdc28 as cells exit mitosis, nor evidence of Swe1-independent tyrosine phosphorylation of Cdc28.

hct1Δ cells can induce high levels of inhibitory phosphorylation

During the time I thought inhibitory phosphorylation of Cdc28 was important for adaptation to checkpoint arrest, the role of Hct1 in degrading Clb proteins was discovered (Schwab et al., 1997; Visintin et al., 1997). *hct1Δ* cells are viable and quite healthy, despite the fact that levels of the Clb2 protein don't fluctuate (although more recent reports suggest that some Clb2 protein is degraded by the Cdc20-dependent APC). The ability of *hct1Δ* cells to exit mitosis in the absence of proteolysis of Clb2 was thought to be due to inhibition of Cdc28 by Sic1. The primary evidence being that *sic1Δ* cells are viable in combination with APC mutants, but not in combination with *hct1Δ* (although *sic1Δ apc* mutants are viable, they are incredibly sick (data not shown)).

I wondered if inhibitory phosphorylation on Cdc28, like Sic1, might contribute to mitotic exit in *hct1Δ* cells. I examined the levels of inhibitory phosphorylation in *cdc15-2* and *cdc15-2 hct1Δ* mutants released from the anaphase arrest of *cdc15-2*. There was little detectable phosphotyrosine on Cdc28 in *cdc15-2* cells during the release from arrest, but some *cdc15-2 hct1Δ* cells had high levels of inhibitory phosphorylation (Figure 9A and 9C), while others did not (Figure 9B and 9C). I don't know if this difference was the result of a common suppresser of *hct1Δ* or an epigenetic phenomenon. The starting strains that were crossed to make the double mutants were tested carefully and no other mutation is harbored in these strains.

Although inhibitory phosphorylation on Cdc28 is induced in some of these strains, it is not required for *hct1Δ* cells to live because *hct1Δ swe1Δ* cells are viable (data not shown). I was surprised to discover that *mih1Δ* is synthetically lethal in combination with *hct1Δ*, and this lethality is suppressed by *swe1Δ*. Why are *mih1Δ hct1Δ* cells inviable? Perhaps for the opposite reason than the reason that I began doing these experiments. *mih1Δ* cells, like some *hct1Δ* cells induce high levels of inhibitory phosphorylation on Cdc28 (Chapter 1, Figure 4), and the preliminary evidence in Chapter 4 suggests that high

levels of inhibitory phosphorylation inhibit the Cdc20-dependent APC. *hct1Δ* cells have a greater reliance on Cdc20 for viability, and perhaps the slightly inhibited Cdc20-dependent APC in *mih1Δ* cells provides insufficient activity to keep *hct1Δ* cells alive.

Part 3

Once I learned that the defect of *CDC28-VF* was not solely caused by a lack of inhibitory phosphorylation (Chapter 1), I began to investigate what other defects *CDC28-VF* cells might have.

Sic1 bind normally to Cdc28-VF

One possibility was that the Sic1 inhibitor, which has been shown to aid mitotic exit, cannot bind well to Cdc28-VF (Nugroho and Mendenhall, 1994; Schwab et al., 1997). It is hard to investigate Sic1 binding to Cdc28 during mitosis because Sic1 levels are undetectable in mitosis and then rapidly accumulate as cells enter G1. I therefore investigated Sic1 binding in cells that were arrested by the *cdc34-2* mutation, which arrests cells before S-phase, because Sic1 cannot be degraded (Dahmann et al., 1995). *cdc34-2* and *cdc34-2 CDC28-VF* cells were arrested at 37°C, and the amount of Sic1 bound to immunoprecipitates of Cdc28 was determined. Cdc28-VF binds to the same amount or a greater amount of Sic1 than Cdc28 (Figure 10A).

Because Sic1 binding to Cdc28 is what prevents S-phase during the *cdc34-2* arrest, I also investigated if S-phase occurred in *CDC28-VF* cells during this arrest as a second method to detect poor Sic1 binding to Cdc28-VF. No premature entry into S-phase was seen in *cdc34-2 CDC28-VF* cells (data not shown).

I also explored the possibility that the defect in *CDC28-VF* cells was due to both a lack of inhibitory phosphorylation on Cdc28 and poor Sic1 binding. High levels of Mps1

do not cause lethality in *sic1Δ swe1Δ* and *sic1Δ swe1Δ CDC28-V* mutants (Figure 10B and data not shown), suggesting that additional defects must cause the mitotic phenotypes of *CDC28-VF*.

Clb2 binds normally to Cdc28-VF

Another possible defect of Cdc28-VF is that it binds its cyclin subunits tighter than wild type Cdc28. *CDC28-VF* cells have higher steady state levels of Clb2, and Cdc28-VF binds slightly more Clb2 than Cdc28 (see Chapters 1 and 2), so this seemed a possible defect. A simple prediction of such a model is that deletion of cyclins might suppress *CDC28-VF* phenotypes. *CDC28-VF clb2Δ* cells are quite sick, and display greater sensitivity to high levels of Mps1 (data not shown). As shown in Chapters 1 and 2, *clb2Δ* cells alone are as sensitive to Mps1 overexpression, and as defective in APC phosphorylation and Cdc20-dependent APC activity as *CDC28-VF* cells.

During the time I was investigating APC phosphorylation by Cdc28, I noticed that Cks1 pulldowns, or immunoprecipitates of Cdc28, when incubated with ATP, would phosphorylate Clb2 extensively (Figure 11A and 11B). Cdc28-VF is less effective at phosphorylating Clb2 in vitro in these reactions (Figure 11A). This defect appears more severe than the defect in APC phosphorylation because not only is the total amount of phosphorylation reduced, but certain sites in Clb2 appear resistant to phosphorylation by Cdc28-VF.

I also see variations in Clb2 phosphorylation when purified Cdc28 and Clb2 are incubated with ATP in the absence or presence of purified Cks1, the small Cdc28 binding protein. Cks1 not only increases the quantity, but also the quality of phosphorylation (Figure 11C). In these experiments I do not know if Cdc28 phosphorylates its own cyclin partner, or a neighboring Cdc28's cyclin partner, and I don't know if the defect in Cdc28-

VF simply reflects its lower specific activity (see Chapter 1, Figure 4), or a defect in substrate binding.

I also investigated if there are differences in the extent of Clb2 phosphorylation in vivo. wild type and *CDC28-VF* cells were arrested in mitosis with nocodazole, and were labeled with $^{32}PO_4$ and Clb2 was immunoprecipitated using rabbit polyclonal anti-Clb2 antibodies. Slightly more Cdc28-VF/Clb2 was precipitated than Cdc28/Clb2, but slightly greater labeling of Cdc28-VF/Clb2 was also seen (Figure 12). This experiment suggests that Cdc28-VF may have a slight defect in Clb2 phosphorylation, but the relevance of this finding remains unknown. Because Cdc28 is not phosphorylated on tyrosine 19 during mitosis, the Cdc28 phosphorylation in this labeling almost certainly reflects threonine 169 phosphorylation.

In Chapter 1 I concluded that the defect in *CDC28-VF* is probably due to lower specific activity of Cdc28-VF. *CDC28-F88G* has defects similar to *CDC28-VF* (see Chapter 1, Figure 4) and when the kinase activity of Cdc28-F88G is assayed in vitro, it has 6-fold lower specific activity than wild type Cdc28 (J. Ubersax and D. Morgan, personal communication), I never purified Cdc28-VF to do careful kinetic measurements, but I did attempt to measure its K_d for ATP using immunoprecipitates of Cdc28 and Cdc28-VF. Although the exact enzyme concentrations can't be known in immunoprecipitates, one can still roughly calculate the K_d for ATP. Parallel immunoprecipitates of Cdc28 and Cdc28-Vf were incubated with varying concentrations of ATP and an excess of histone H1 as a substrate. The rate of phosphorylation at each of these ATP concentrations was determined and then a Kd for ATP was determined for Cdc28 and Cdc28-VF (data not shown). The K_d for Cdc28 was calculated as 89μM+/-25, which is higher than the value determined using purified Cdc28 (J. Ubersax and D. Morgan, personal communication). The K_d for Cdc28-VF was determined to be 80μM+/-4. If anything Cdc28-VF binds ATP tighter than Cdc28, therefore the difference in specific activity between Cdc28 and Cdc28-VF is likely caused by a defect in substrate binding.

Cks1 binds normally to Cdc28-VF

The small Cdk binding protein, Cks1, is required for the phosphorylation of frog and(Kotani et al., 1999; Patra and Dunphy, 1998) mammalian APC in vitro . It is also required for APC phosphorylation in vivo in budding yeast (Chapter 2, Figure 4). I therefore wondered if Cdc28-VF is defective in binding to Cks1. I compared the amount of epitope tagged Cks1 bound to immunoprecipitates of Cdc28 and Cdc28-VF. If anything, more Cks1 binds to Cdc28-VF than to Cdc28 (Figure 13A). In addition, equal amounts of Cdc28 and Cdc28-VF bind to immunoprecipitates of Cks1. Although an attractive hypothesis, this appears not to be the defect in *CDC28-VF*.

Cdc20 does not bind to Cdc28 or Cdc28-VF

Once I learned that *CDC28-VF* cells are defective in activating the Cdc20-dependent APC, I wondered if Cdc28 might bind directly to Cdc20, and if this binding is defective in *CDC28-VF* cells. No epitope tagged Cdc20 is ever detected in immunoprecipitates of Cdc28 or Cdc28-VF (Figure 13B).

A mysterious modification on Cdc28

When an HA tagged Cdc28 is overexpressed from the *GAL1* promoter a small amount of slower migrating forms of Cdc28 are visible on western blots (Figure 14A and 14B). The major modified form of Cdc28 is roughly 5-10 kD larger than Cdc28, and is Cdc28 because this modified form is visible when Cdc28 is blotted with an anti-HA antibody, or with a anti-PSTAIRE antibody, which recognizes a number of protein kinases, but Cdc28 is the predominant band recognized in this area of the gel. Modified

Cdc28-VF is not detected when Cdc28-VF is overexpressed from the *GAL1* promoter (Figure 14B). This modified form is also not detected in blots of the endogenous Cdc28 or Cdc28-VF proteins, presumably because the modified Cdc28 is present in small amounts. What is the nature of this modification? The modification is not caused by any of the known phosphorylations of Cdc28 because these forms run on protein gels at the same size as unphosphorylated Cdc28 (data not shown). The modification is probably not due to a moiety linked to Cdc28 by a labile di-sulfide bond, because making protein extracts in the absence of reducing agents does not increase the amount of the modified form (data not shown). I began to exlpore if the modification might be a ubiquitin protein by transforming a tagged ubiquitin into these strains, but never obtained a conclusive answer (data not shown).

Cdc28/Clb2 localization

Cdk1 and cyclin B are predominantly nuclear proteins and the mechanism of cyclin B nuclear localization has recently been determined. In these studies it has been assumed that Cdk1 is carried along with cyclin B, but it is not known if Cdk1 affects the localization of cyclin (Moore et al., 1999; Takizawa et al., 1999; Yang and Kornbluth, 1999). Very little is known about how Cdc28 and Clb2 localize to the nucleus in budding yeast, only that mutants in importin-beta (*CSE1*) and importin (*SRP1*) arrest in mitosis (Loeb et al., 1995; Solsbacher et al., 1998). This arrest phenotype is similar to the arrest point of APC mutants and it has been speculated that a key regulator of mitotic exit, like Cdc20, needs to be imported into the nucleus to trigger anaphase and mitotic exit.

Another possibility is that Clb2 itself need to be imported into the nucleus to activate the Cdc20-dependent APC in the nucleus. If this were true, than another possible defect of Cdc28-VF could be an inability to localize itself and Clb2 to the nucleus. A large difference in localization would help explain why *CDC28-VF* cells have such a severe defect in Pds1

degradation, though a minor defect in specific activity and in APC phosphorylation (see Chapters 1 and 2). It is intriguing that Clb2 phosphorylation appears to be somewhat defective in *CDC28-VF* cells (Figures 11A and 12), because in mammalian cells and frog egg extracts cyclin B nuclear export is regulated by cyclin B phosphorylation.

Figure 1 *CDC28-VF* is sensitive to spindle depolymerization

(A) Wild type (ADR21), *CDC28-VF* (JM434) and *mad2Δ* (ADR55) were grown to saturation for two days in YEP+ 2% glucose at 30°C, diluted ten-fold and four-fold serial dilutions were prepared in a multi-well dish and spotted onto YEP+2% glucose plates containing (right) or not containing (left) 7.5μg/ml benomyl. The plates were incubated at 23°C for 2-3 days.

(B) Wild type (ADR21) and *CDC28-VF* (JM434) were grown overnight at 23°C in YEP+2% glucose to mid log-phase and at t=0, unbudded cells were picked out onto YEP+2% glucose containing 10μg/ml benomyl. As each cell divided, the number of cells in each microclony were counted at the indicated times. Large budded cells were counted as two cells and the original cells which did not bud were not included in the analysis. Each strain has been tested at least three times, following 20-30 cells of each strain in each experiment.

(C) Wild type (—□—, ADR21) and *CDC28-VF* (—○—, JM434) were grown overnight at 23°C in YPD to mid log phase, and the cultures were transferred into fresh YPD containing 15μg/ml nocodazole. Samples were harvested at the indicated times, diluted and plated onto YPD plates. The number of colonies at each time was compared with the number immediately before nocodazole addition to determine the % viable cells.

Figure 1

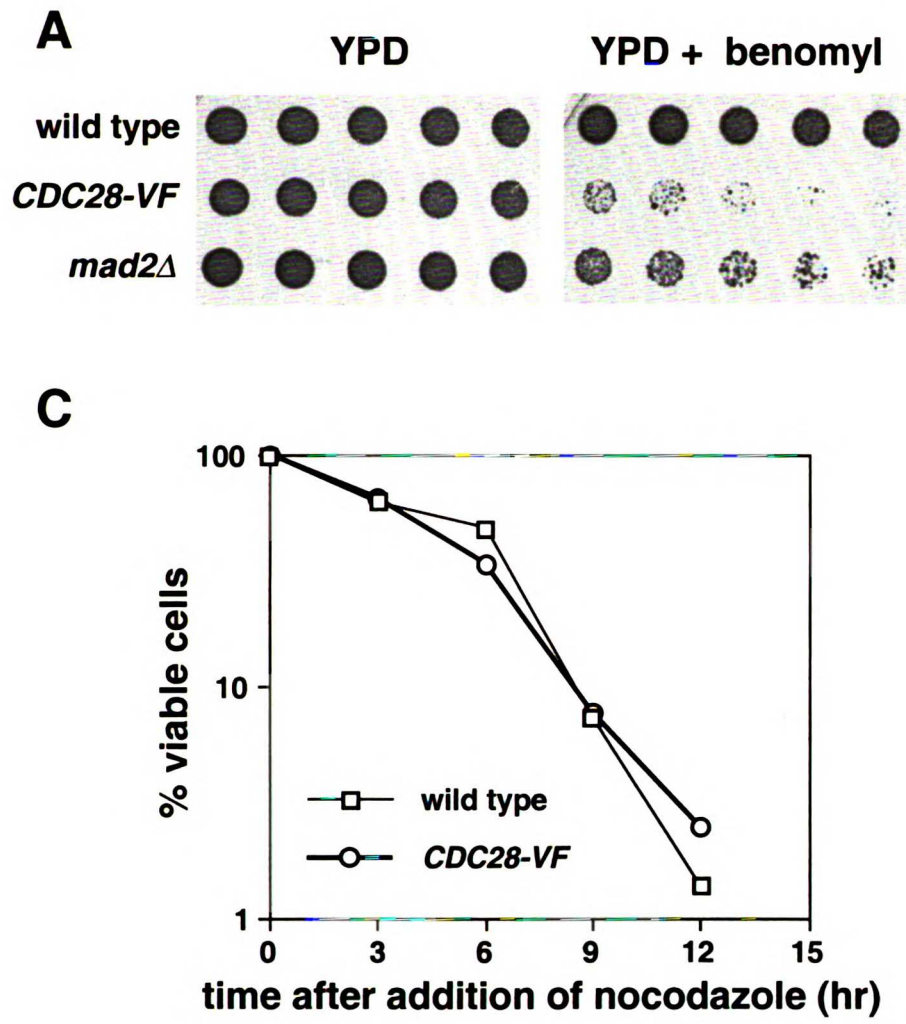

A
YPD
YPD + benomyl
wild type
CDC28-VF
mad2Δ
B
size of microcolony:
1-10 cells
11-20 cells
21+ cells
wild type
CDC 28-VF
0 hrs
12 hrs
24 hrs
74% form colonies
3% form colonies
C
100
10
1
% viable cells
wild type
CDC28-VF
0
3
6
9
12
15
time after addition of nocodazole (hr)

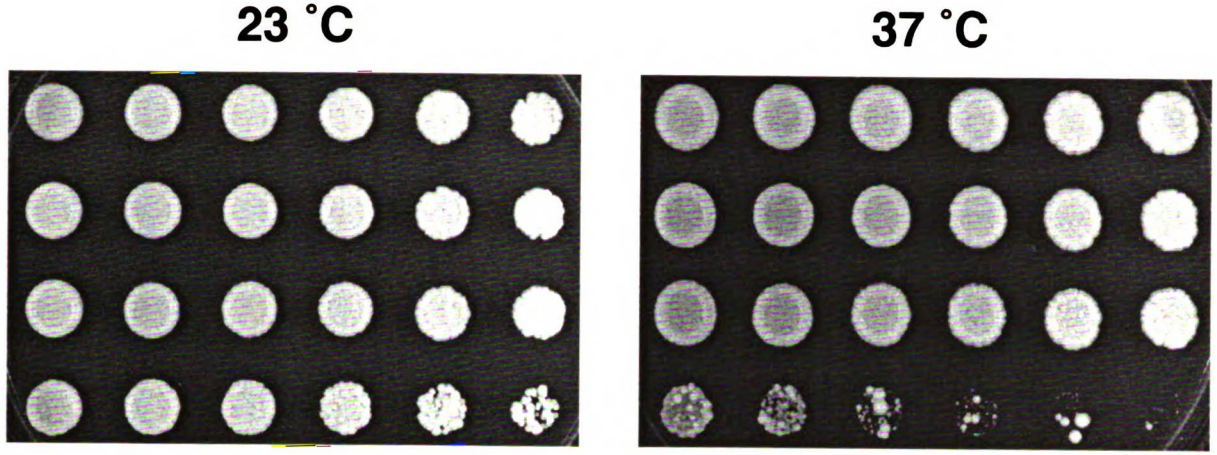
Figure 2 *cin8Δ CDC28-VF* cells are temperature sensitive

(A) Wild type (JM425), *CDC28-VF* (JM434), *cin8Δ* (ADR1171) and *cin8Δ CDC28-VF* (ADR1174) were grown to saturation for two days in YEP+ 2% glucose at 23°C, diluted ten-fold and four-fold serial dilutions were prepared in a multi-well dish and spotted onto YEP+2% glucose plates. Both plates shown were incubated for three days, at either 23°C (left) or 37°C (right). The severe mitotic delay of *cin8Δ CDC28-VF* at 23° observed in Table I causes this strain to grow noticeably slower than the other three strains (left). Note that although *cin8Δ* is temperature sensitive in the S228C strain background (Hoyt et al., 1992) it grows at 37°C in W303.

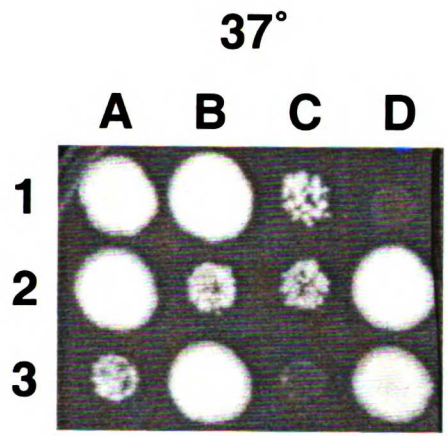
(B) *mps2-1* (ADR1256) was crossed to *CDC28-VF*(JM434), diploids were sporulated and tetrads dissected. 3 representative tetrads were grown to saturation for two days in YEP+ 2% glucose at 23°C, diluted ten-fold and four-fold serial dilutions were prepared in a multi-well dish and spotted onto a YEP+2% glucose plate.which was grown at 37°C for 3 days. *mps2-1 CDC28-VF* (ADR1258) cells are noticeably more temperature sensitive than *mps2-1* cells.

Figure 2

A



B



1A: wild type
1B: *CDC28-VF*
1C: *mps2-1*
1D: *CDC28-VF mps2-1*

2A: *CDC28-VF*
2B: *mps2-1*
2C: *mps2-1*
2D: *CDC28-VF*

3A: *mps2-1*
3B: *CDC28-VF*
3C: *CDC28-VF mps2-1*
3D: wild type

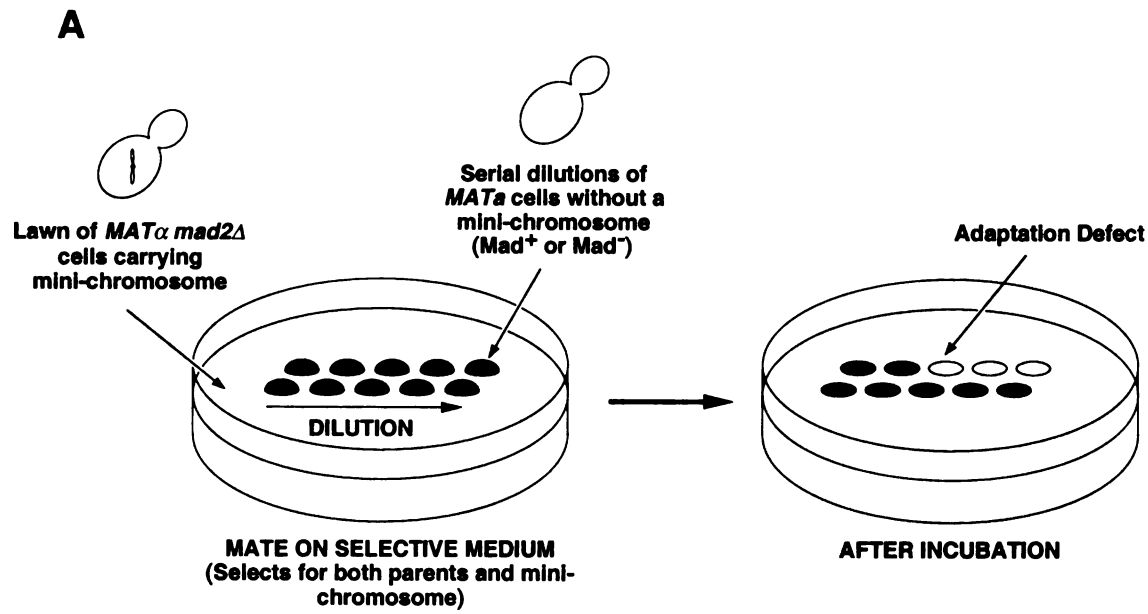
Figure 3 *CDC28-VF* cells cannot maintain short linear centromeric mini-chromosomes

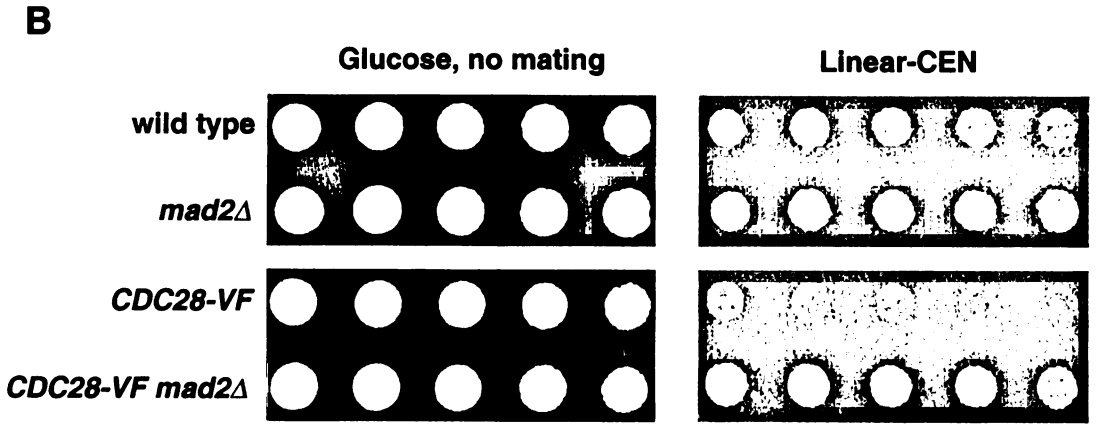
(A) Experimental design.

(B&C) Plate matings with short linear centromeric mini-chromosome. Wild type (ADR413), *mad2Δ* (ADR1123), *CDC28-VF* (ADR1117) and *mad2Δ CDC28-VF* (ADR1121) yeast were grown overnight at 30°C to mid log phase and diluted to A_{600} 0.35. Two-fold serial dilutions of the strains were spotted onto YEP+2% dextrose or lawns of *mad2Δ* or *mad2Δ CDC28-VF*. The lawns of cells contained either a short linear centromeric mini-chromosome (pVL106-L, ADR887 and ADR891), a circular centromeric mini-chromosome (pVL106-C, ADR888 and ADR893) or a short linear acentric mini-chromosome (pVL111-L, ADR889 and ADR925). The serially diluted strains were all *leu⁻ HIS⁺* and the lawns were *LEU⁺ his⁻*. The matings were performed on -leu -his plates, so only diploids formed by mating grow. The *CDC28-VF MAD⁺* diploids containing the short linear centromeric mini-chromosome are severely inhibited for growth.

(D) Similar matings were performed as in (B&C), except instead of matings the strains in lawns, they were mixed with a toothpick on CSM-leu+2% glucose plates and allowed to mate overnight for 8-10 hours. Zygotes were were picked out onto CSM-leu+2% glucose plates and as each cell divided, the number of cells in each microclony were counted at the indicated times. Large budded cells were counted as two cells and the original cells which did not bud were not included in the analysis. The *CDC28-VF mad2Δ* strain harboring the 2μ plasmid (yEPlac181) is ADR923.

Figure 3


A
Lawn of MATα mad2Δ cells carrying mini-chromosome
Serial dilutions of MATa cells without a mini-chromosome (Mad+ or Mad-)
DILUTION
MATE ON SELECTIVE MEDIUM (Selects for both parents and mini-chromosome)
Adaptation Defect
AFTER INCUBATION


B
Glucose, no mating
Linear-CEN
wild type
mad2Δ
CDC28-VF
CDC28-VF mad2Δ

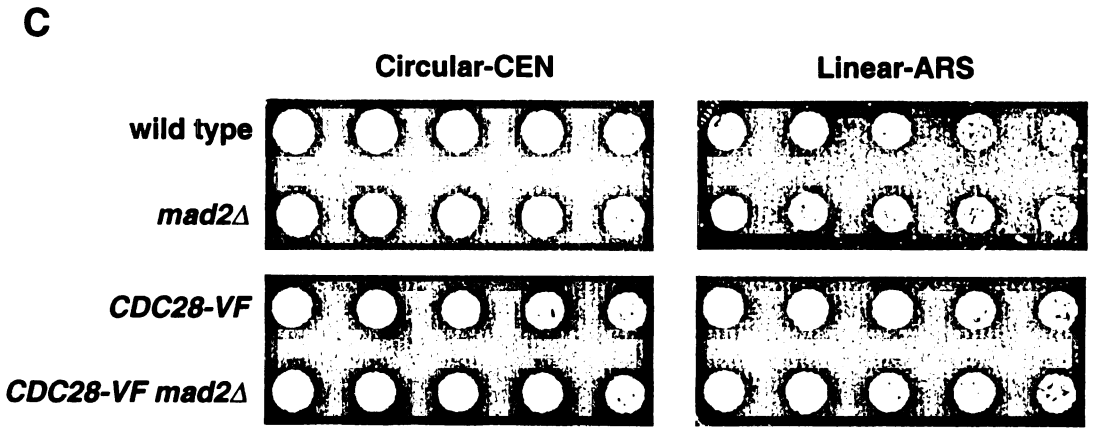

C
Circular-CEN
Linear-ARS
wild type
mad2Δ
CDC28-VF
CDC28-VF mad2Δ

Figure 3

D

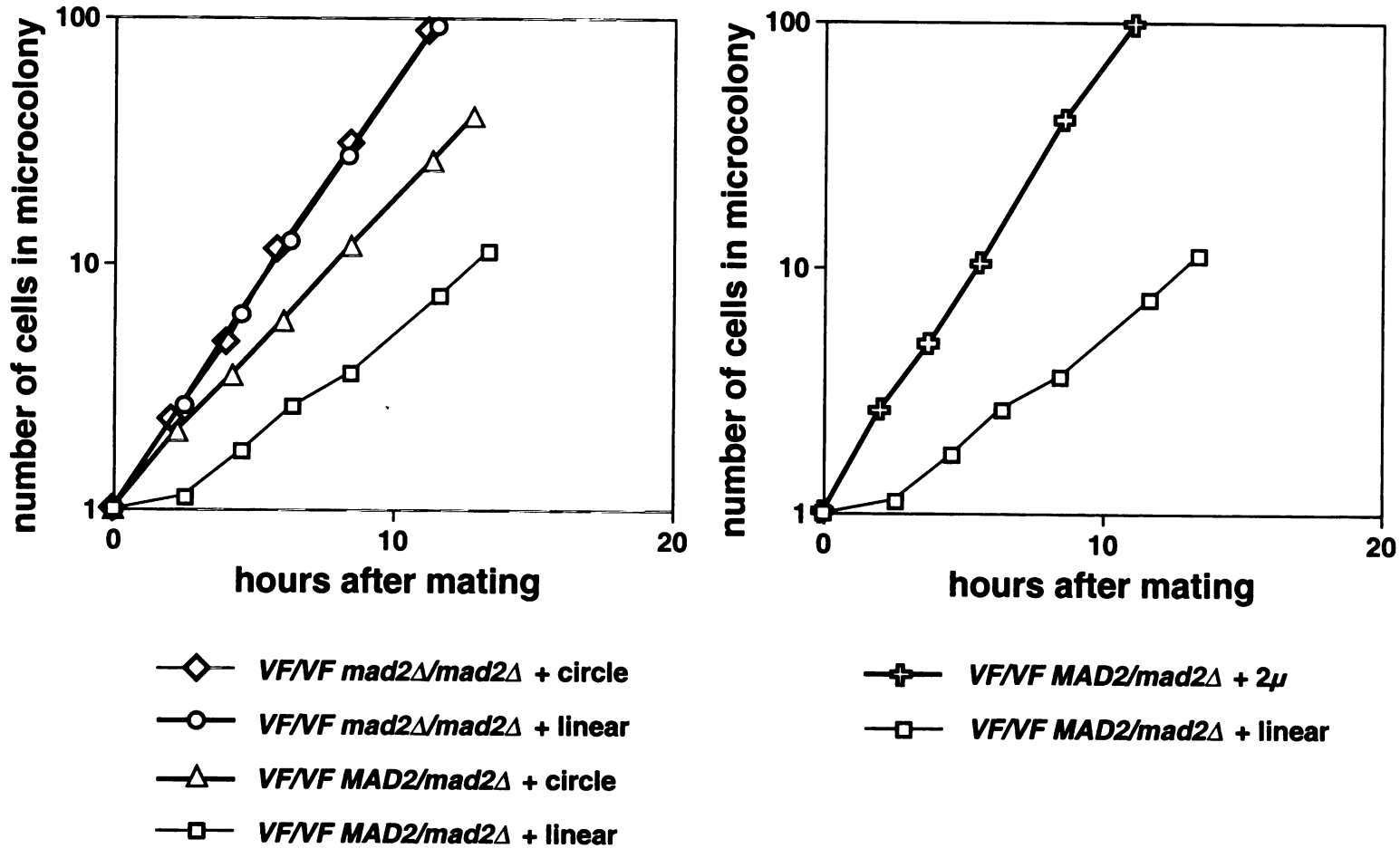

number of cells in microcolony
100
10
1
0
10
20
hours after mating
number of cells in microcolony
100
10
1
0
10
20
hours after mating
VF/VF mad2Δ/mad2Δ + circle
VF/VF mad2Δ/mad2Δ + linear
VF/VF MAD2/mad2Δ + circle
VF/VF MAD2/mad2Δ + linear
VF/VF MAD2/mad2Δ + 2μ
VF/VF MAD2/mad2Δ + linear

Figure 4 Genetic analysis of the *CDC28-VF* defect

(A) Serial dilution of diploids showing that *CDC28-VF* is semi-dominant. *pGAL-MPS1/pGAL-MPS1* (ADR1193), *CDC28/CDC28-VF pGAL-MPS1/pGAL-MPS1* (ADR1195) and *CDC28-VF/CDC28-VF pGAL-MPS1/pGAL-MPS1* (ADR1197) diploids were grown overnight in YEP+2% dextrose to mid-log phase, six two-fold serial dilutions of each strain were prepared in a multi-well dish and the strains were spotted onto YEP+2% dextrose (left) or YEP+4% galactose (right).

(B) Serial dilution of strains showing that overexpressed *CDC28-VF* is fully dominant. *pGAL-MPS1* (KH153), *pGAL-MPS1 pGAL-CDC28* (ADR1207), *pGAL-MPS1 pGAL-CDC28-VF* (ADR1215) yeast in the top two panels and wild type (AFS35), *pGAL-CDC28* (ADR1024) and *pGAL-CDC28-VF* (ADR1022) yeast in bottom two panels, were grown, serially diluted and spotted exactly as described in (A) The two left plates are plates are YEP+2% dextrose and the two right plates are YEP+4% galactose. The yeast co-expressing *pGAL-MPS1* and *pGAL-CDC28-VF* cannot grow when both genes are induced on galactose (top right panel), while the yeast co-expressing *pGAL-MPS1* and *pGAL-CDC28* only show a mild growth defect on galactose (top right panel).

(C) *CDC28-VF* is not haplo-insufficient. *CDC28/CDC28 pGAL-MPS1/ura3-1*(—□—, *28/28*, ADR935), *CDC28/cdc28Δ pGAL-MPS1/ura3-1*(—○—, *28/Δ*, ADR1080), *CDC28-VF/CDC28-VF pGAL-MPS1/ura3-1* (—△—, *28-VF/28-VF*, ADR939) and *CDC28-VF/cdc28Δ pGAL-MPS1/ura3-1*(—◇—, *28-VF/Δ*, ADR1083) were grown overnight at 30°C in YEP+2% raffinose to log-phase and at t=0, unbudded cells were picked out onto YEP+2% galactose plates. As each cell divided, the number of cells in each microcolony were counted at the indicated times. Large budded cells were counted as two cells and the original cells which did not bud were not included in the analysis. In the experiment shown, thirty cells of each strain were followed.

(D) There is no dosage compensation for *CDC28* in *CDC28/cdc28Δ* cells. *CDC28/CDC28* (*28/28*, ADR941), *CDC28/cdc28Δ* (*28/Δ*, ADR951), *CDC28-VF/CDC28-*

VF (*VF/VF*, ADR949) and *CDC28-VF/cdc28Δ* (*VF/Δ*, ADR953) were grown overnight at 23°C in YEP+2% glucose to mid log-phase and then arrested in mitosis with nocodazole (10μg/ml) for 3 hours. Cells were harvested, lysed and immunoblotted for Clb2, Cdc28-HA and Cdc28. The *28/28* and *VF/VF* samples were diluted two and four fold to aid in quantification.

Figure 4

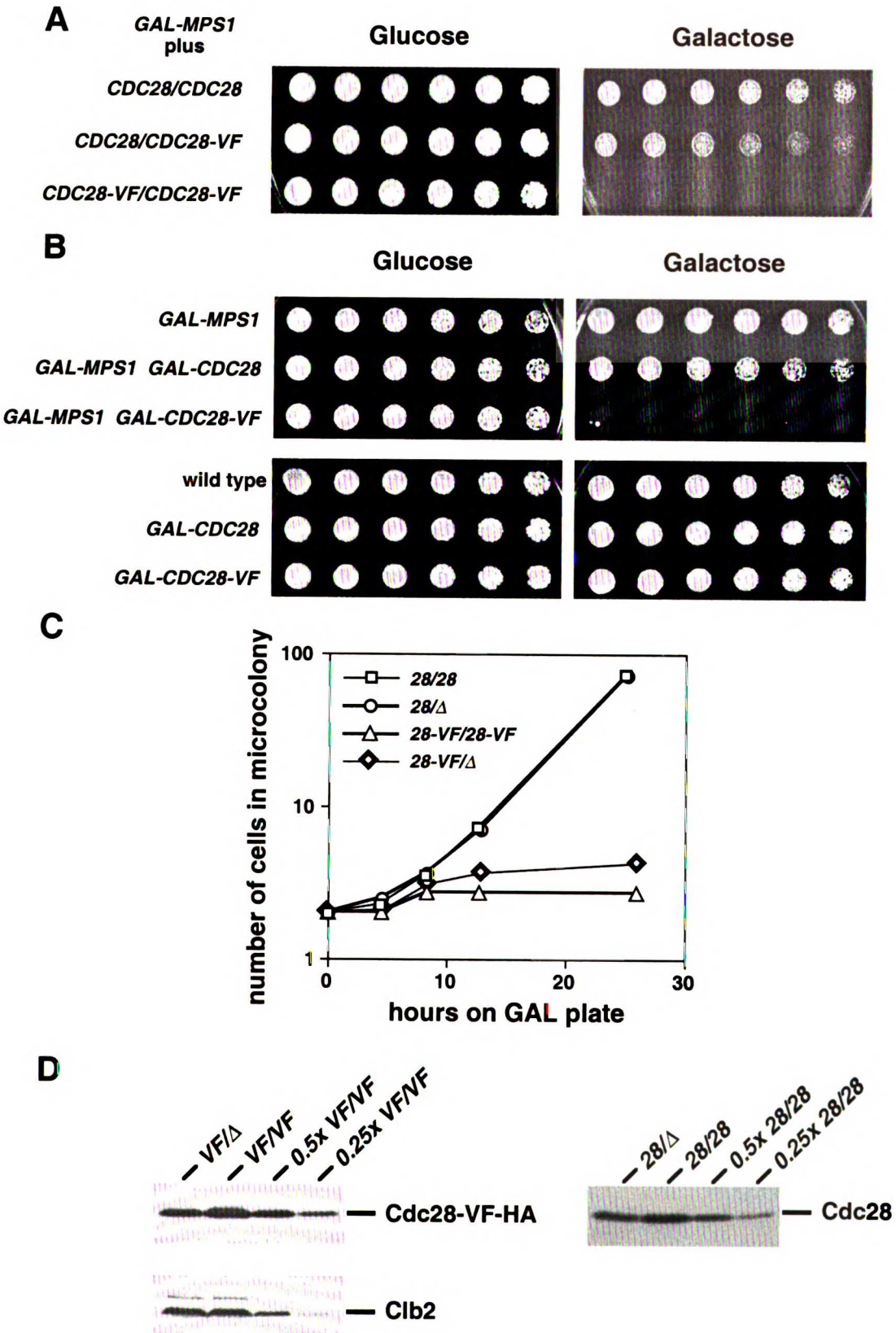

A
GAL-MPS1 plus
Glucose
Galactose
CDC28/CDC28
CDC28/CDC28-VF
CDC28-VF/CDC28-VF
B
Glucose
Galactose
GAL-MPS1
GAL-MPS1 GAL-CDC28
GAL-MPS1 GAL-CDC28-VF
wild type
GAL-CDC28
GAL-CDC28-VF
C
number of cells in microcolony
100
10
1
0
10
20
30
hours on GAL plate
28/28
28/Δ
28-VF/28-VF
28-VF/Δ
D
VF/Δ
VF/VF
0.5x VF/VF
0.25x VF/VF
Cdc28-VF-HA
Clb2
28/Δ
28/28
0.5x 28/28
0.25x 28/28
Cdc28

Figure 5 *CDC28-VF sic1Δ* cells overexpressing Mps1 maintain Cdc28-associated kinase activity and cyclin protein levels

Timecourses of Clb2 and Clb2-associated kinase activity (—□—), and Clb3 and Clb3-associated kinase activity (—○—), during recovery from checkpoint arrest in four strains: (A) *GAL-MPS1* (ADR1088), (B) *CDC28-VF GAL-MPS1* (ADR1090), (C) *sic1Δ GAL-MPS1* (ADR1276) and (D) *CDC28-VF sic1Δ GAL-MPS1* (ADR1270). At t=0 cells were released from a mating-pheromone induced G1 arrest into galactose-containing medium to induce overexpression of Mps1. At 6 hours, alpha factor (1.5μg/ml) was added back to the cultures to prevent cells which had exited mitosis from continuing in the cell cycle. Samples were taken at the indicated times, lysed and either immunoblotted for Clb2 and Clb3, or Clb2 and Clb3 were immunoprecipitated with anti-Clb2 and anti-Clb3 antibodies and the immunoprecipitates tested for their ability to phosphorylate histone H1. The kinase reactions were run on a 15% polyacrylamide gel and radiolabeled phosphate incorporation was measured using a Phosphorimager, and graphed.

Figure 5

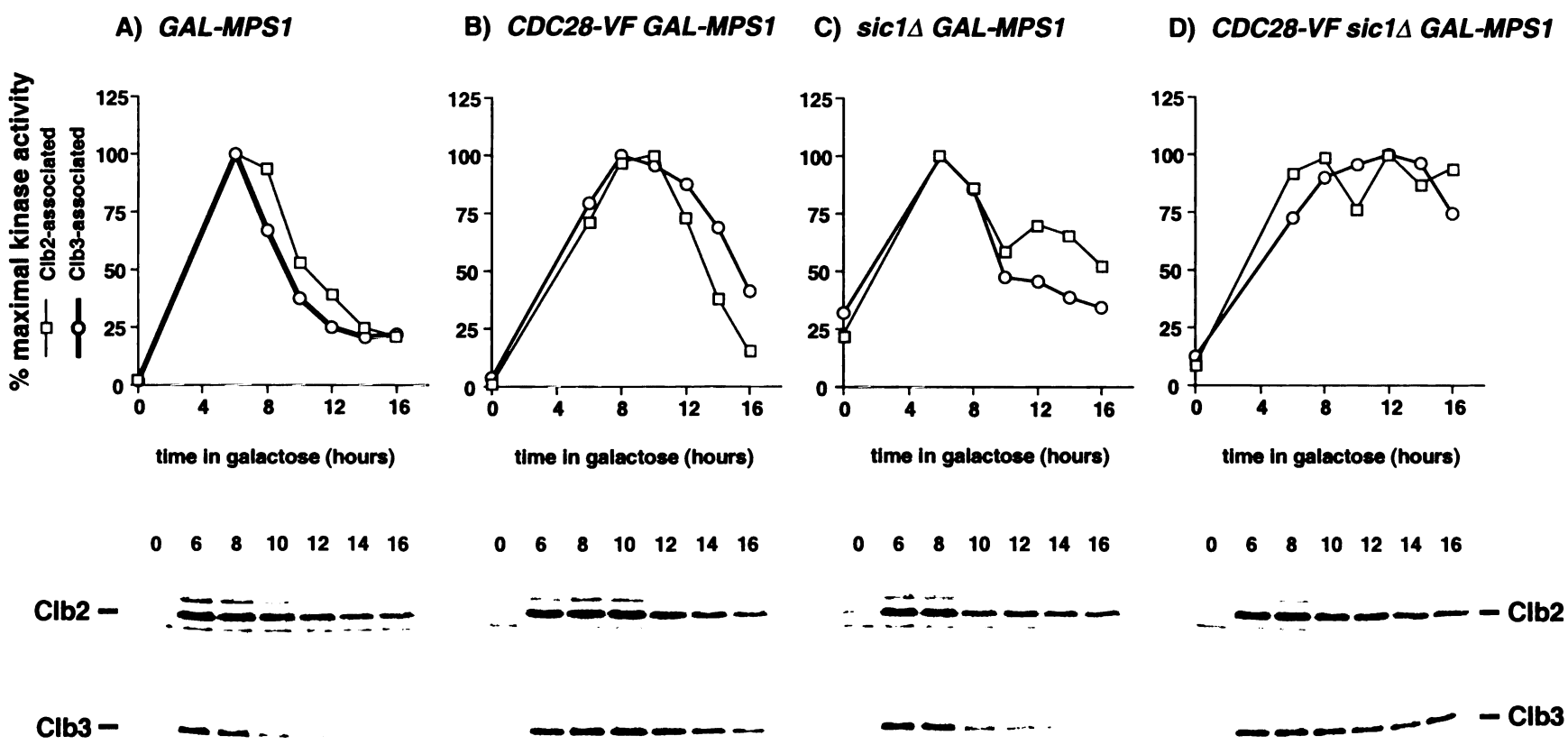

A) GAL-MPS1
B) CDC28-VF GAL-MPS1
C) sic1Δ GAL-MPS1
D) CDC28-VF sic1Δ GAL-MPS1
% maximal kinase activity
Clb2-associated
Clb3-associated
125
100
75
50
25
0
0 4 8 12 16
time in galactose (hours)
0 6 8 10 12 14 16
Clb2
Clb3

Figure 6. *apc* mutants are hypersensitive to spindle checkpoint activation

cdc16-1 is hypersensitive to checkpoint arrest and this sensitivity is abrogated in a *cdc16-1 mad3Δ* double mutant. *GAL-MPS1* (KH153), *CDC28-VF GAL-MPS1* (KH181), *cdc16-1 GAL-MPS1* (ADR1280), *cdc16-1 mad3Δ GAL-MPS1* (ADR1283) *mad3Δ GAL-MPS1* (ADR1248) and*CDC28-VF mad3Δ GAL-MPS1* (KH183) were grown, serially diluted and spotted exactly as described in Figure 4A. The plates were incubated at 30°C for 1.5 days.

Figure 6

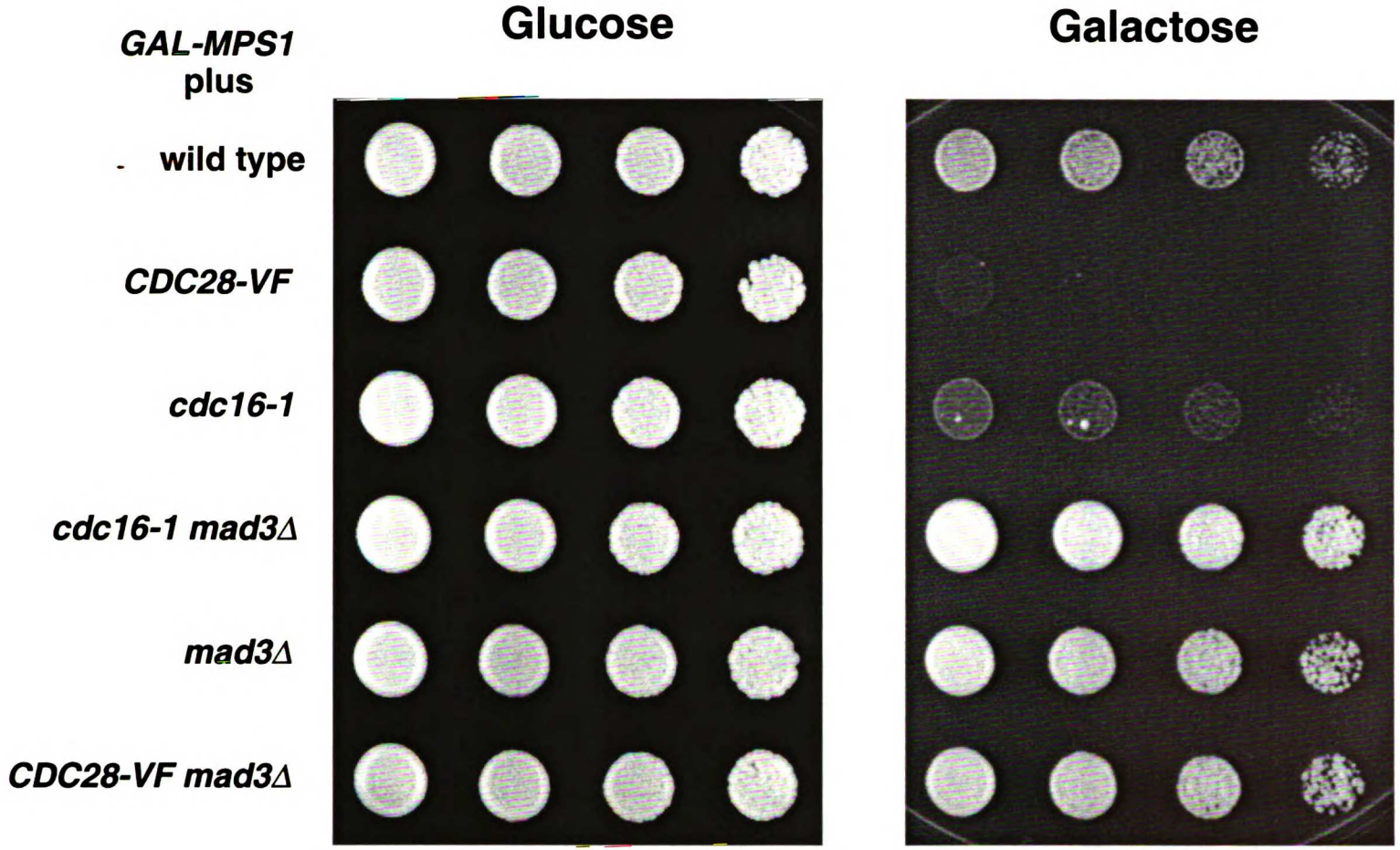

GAL-MPS1
plus
-
wild type
CDC28-VF
cdc16-1
cdc16-1 mad3Δ
mad3Δ
CDC28-VF mad3Δ
Glucose
Galactose

Figure 7 *CDC28-VF* does not activate the Mad or Bub2 checkpoints

cdc23-1 GAL-CDC28 (ADR2014), *cdc23-1 GAL-CDC28 mad2Δ bub2Δ* (ADR2019), *cdc23-1 GAL-CDC28-VF* (ADR2015), *cdc23-1 GAL-CDC28-VF mad2Δ bub2Δ* (ADR2021) and *cdc23-1 GAL-CDC28-VF mad2Δ* (ADR2072) were grown overnight at 23°C in YEP+2% raffinose to log phase, arrested in G1 with alpha factor (10μg/ml) for 3.5 hours and at t=0 released from the G1 arrest into fresh YEP+2% galactose. After cells had budded (t=2), alpha factor (15μg/ml) was added back to the cultures to re-arrest the cells in the next G1. Samples were taken at the indicated times, lysed and immunoblotted for Clb2 and Sic1.

Figure 7

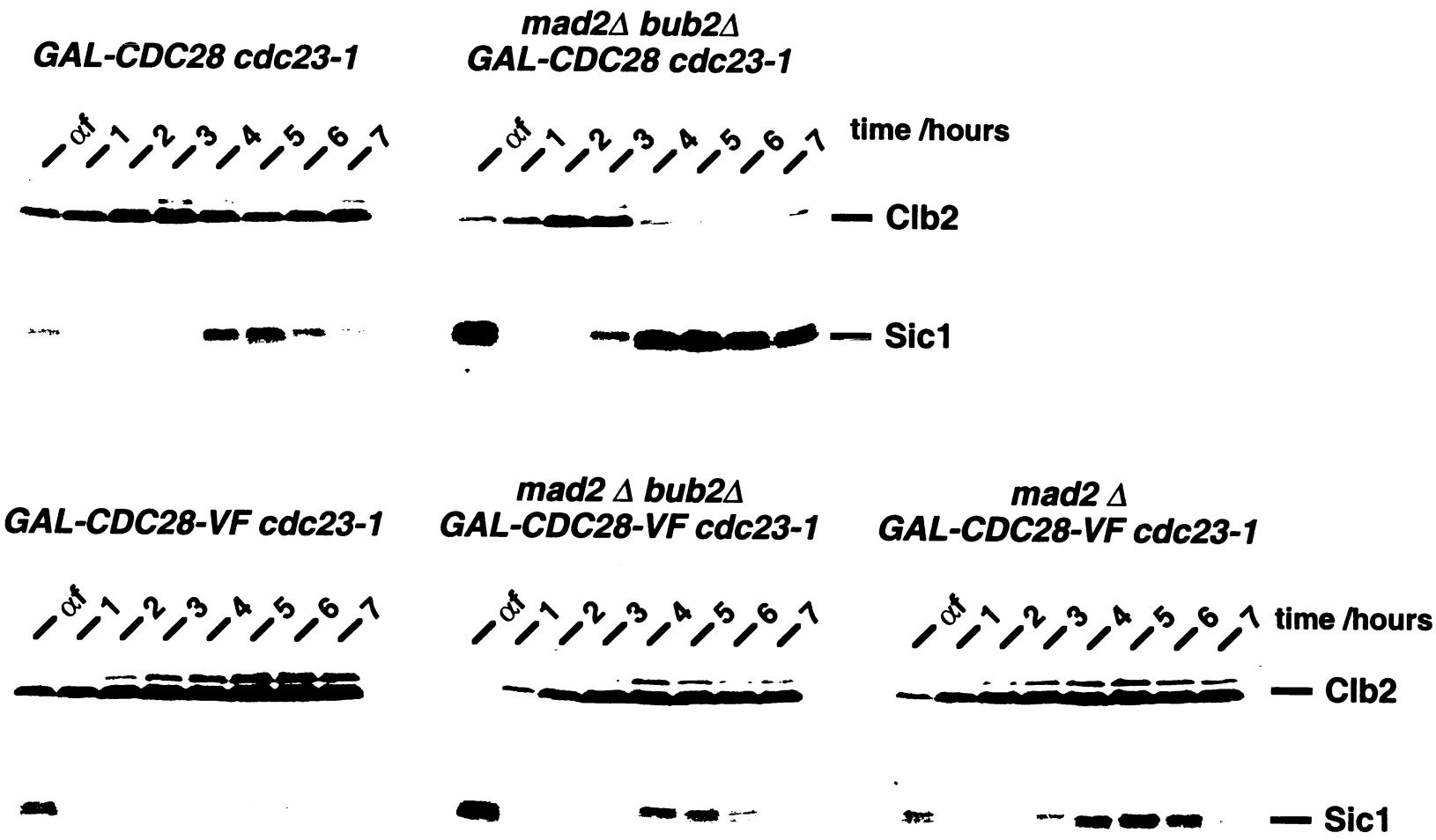
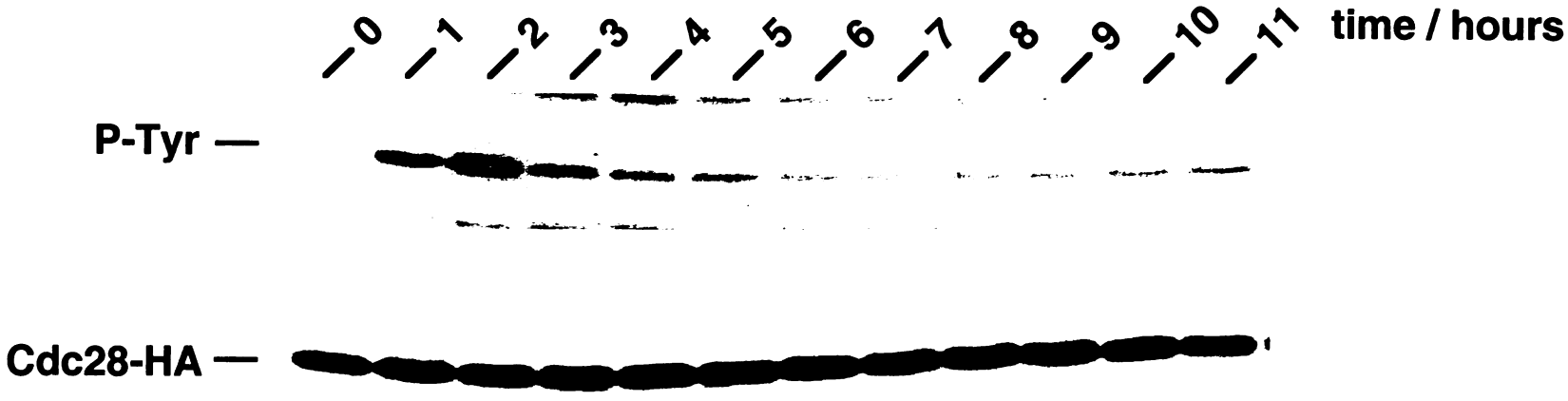

GAL-CDC28 cdc23-1
mad2Δ bub2Δ
GAL-CDC28 cdc23-1
α f 1 2 3 4 5 6 7
time /hours
Clb2
Sic1
GAL-CDC28-VF cdc23-1
mad2 Δ bub2Δ
GAL-CDC28-VF cdc23-1
mad2 Δ
GAL-CDC28-VF cdc23-1
α f 1 2 3 4 5 6 7
time /hours
Clb2
Sic1

Figure 8 Tyrosine phosphorylation on Cdc28 is not induced as cells exit mitosis

GAL-MPS1 (ADR1088) cells were grown overnight at 23°C in YEP+2% raffinose to log phase, arrested in G1 with alpha factor (1μg/ml) for 3.5 hours and at t=0 released from the G1 arrest into fresh YEP+4% galactose. After cells had budded (t=3), alpha factor (1.5μg/ml) was added back to the cultures to re-arrest the cells in the next G1. Cells were harvested at the indicated times, lysed and Cdc28-HA was immunoprecipitated with 12CA5. The immunoprecipitates were run out on a polyacrylamide gel and probed either with a phosphotyrosine antibody (top) or with 12CA5 (bottom) as a control for equal loading.

Figure 8



arrested in raffinose and alpha factor → alpha factor washed out and galactose added (t=0) → alpha factor added back (t=3)

Figure 9 Some *hct1Δ* cells induce inhibitory phosphorylation on Cdc28

(A) *cdc15-2* (ADR1459) and *hct1Δ cdc15-2* (ADR1458) were grown overnight at 23°C in YPD to mid log phase and the cultures were shifted to 37°C for 3.5 hours to arrest the cells in anaphase. The cultures were then rapidly cooled to 23°C (t=0) and alpha factor (10μg/ml) to the cultures to arrest cells that had exited mitosis. Duplicate samples of cells were harvested at 10 minute intervals. One set of samples was lysed and Cdc28-HA was immunoprecipitated with 12CA5. The immunoprecipitates were run out on a polyacrylamide gel and probed either with a phosphotyrosine antibody, 12CA5 antibody or an anti-Clb2 antibody. The other set of samples was lysed and Clb2 was immunoprecipitated with an anti-Clb2 antibody and the immunoprecipitates tested for their ability to phosphorylate histone H1.

(B) This experiment is identical to the experiment in (A), but *hct1Δ cdc15-2* (ADR1460) was used instead of *hct1Δ cdc15-2* (ADR1458)

(C) This experiment is identical to the experiment in (A), but samples were harvested only at t=0,30 and 60 minutes. Three different *hct1Δ cdc15-2* strains were used (ADR1458, ADR1460 and ADR1461).

Figure 9

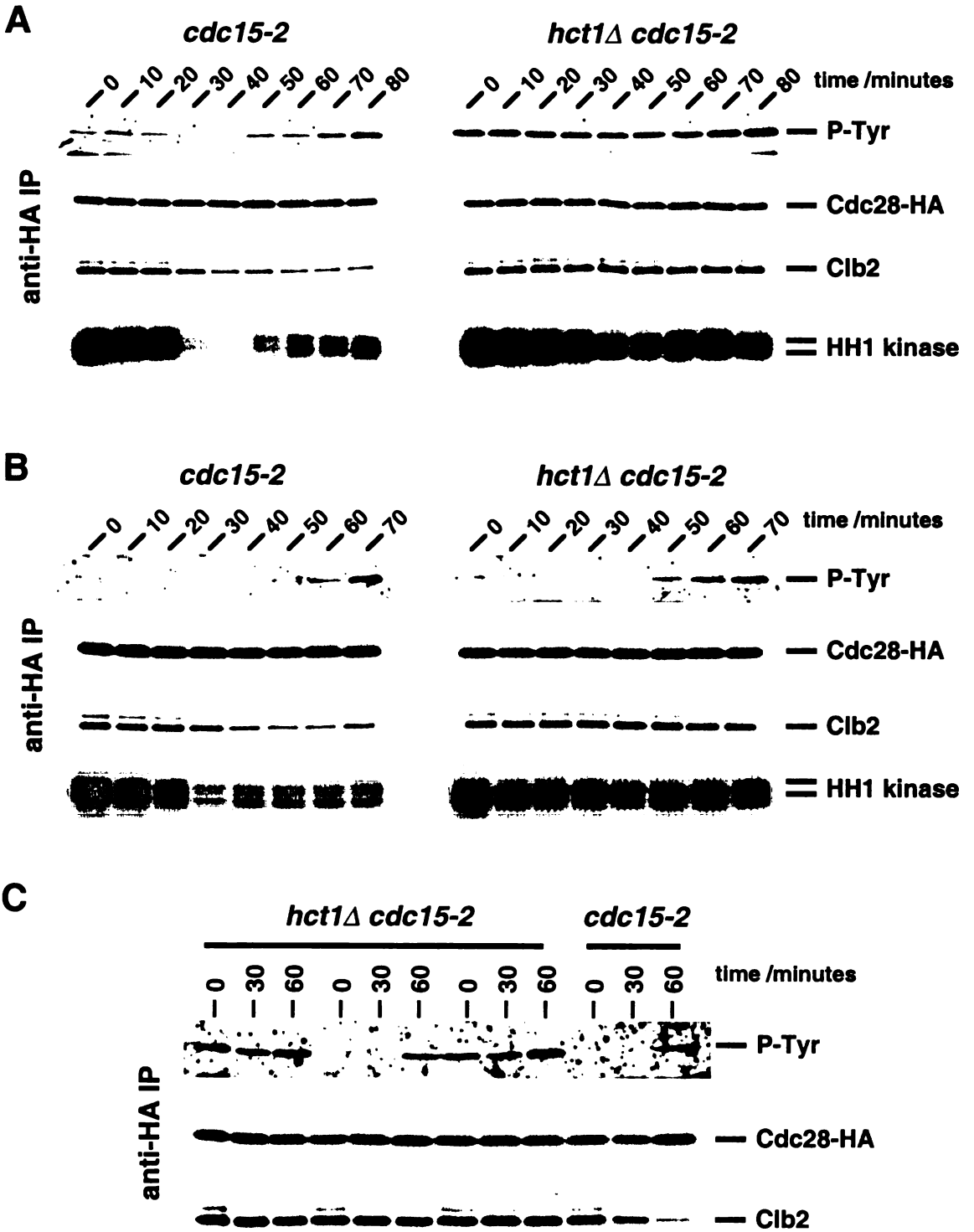
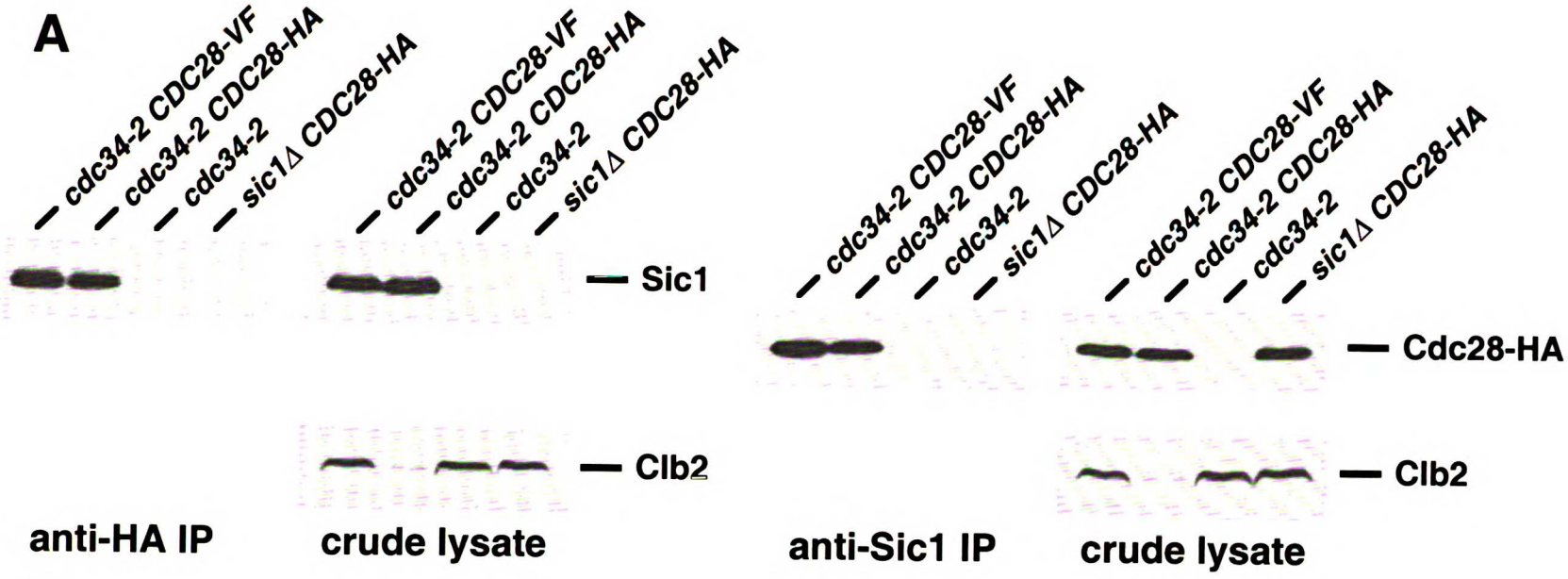

A
cdc15-2
hct1Δ cdc15-2
0 10 20 30 40 50 60 70 80
time /minutes
P-Tyr
anti-HA IP
Cdc28-HA
Clb2
HH1 kinase
B
cdc15-2
hct1Δ cdc15-2
0 10 20 30 40 50 60 70
time /minutes
P-Tyr
anti-HA IP
Cdc28-HA
Clb2
HH1 kinase
C
hct1Δ cdc15-2
cdc15-2
0 30 60 0 30 60 0 30 60 0 30 60
time /minutes
P-Tyr
anti-HA IP
Cdc28-HA
Clb2

Figure 10 Cdc28-VF is not defective in binding to Sic1

(A) Cdc28-VF binds to Sic1. *cdc34-2 CDC28-VF-HA* (ADR1575), *cdc34-2 CDC28-HA* (ADR1571), *cdc34-2* (AFS129) and *sic1Δ CDC28-HA* (ADR1567) were grown overnight at 23°C in YPD to mid log phase and the cultures were shifted to 37°C for 3.5 hours to arrest the cells before S-phase. Cells were harvested, lysed and Cdc28-HA was immunoprecipitated with 12CA5 and Sic1 was immunoprecipitated with anti-Sic1 antibodies. The immunoprecipitates were then immunoblotted for Sic1 and Cdc28-HA respectively. The crude lysate used in the immunoprecipitations was also immunoblotted for Sic1, Clb2 and Cdc28-HA.

(B) Checkpoint hypersensitivity in *CDC28-VF* is not caused by a combination of defects in inhibitory phosphorylaiton and Sic1 binding. *GAL-MPS1* (KH153), *CDC28-VF GAL-MPS1* (KH181), *swe1Δ* (KH207), *mih1Δ* (ADR1378), *sic1Δ* (ADR909), *swe1Δ sic1Δ* (ADR1612) were grown to saturation for two days in YEP+ 2% glucose at 30°C , diluted ten-fold and four-fold serial dilutions were prepared in a multi-well dish and spotted onto YEP+2% glucose (left) or YEP+2% galactose (right). The plates were incubated at 30°C for 2 days.

Figure 10


A
cdc34-2 CDC28-VF
cdc34-2 CDC28-HA
cdc34-2
sic1Δ CDC28-HA
Sic1
Clb2
anti-HA IP
crude lysate
Cdc28-HA
Clb2
anti-Sic1 IP
crude lysate

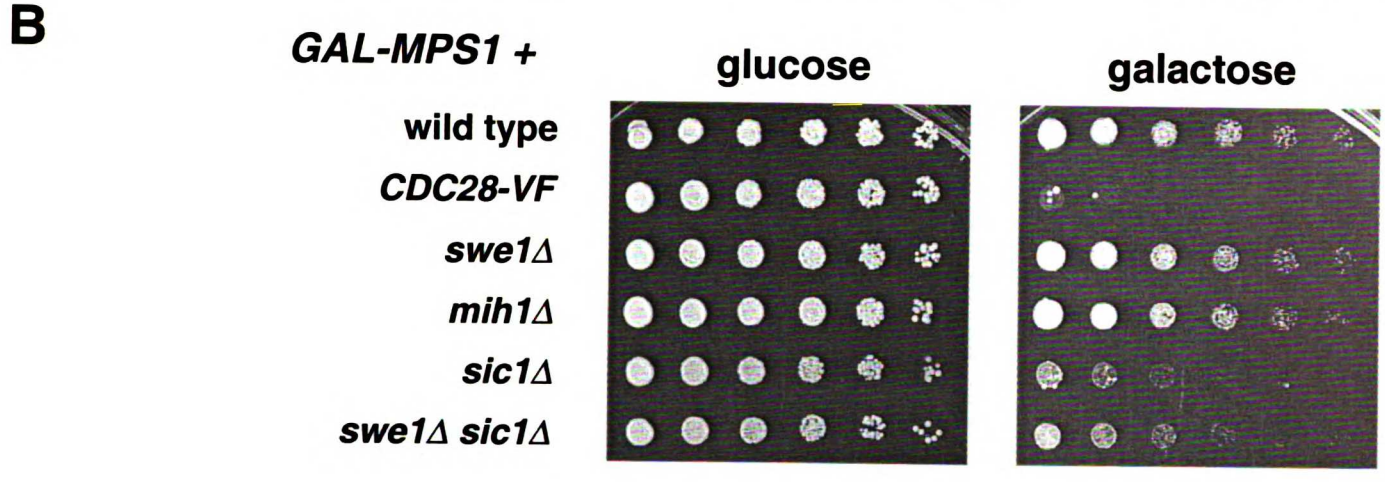

B
GAL-MPS1 +
glucose
galactose
wild type
CDC28-VF
swe1Δ
mih1Δ
sic1Δ
swe1Δ sic1Δ

Figure 11 Cdc28-VF is defective in Clb2 phosphorylation

(A) Clb2 is modified on Cdc28 immunoprecipitates. Wild type (JM425) and *CDC28-VF* (JM434) were grown overnight at 23°C in YPD to mid log phase, arrested in mitosis with nocodazole (10μg/ml) for 3 hours, harvested, lysed and Cdc28-HA/Clb2 was precipitated with either Cks1-coupled beads (left) or 12CA5 (right). Immunoprecipitates were washed extensively, incubated with or without ATP and immunoblotted for Clb2.

(B) Clb2 modifications are phosphorylation. Cdc28-HA/Clb2 was precipitated with Cks1-coupled beads and incubated with ATP as in (A). The beads were then washed and treated with lambda phosphatase, lambda phosphatase and inhibitors alone. The precipitates were then immunoblotted for Clb2.

(C) Cks1 stimulates Clb2-MBP phosphorylation by Cdc28. Purified Cdc28-His_6 and Clb2-MBP were incubated with [γ-^{32}P]-ATP with or without Cks1 protein, and run on a polyacrylamide gel which was subjected to autoradiography.

Figure 11

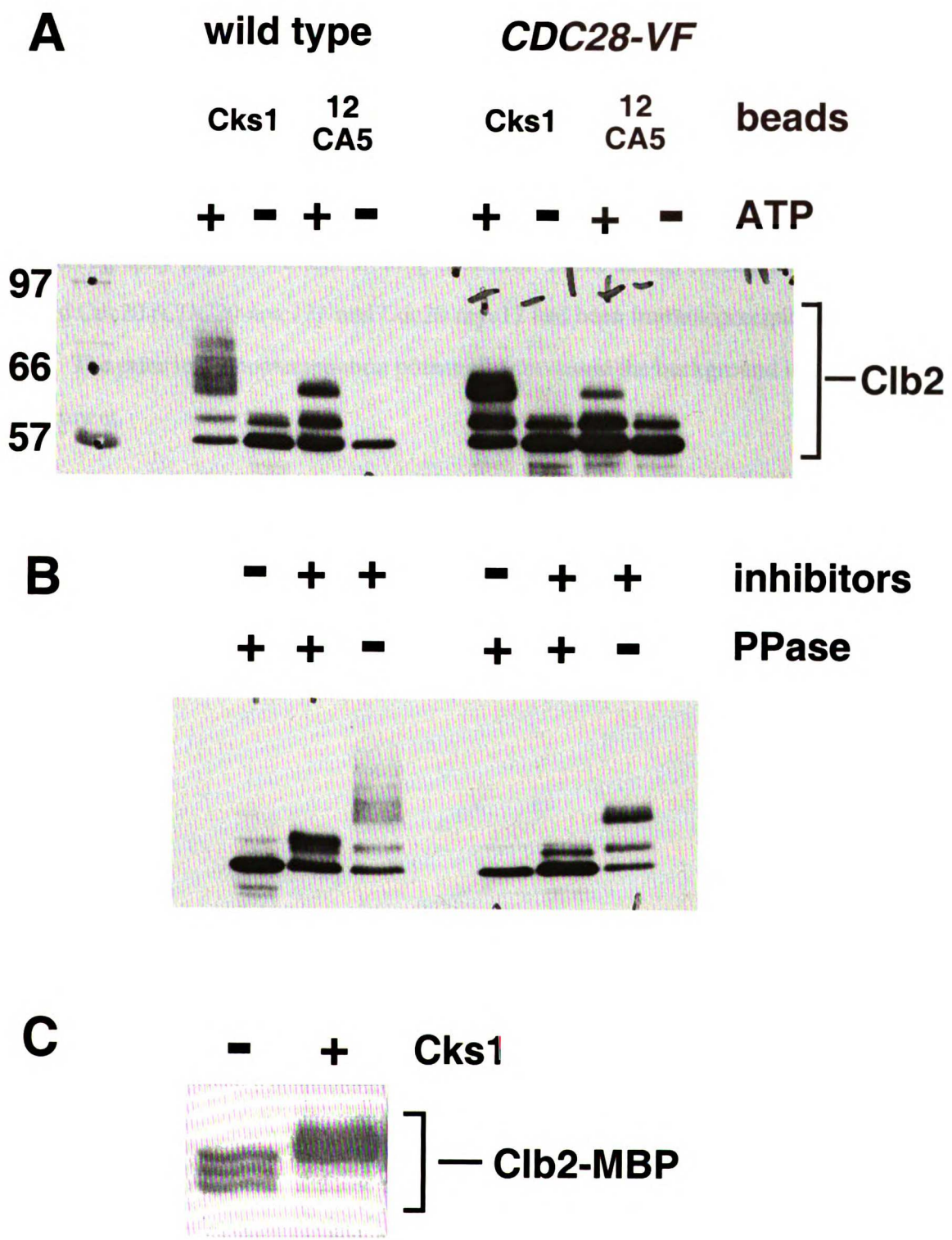
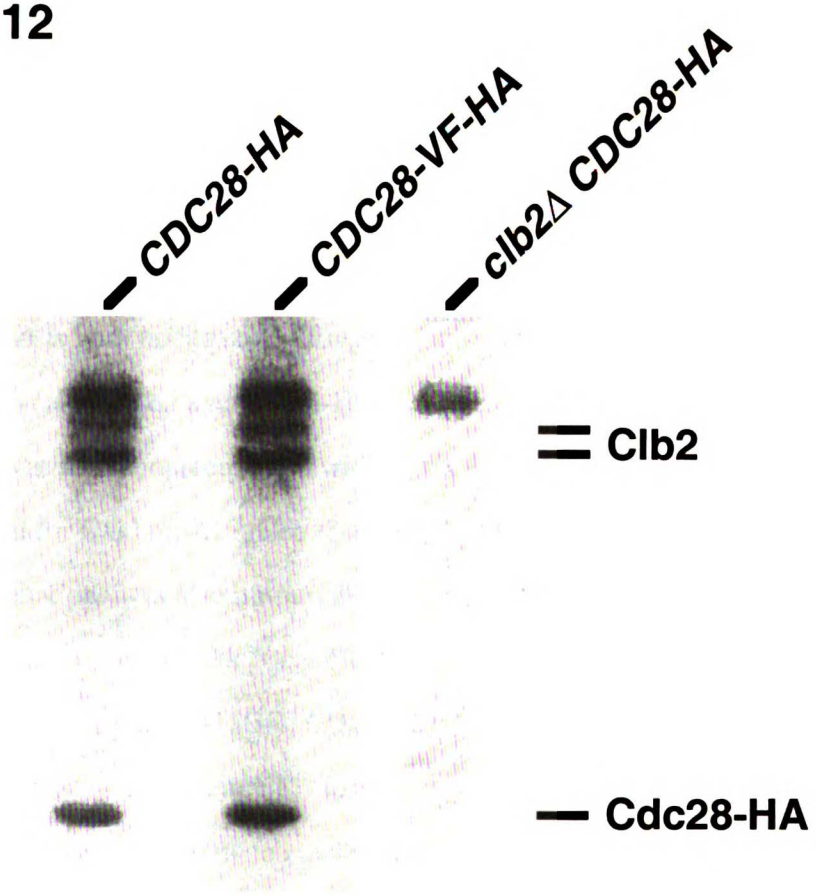

A
wild type
CDC28-VF
Cks1
12 CA5
Cks1
12 CA5
beads
+ - + - + - + - ATP
97
66
57
Clb2
B
- + + - + + inhibitors
+ + - + + - PPase
C
- + Cks1
Clb2-MBP

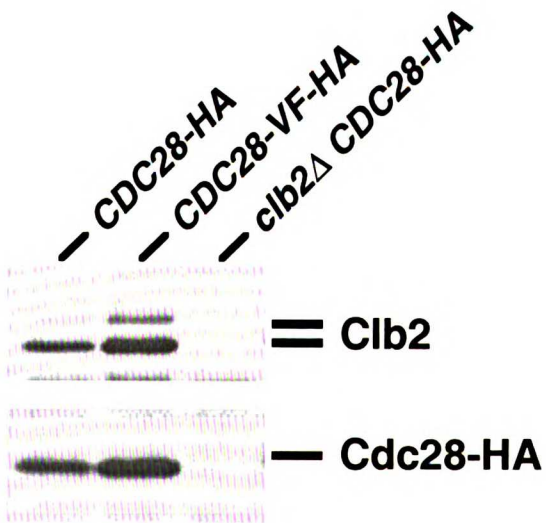
Figure 12 Clb2 is phosphorylated in vivo

cdc15-2 CDC28-HA (ADR1790), *cdc15-2 CDC28-VF* (ADR1793) and *clb2Δ CDC28-HA cdc15-2* (ADR1988) were grown overnight in YEP+2% glucose at 23°C to log phase. The cultures were shifted to 37°C and for four hours and then the cells were transferred to phosphate free-CSM+2% glucose containing $^{32}PO_4$ and were kept at 37°C. After 1 hour cells were harvested, lysed and Clb2 immunoprecipitated with anti-Clb2 antibodies. Immunoprecipitates were run on a polyacrylamide gel which was subjected to either autoradiography (top) or western blotting (bottom). These strains contain an epitope tagged Cdc20 *(CDC20-myc12)* and Cdc20-myc12 had been immunoprecipitated prior to Clb2. The prior immunoprecipitation potentially decreased the background in this experiment.

Figure 12



in vivo labeling



IP western

Figure 13 Cdc28-VF is not defective in Cks1 binding and does not bind Cdc20

(A) Cdc28-VF binds to Cks1. *CDC28-HA CKS1-myc13* (ADR1707), *CDC28-VF-HA CKS1-myc13* (ADR1711), *CKS-myc13* (ADR1708) and *CDC28-HA* (JM425) were grown overnight at 23°C in YPD to mid log phase and the cultures were split and either arrested in mitosis with nocodazole (10μg/ml) for 3 hours or left to grow asynchrounously. Cells were harvested, lysed and Cdc28-HA was immunoprecipitated with 12CA5 and Cks1-myc13 was immunoprecipitated with 9E10. The immunoprecipitates were then immunoblotted for Cks1-myc13, Cdc28-HA and Clb2. The crude lysate used in the immunoprecipitations was also immunoblotted for Sic1 and Cdc28-HA.

(B) Cdc28 does not bind to Cdc20. *CDC28-HA CDC20-myc12* (ADR1802), *CDC28-VF-HA CDC20-myc12* (ADR1804), *CDC20-myc12* (ADR1689) and *CDC28-HA* (JM425) were grown overnight at 23°C in YPD to mid log phase and the cultures were split and either arrested in mitosis with nocodazole (10μg/ml) for 3 hours or left to grow asynchrounously. Cells were harvested, lysed and Cdc28-HA was immunoprecipitated with 12CA5 and CDc20-myc12 was immunoprecipitated with 9E10. The immunoprecipitates were then immunoblotted for Cdc20-myc12, Clb2 and Cdc28-HA. The crude lysate used in the immunoprecipitations was also immunoblotted for Cdc20-myc12, Clb2 and Cdc28-HA.

Figure 13

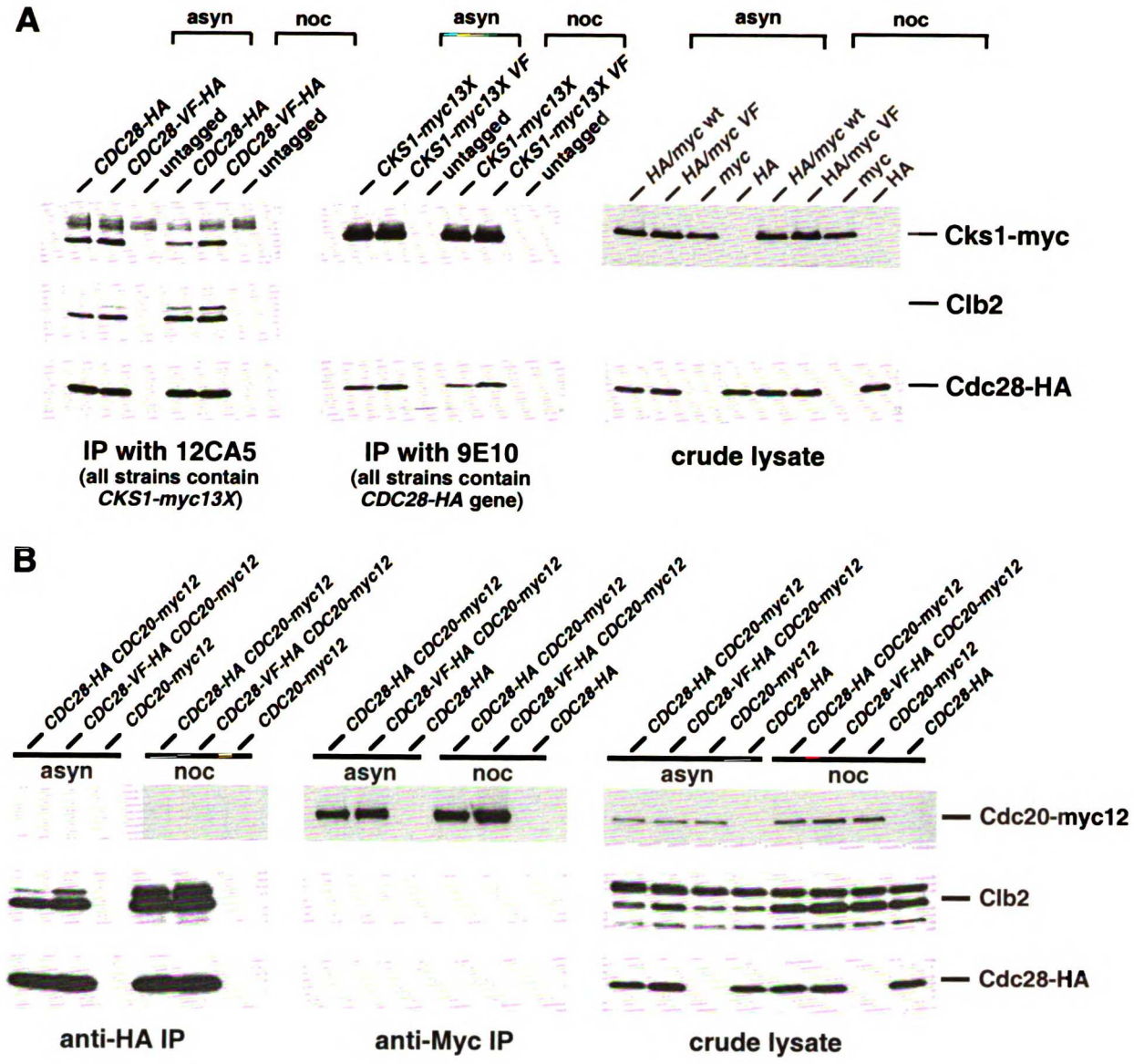

A
asyn
noc
asyn
noc
asyn
noc
CDC28-HA
CDC28-VF-HA
untagged
CDC28-HA
CDC28-VF-HA
untagged
CKS1-myc13X
CKS1-myc13X VF
untagged
CKS1-myc13X
CKS1-myc13X VF
untagged
HA/myc wt
HA/myc VF
myc
HA
HA/myc wt
HA/myc VF
myc
HA
Cks1-myc
Clb2
Cdc28-HA
IP with 12CA5
(all strains contain
CKS1-myc13X)
IP with 9E10
(all strains contain
CDC28-HA gene)
crude lysate
B
CDC28-HA CDC20-myc12
CDC28-VF-HA CDC20-myc12
CDC20-myc12
CDC28-HA
asyn
noc
Cdc20-myc12
Clb2
Cdc28-HA
anti-HA IP
anti-Myc IP
crude lysate

Figure 14 A curious modification on Cdc28 that is not found on Cdc28-VF

(A) Cdc28-HA is modified. Four isolates of *pGAL-CDC28-HA* (ADR1054) were grown overnight at 23°C in YEP+2% raffinose to log phase, transferred to fresh YEP+2% galactose to induce expression of *pGAL-CDC28-HA*. Cells were harvested after four hours of growth, lysed and immunoblotted for Cdc28-HA using 12CA5 and anti-PSTAIRE antibody.

(B) Cdc28-VF-HA is not modified. *pGAL-CDC28-HA* (ADR1054) and *pGAL-CDC28-VF-HA* (ADR1052) were grown as in (A) and cell lysates were immunoblotted with 12CA5.

Figure 14

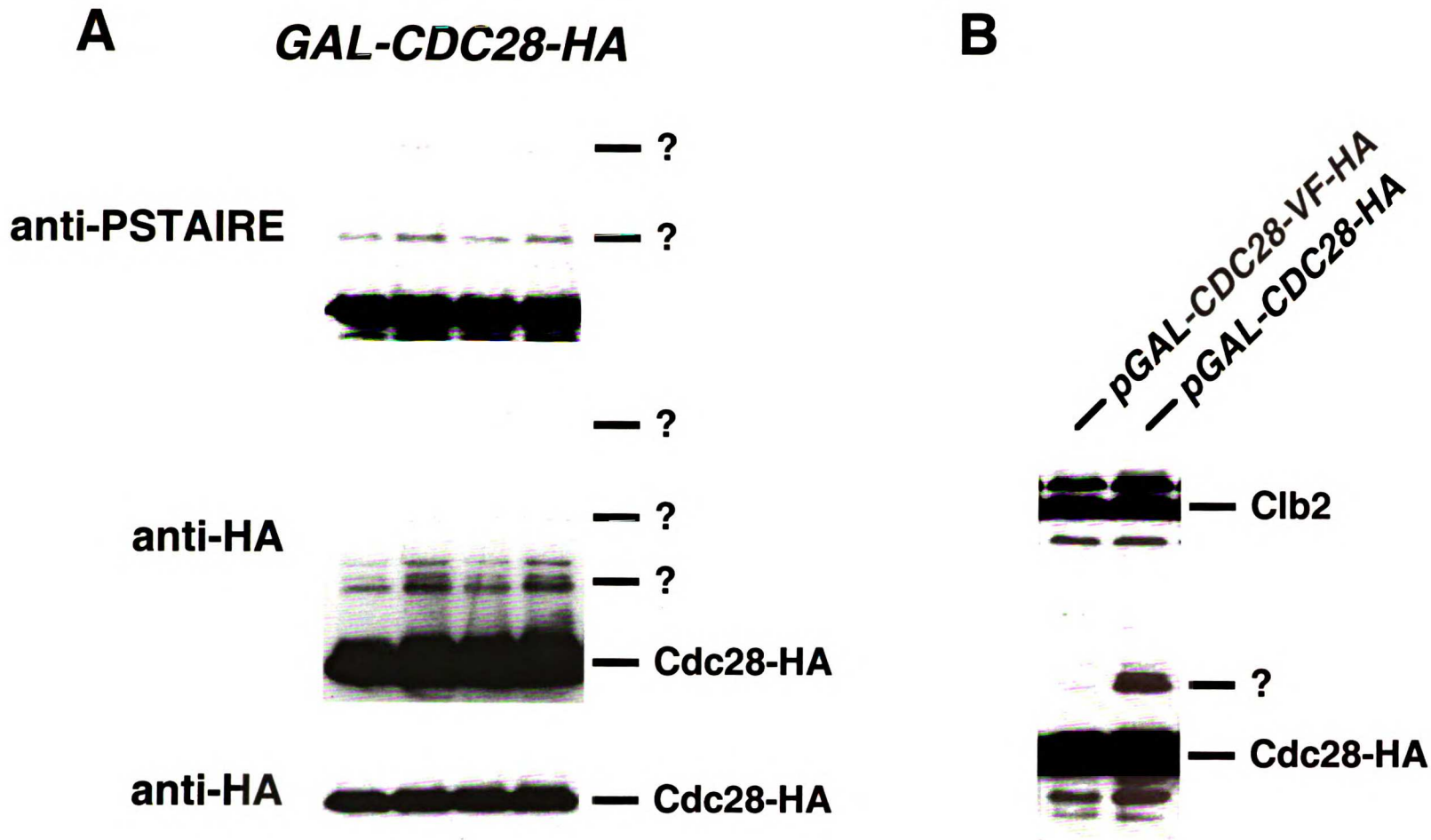

A
GAL-CDC28-HA
anti-PSTAIRE
anti-HA
anti-HA
?
?
?
?
Cdc28-HA
Cdc28-HA
B
pGAL-CDC28-VF-HA
pGAL-CDC28-HA
Clb2
?
Cdc28-HA

Table 1. Interactions between *CDC28-VF* and spindle mutants

STRAIN	Temperature	Un-budded	Small Budded	**Large Budded[a]**	No Spindle	**Short Spindles[b]**	Long spindles
wild type	23°C	28 ± 2.6	53 ± 0.0	**16 ± 3.0**	63 ± 1.8	**21 ± 0.5**	14 ± 1.0
	37°C	36 ± 8.1	49 ± 6.2	**15 ± 2.2**	67 ± 7.6	**20 ± 5.8**	12 ± 3.5
cdc28-VF	23°C	21 ± 2	48.3 ± 2.5	**29 ± 1.0**	48 ± 6.9	**40 ± 4.0**	10 ± 3.4
	37°C	33 ± 3.3	40 ± 1.3	**27 ± 4.2**	60 ± 6.7	**34 ± 7.8**	4 ± 1.7
cin8Δ	23°C	20 ± 4.0	42 ± 7.5	**35 ± 5.6**	49 ± 5.2	**43 ± 6.1**	7 ± 0.9
	37°C	30 ± 6.6	39 ± 0.9	**30 ± 5.2**	62 ± 5.8	**35 ± 4.0**	2 ± 1.8
cin8Δ cdc28-VF	23°C	18 ± 4.9	22 ± 2.2	**56 ± 5.2**	30 ± 3.6	**62 ± 2.0**	3 ± 0.5
	37°C	24 ± 5.2	15 ± 3.6	**61 ± 4.7[c]**	7 ± 2.5	**92 ± 3.1[c]**	2 ± 1.2
kip1Δ	23°C	28 ± 6.4	51 ± 3.1	**20 ± 3.5**	64 ± 6.8	**27 ± 4.0**	7 ± 2.7
kip1Δ cdc28-VF	23°C	21 ± 7.0	39 ± 2.4	**38 ± 4.8**	46 ± 1.7	**43 ± 2.4**	9 ± 2.0
cin1Δ	23°C	25 ± 6.3	44 ± 2.1	**30 ± 6.6**	61 ± 4.1	**31 ± 3.3**	6 ± 1.8
cin1Δ cdc28-VF	23°C	18 ± 3.3	23 ± 3.7	**55 ± 3.7**	41 ± 4.8	**48 ± 7.0**	7 ± 5.2

[a] Large budded cells have mother and daughter of equal size.

[b] Short spindles are bipolar spindles less than 2 μm long. Collapsed spindles sometimes seen in *cin8Δ* and *cin8Δ CDC28-VF* cells are counted as short spindles.

[c] *cin8 CDC28-VF* cells at 37°C contain many anucleate un-budded and small budded cells that contain no microtubule structures. This accounts for the large discrepancy in cells with short spindles (92%) and cells which are large budded (61%).

Asynchronous exponentially growing cultures in rich medium (YEP+2% dextrose) were fixed and scored for tubulin immunofluorescence. Some data sets total less then 100% because of cells that could not be scored because of clumping. Each data point represents at least three sets of 100 cells counted and the error shown is two standard errors from the mean.

Appendix 2

Supplemental data for Chapter 2

Introduction

This appendix contains supplemental data for Chapter 2. Phosphorylation of the APC is important for its activation by Cdc20. The next step in understanding the role of APC phosphorylation will be to explain how Cdc5 and Cdc28 work together to phosphorylate the APC and to understand the Cks1-dependence of APC phosphorylation. In addition, developing in vitro assays for Cdc20 binding to and activation of the APC, will allow a more detailed understanding of how Cdc20 activates the APC, and could also be used to understand how Mad2 inhibits Cdc20-dependent APC activity.

Results

Apc9 is phosphorylated in vivo

In Figure 8 of Chapter 2, I showed that purified Cdc5 phosphorylates a protein that migrates at 42 kD which is Apc9. I have also shown that purified Cdc28/Clb2/Cks1 complexes phosphorylate the same protein at 42kD. A myc tagged Apc9, as well as a HA tagged Apc9, immunoprecipitates with other APC subunits in an anti-Cdc26 immunoprecipitate (Figure 1A). Figure 1B shows that Cdc28 phosphorylation of an APC containing an epitope tagged Apc9 causes the phosphorylated 42kD protein to now run at slower mobility, confirming its identity as Apc9. Less APC immunoprecipitates from the *APC9-HA3* cells in this experiment, suggesting that it does not supply full Apc9 activity, and APC assembly is compromised. This has been reported as the phenotype of the *apc9Δ* (Zachariae et al., 1996).

Is Apc9 phosphorylated in vivo? I have detected weak labeling of Apc9 in in vivo labelings of the APC (Figure 3; and data not shown). I wondered if this small of phosphorylation could be detected by western blotting of the tagged Apc9. *APC9-myc13* cells were arrested in G1 with alpha factor and then released into fresh medium. Alpha factor was re-added after cells had budded to arrest the cells in the subsequent G1. In addition, a small sample of cells were released in fresh medium containing nocodazole to arrest the cells by the spindle checkpoint. No changes in the mobility of Apc9-myc13 are seen through the cell cycle, though Apc9-myc13 does migrate with slower mobility in nocodazole treated cells (Figure 2). I suspect this modification is phosphorylation, but this has not been confirmed.

Apc1, 4 and 5 phosphorylation may be reduced in *cdc5-1* mutants.

Figure 8 of Chapter 2 shows that purified Cdc5 phosphorylates proteins that may be Apc1, Apc4 and Apc5. In support of this idea, in one in vivo labeling I detected weak phosphorylation of proteins that migrate at the molecular weights of Apc1, 4 and 5, and are not phosphorylated in a *cdc5-1* mutant (Figure 3A). Phosphorylation of these proteins was not detected in all in vivo labelings, though resolving the phosphorylated proteins on a long protein gel appears to help (Figure 9A, Chapter 3). In vivo labeling of cells overexpressing Cdc5 may allow confirmation of the identities of these proteins.

Cdc5 binds Cks1 beads

By chance I discovered that Cks1-coupled beads bind Cdc5 (Figure 3A). Cdc5 binding does not depend on APC binding to the Cks-coupled beads, because the same amount of Cdc5 binds when it is isolated from a strain containing Cdc27-5A, which prevents APC binding to the Cks1-coupled beads (see Chapter 2, Figure 3 and 4). The relevance of this binding is unknown. Cks1 does not enhance Cdc5 phosphorylation of the APC in vitro (data not shown), but it may for other substrates. *cdc5-ad* is defective in adaptation to the DNA damage checkpoint, but has wild type levels of Cdc5 kinase activity in vitro (Charles et al., 1998; Toczyski et al., 1997). Might its binding to Cks1 be defective?

Cdc28 purified from yeast phosphorylates the APC

Before I used insect cell purified Cdc28 and bacterial expressed Clb2 for in vitro phosphorylation of the APC, I developed a protocol for isolating a small amount of soluble active Cdc28 from yeast cells. Cdc28/Clb and Cdc28-VF/Clb complexes are isolated from

nocodazole arrested cells and purified on Cks1 beads. The beads were washed extensively, and then Cdc28 was eluted with excess pure Cks1. The soluble kinase was active against Histone H1 and contained Clb2 (Figure 4A). Silver staining of the purified kinase, though, revealed that many contaminating proteins were also present (data not shown). The purified Cdc28 and Cdc28-VF were used to phosphorylate anti-Cdc26 immunoprecipitates, and like later experiments (for example, Chapter 2, Figure 1), Cdc16, Cdc27 and Apc9 were well phosphorylated (Figure 4B). This experiment also allowed me to test whether Cdc28-VF can phosphorylate the APC in vitro. Equal amounts of histone H1 kinase activity were used to phosphorylate the APC, and roughly equal amounts of APC phosphorylation was detected. Thus, histone H1 and the APC behave as similar substrates for Cdc28 and Cdc28-VF.

CDC27-5A is sensitive to checkpoint arrest

In Figure 6 of Chapter 2, I showed that phosphorylation site mutants in APC subunits delay passage through mitosis and are sensitive to high levels of Mps1. I have also examined whether *CDC27-5A* cells delay exit from an Mps1 induced checkpoint arrest. Wild type, *CDC28-VF*, *CDC27-5A* and *CDC28-VF CDC27-5A* cells were arrested in G1 by alpha factor, and then released into the cell cycle on galactose plates, which cause induction of high levels of Mps1. All four strains initially arrest in mitosis for eight hours, after which wild type begins to re-bud (Figure 5). *CDC28-VF* and *CDC27-5A* are delayed relative to wild type by about 3-4 hours, and *CDC28-VF CDC27-5A* cells are delayed by an additional 2 hours. Although *CDC28-VF* and *CDC27-5A* appear to have different sensitivities to high levels of Mps1 on plates (Figure 6B, Chapter 3), by this assay, they appear identical. Why does *CDC28-VF CDC27-5A* have a stronger phenotype than *CDC28-VF* or *CDC27-5A*? The phosphorylation of other APC subunits are educed in the *CDC28-VF* mutant, and this probably contributes, but *CDC28-VF* probably has additional

Figure 1 Apc9 is phosphorylated by Cdc28 in vitro

(A) Tagged Apc9 is functional. Wild type (ADR376), *APC9-HA3* (ADR2042) and *APC9-myc13* (ADR2039) were grown overnight at 30°C in YEP+2% glucose to log phase, arrested in G1 with alpha factor (1μg/ml) for 2.5 hours. The cells were harvested, lysed and the APC immunoprecipitated with anti-Cdc26 antibody. The immunoprecipitates immunoblotted for Apc9-HA3 and Cdc16 (left) and Apc9-myc13 and Cdc23 (right).

(B) The three strains in (A) and*cdc26Δ* (LH307) were treated as in (A). The immunoprecipitates were treated with purified Cdc28-His_6, Clb2-MBP, Cks1 and [γ-^{32}P]-ATP, washed to remove phosphorylated Clb2-MBP, and run on a polyacrylamide gel which was subjected to autoradiography.

Figure 1

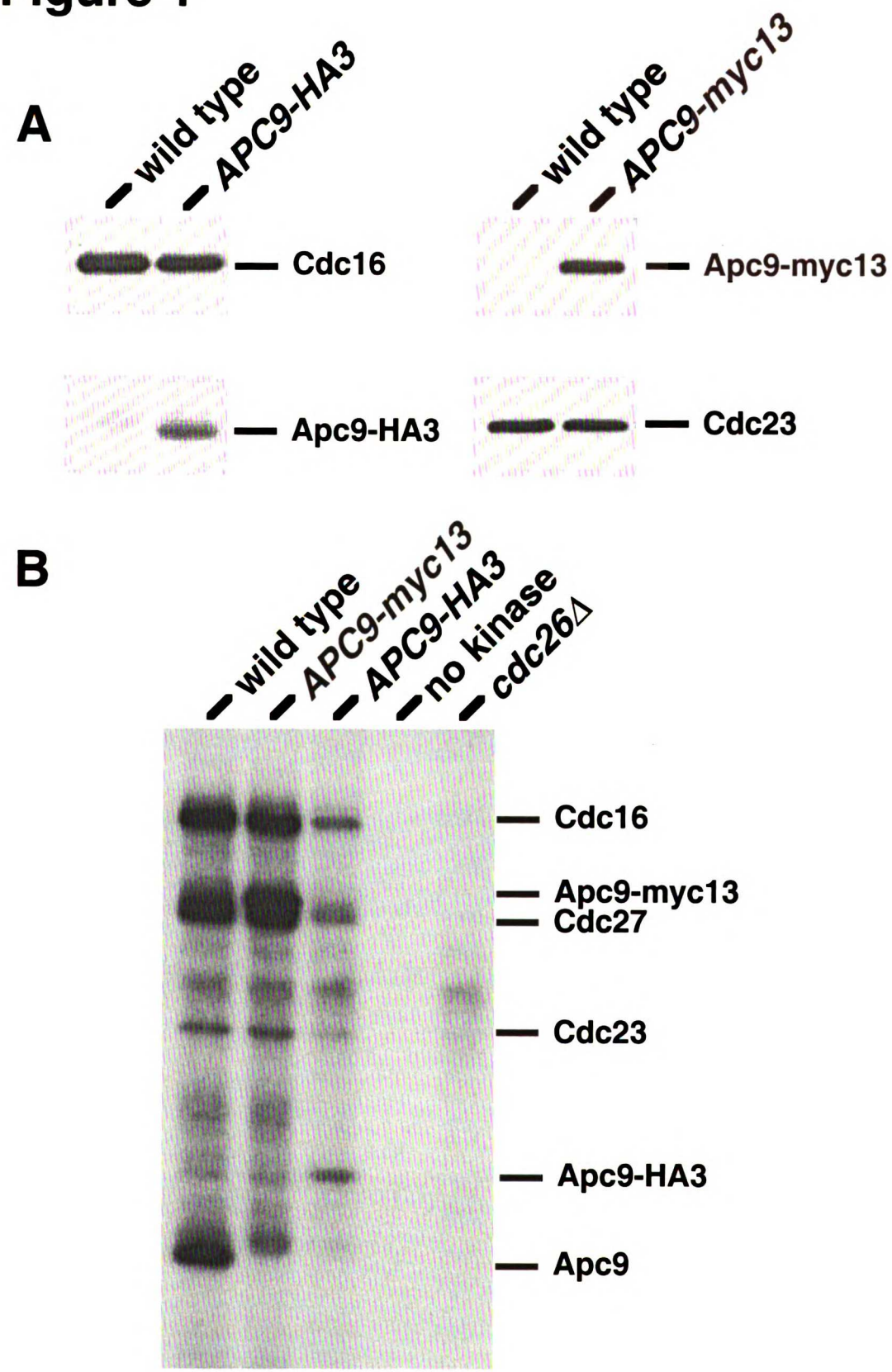

A
wild type
APC9-HA3
Cdc16
Apc9-HA3
wild type
APC9-myc13
Apc9-myc13
Cdc23
B
wild type
APC9-myc13
APC9-HA3
no kinase
cdc26Δ
Cdc16
Apc9-myc13
Cdc27
Cdc23
Apc9-HA3
Apc9

Figure 2 Apc9 may be phosphorylated in vivo

APC9-myc13 (ADR2039) were grown overnight at 23°C in YPD to mid log phase, arrested in G1 with alpha factor (1μg/ml) and at t=0 the cells were released from the G1 arrest. At t=90, alpha factor (1.5μg/ml) was added back to the cultures to re-arrest the cells in the next G1. A portion of the overnight cultures was arrested in mitosis with nocodazole (10μg/ml) for 2.5 hours. Samples were taken at the indicated times, lysed and immunoblotted for Apc9-myc13, Cdc27, Clb2, and Sic1.

Figure 2


-noc
-0
-40
-60
-80
-100
-120
-140
time/minutes
—Apc9-myc13
=Cdc27
—Clb2
—Sic1

Figure 3 Cdc5 may phosphorylate Apc1,4 and 5, and binds to Cks1-coupled beads

(A) The experiment shown in Chapter 2, Figure 2C was subjected to autoradiography for an extended time. Only wild type, *cdc5-1* and *cdc26Δ* lanes are shown. *CDC28-VF* and *cdc28-1N* show phosphorylation of the bands believed to be Apc1, 4 and 5 (data not shown)

(B) *CDC5-3XHA* (ADR1699) and *CDC5-3XHA CDC27-5A* (ADR2053) were grown overnight at 23°C in YPD to mid log phase, arrested in mitosis with nocodazole (10μg/ml), harvested and lysed. The lysate was mixed with Cks1-coupled beads and then the bead bound protein was immunoblotted for Cdc5-3XHA and Cdc27. A portion of the starting lysate was also immunoblotted directly for Cdc5-3XHA and Cdc27.

Figure 3

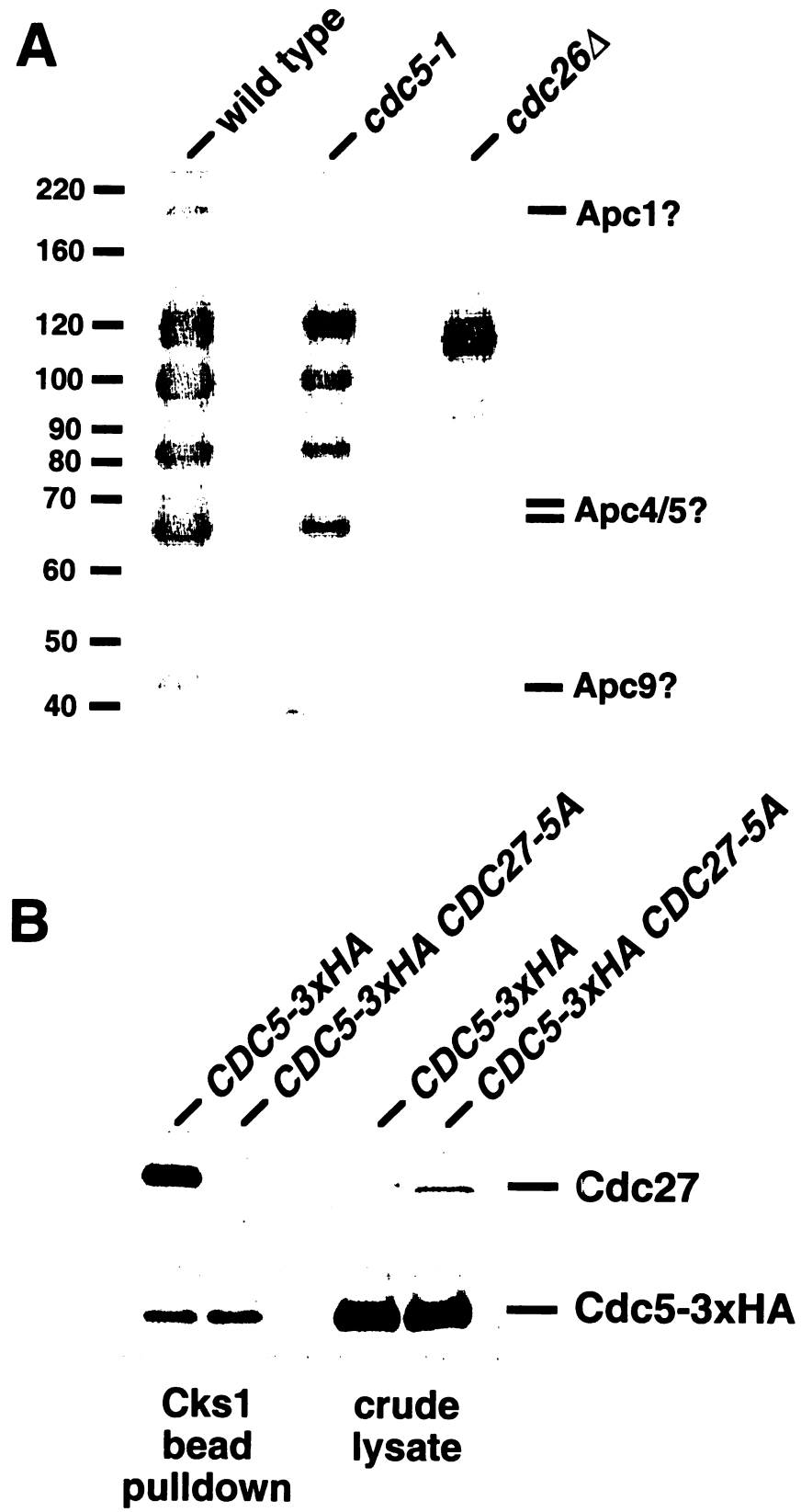
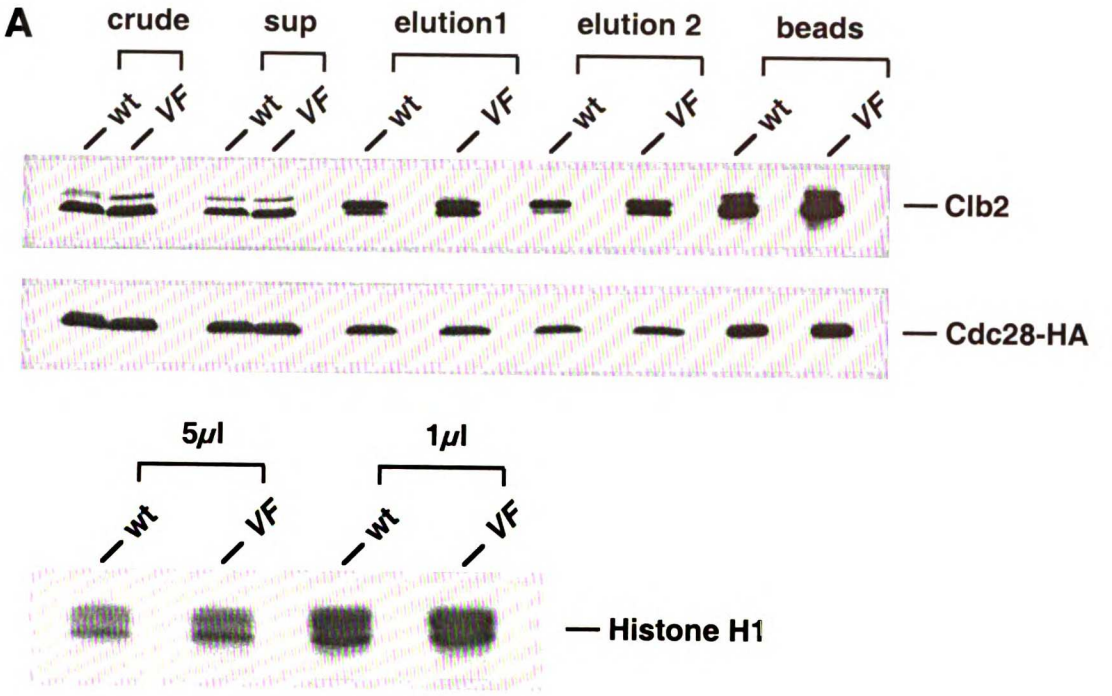

A
wild type
cdc5-1
cdc26Δ
220
160
120
100
90
80
70
60
50
40
Apc1?
Apc4/5?
Apc9?
B
CDC5-3xHA
CDC5-3xHA CDC27-5A
CDC5-3xHA
CDC5-3xHA CDC27-5A
Cdc27
Cdc5-3xHA
Cks1 bead pulldown
crude lysate

Figure 4 Cdc28/Clb2 complexes can be purified from Cks1-coupled beads

(A) *CDC28-HA* (JM425) and *CDC28-VF-HA* (JM434) were grown overnight at 23°C in YPD to mid log phase, arrested in mitosis with nocodazole (10μg/ml), harvested and lysed. The lysate was mixed with Cks1-coupled beads, the beads washed extensively, and eluted twice with pure Cks1 protein. The starting lysate (crude), the supernatant after the bead treatment (sup), the two eluates and the beads were immunoblotted for Clb2 and Cdc28-HA (top). Two concentrations of the eluate was tested for their ability to phosphorylate histone H1 (bottom).

(B) Cdc28 and Cdc28-VF phosphorylate Cdc27 in vitro. The APC was isolated from wild type (ADR376) and *CDC27-MBP* (ADR1705) as described in Figure 1. The purified APC was treated with Cdc28/Clb2 or the Cdc28-VF/Clb2 complexes purified in (A). As controls, no anti-Cdc26 antibody was used to precipitate the APC (-Ab) and no Cdc28 or Cdc28-VF kinase was added to APC (-Cdc28).

Figure 4


A
crude
sup
elution1
elution 2
beads
wt
VF
Clb2
Cdc28-HA
5μl
1μl
Histone H1

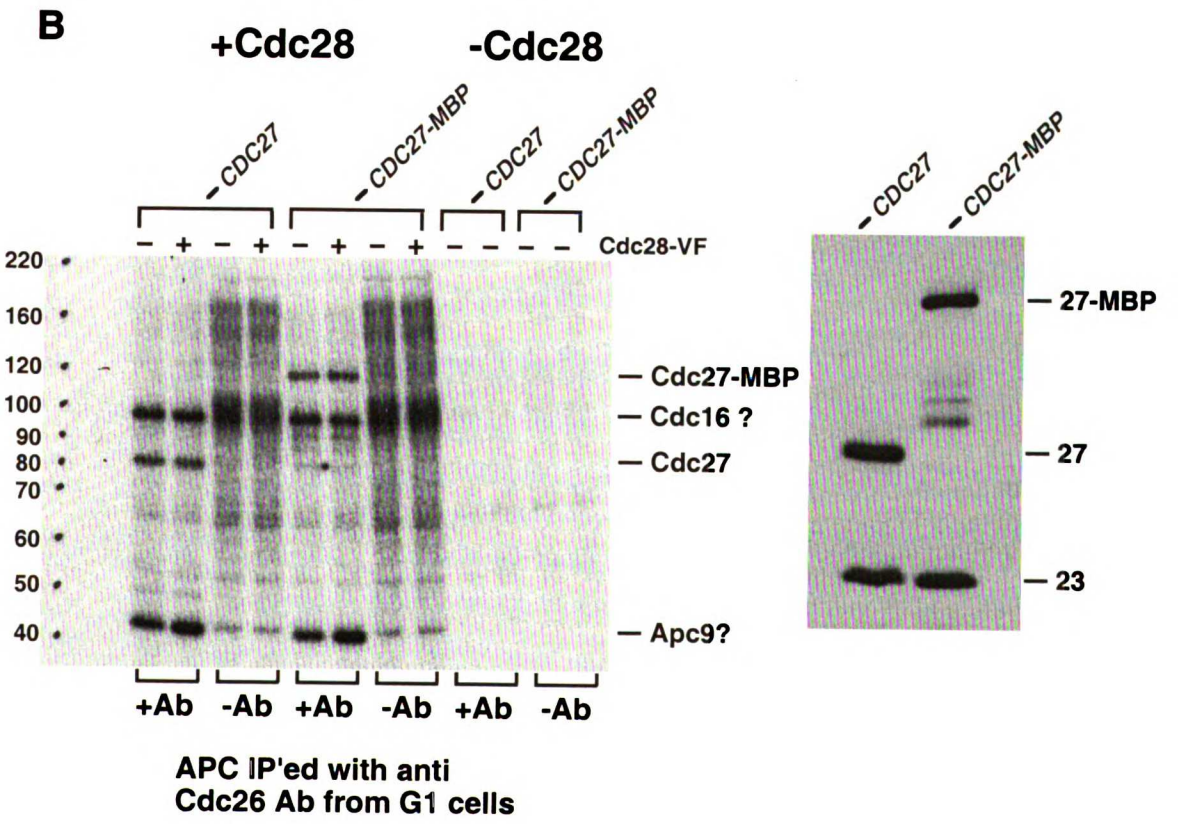
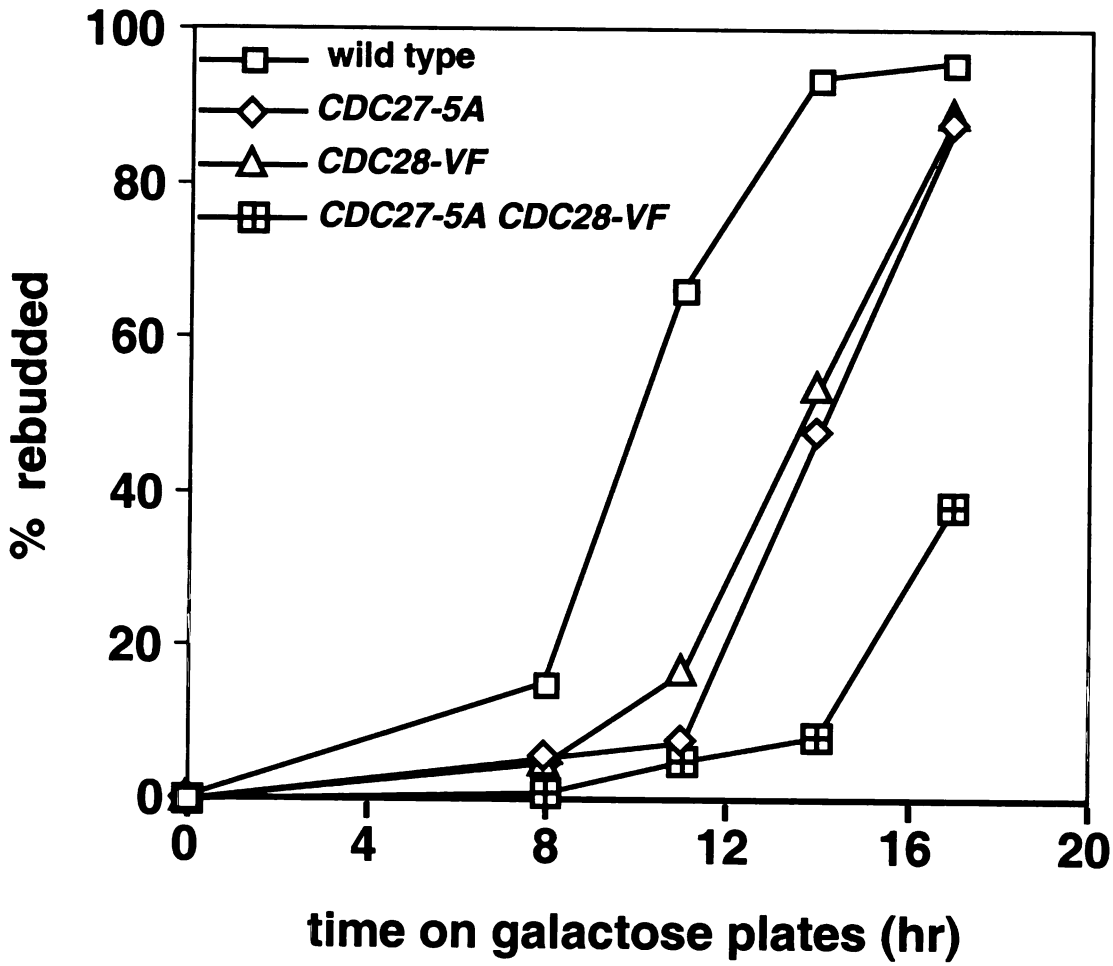

B
+Cdc28
-Cdc28
CDC27
CDC27-MBP
Cdc28-VF
220
160
120
100
90
80
70
60
50
40
Cdc27-MBP
Cdc16 ?
Cdc27
Apc9?
+Ab
-Ab
APC IP'ed with anti
Cdc26 Ab from G1 cells
27-MBP
27
23

Figure 5 ***CDC27-5A*** **is as sensitive to spindle checkpoint arrest as** ***CDC28-VF***

All strains contain *pGAL-MPS1*. Wild type (—□—, KH153), *CDC27-5A* (—◇—, ADR1974), *CDC28-VF* (—△—, KH181) and *CDC27-5A CDC28-VF* (—⊞—, ADR2073) were grown overnight at 30°C in YEP+2% raffinose to mid log phase and arrested in G1 with alpha factor (10μg/ml) for 2.5 hours. Arrested cells were placed on plates containing YEP+2% galactose to induce *pGAL-MPS1* and the rate of division of individual cells was followed. When a microcolony consisted of three or more cells, it was scored as rebudded. Cells that did not bud after the initial G1 arrest were not included in the analysis. 100 microcolonies were followed for each strain.

Figure 5


100
80
60
40
20
0
% rebudded
wild type
CDC27-5A
CDC28-VF
CDC27-5A CDC28-VF
0
4
8
12
16
20
time on galactose plates (hr)

Appendix 3

Other targets of Cdc28 that may regulate mitotic exit

Introduction

Cdc28 phosphorylates and activates the APC, but does Cdc28 phosphorylate other proteins that regulate the exit from mitosis? *CDC28-VF* and *CDC16-6A CDC27-5A CDC23-A* have similar phenotypes even though *CDC28-VF* is much less defective in APC phosphorylation (Chapter 2) and *CDC28-VF* and *CDC16-6A* are synthetically lethal in combination (data not shown). These two observations suggest that either the phosphorylation of APC subunits other than Cdc16, Cdc23 and Cdc27 is defective in *CDC28-VF*, or more likely, that Cdc28-VF is defective in the phosphorylation and activation of other proteins. I initially examined APC phosphorylation in *CDC28-VF* because it seemed a likely target, but both Cdc20 and Esp1/Pds1 are other targets that could influence the exit from mitosis. This appendix will present preliminary data that Cdc20 and Pds1 activity might be regulated by Cdc28.

Results

Cdc28 regulates Cdc20 stability

A possible substrate of Cdc28 is Cdc20. Homologues of Cdc20 are phosphorylated in vivo and although an Aurora kinase binds to Cdc20 (Farruggio et al., 1999; Weinstein et al., 1994), recent work has also shown that Cdc2 and Cdk2 can phosphorylate human Cdc20 in vitro . Human Cdc20 is also a substrate of the APC in vitro, but once phosphorylated by Cdc2, it becomes resisitant to ubiquitination (J. Nourse and D. Morgan, personal communication). In budding yeast, Cdc20 degradation is mediated by the APC in vivo, and it stability and protein levels are greatest in mitosis. I therefore wondered if Cdc28 phosphorylation of Cdc20 might be required to stabilize Cdc20 during mitosis, allowing Cdc20 to activate the APC.

I first examined if the accumulation of Cdc20 was impaired in *CDC28-VF* and *clb2Δ* cells. Wild type, *CDC28-VF clb2Δ* cells that contain an epitope tagged Cdc20 were arrested in G1 by alpha factor, released into the cell cycle and the expression of the Cdc20 protein was examined. Cdc20 levels rise as cells enter mitosis and then decline as they exit (Figure 1) Cdc20 reaches its peak expression 40 minutes later in *CDC28-VF* cells compared to wild type cells, and this delay parallels the delay in Clb2 accumulation. Although *CDC28-VF* delays accumulating Cdc20, the peak levels of Cdc20 are similar in wild type and *CDC28-VF* cells, suggesting that Cdc20 stability is not obviously impaired in *CDC28-VF*. As expected, Cdc20 protein, like Clb2 protein, remains at its peak levels far longer in *CDC28-VF* than wild type, indicating a delay in mitosis (Figure 1 and Chapter1, Figure1). *clb2Δ* cells also delay accumulating Cdc20, and the peak level of Cdc20 is slightly lower than seen in wild type (Figure 1).

I also examined Cdc20 in *cdc28-1N* cells, which have similar defects to *CDC28-VF* (Chapters 1 and 2). At the *cdc28-1N* arrest point almost no Cdc20 is present, especially

compared to the amount of Cdc20 present in wild type cells arrested by nocodazole or *cdc15-2* cells arrested in anaphase (Figure 2A and data not shown). This was surprising because *cdc28-1N* cells have high levels of Cdc28-asociated kinase activity, and are arrested in mitosis (Surana et al., 1991). This defect in Cdc20 accumulation could be due to a transcriptional defect or decreased Cdc20 protein stability. I was interested in the possibility that Cdc20 was less stable at the *cdc28-1N* arrest. To test this model, I arrested *cdc28-1N* cells at 23°C in mitosis with nocodazole, and then washed out the nocodazole and shifted the culture to 37°C at the same time. Cdc20 protein levels slowly dropped after the shift to 37°C consistent with Cdc20 protein stability being reduced (Figure 2B, left).

I also performed the reciprocal experiment in which *cdc28-1N* cells were arrested at 37°C, and then nocodazole was added and the culture shifted to 23°C. Addition of nocodazole causes some accumulation of Cdc20 (Figure 2B, right). Clb2 levels, however, increase during this experiment. This result suggests that checkpoint activation can re-stabilize Cdc20, and that Cdc20 degradation at the *cdc28-1N* arrest is Cdc20-dependent. I have not yet checked to see if *CDC20* transcription is defective at the *cdc28-1N* arrest, and this is an essential control that must be performed.

Clb5 and the exit from mitosis

Clb5 has recently been identified as a key substrate of the Cdc20-dependent APC. A *cdc20Δ pds1Δ clb5Δ* cell is viable and healthy, suggesting that Pds1 and Clb5 are the only two essential targets of the Cdc20-dependent APC (Shirayama et al., 1999). Clb5 destruction has been hypothesized to be critical for cell cycle progression by relieving the inhibition on Hct1 and perhaps other regulators of Hct1 and Sic1. Clb2 is also a target of the Cdc20-dependent APC (Yeong et al., 2000), and Clb2 and Clb5 may have redundant functions in promoting mitotic progresison.

Because *CDC28-VF* is defective in activating the Cdc20-dependent APC I was curious if, like Pds1 destruction (see Chapter 1, Figure 1), Clb5 destruction was impaired in *CDC28-VF* cells. Since *cdc20Δ pds1Δ clb5Δ* is viable, I initially tested if combining *clb5Δ pds1Δ* with *CDC28-VF* might suppress *CDC28-VF* phenotypes. It does not, and in fact *CDC28-VF pds1Δ* cells are quite sick, which is consistent with the finding that *clb2Δ pds1Δ* cells are inviable (data not shown) (Shirayama et al., 1999).

I next examined if Clb5 was stabilized in *cdc23-1 GAL-CDC28-VF* cells. I performed an experiment identical to the one described in Chapter 1, Figure 5 using *cdc23-1 GAL-CDC28-VF* and *cdc23-1 GAL-CDC28* cells that contain an epitope tagged Clb5. As seen previously, *cdc23-1 GAL-CDC28* cells enter mitosis, exit mitosis and then re-arrest in the next G1 (Figure 3). These strains were not *bar1Δ*, so the re-arrest is only temporary. Clb5 accumulation and destruction occurs before Clb2 accumulation and destruction. Unlike Clb2, Clb5 is not stable in *cdc23-1 GAL-CDC28-VF* cells (Figure 3). And quite mysteriously, Clb5 appears to cycle in *cdc23-1 GAL-CDC28-VF* cells in parallel with Clb5 in *cdc23-1 GAL-CDC28* cells. This cycling is paradoxical because Clb2 levels remain high and Sic1 never re-accumulates. This experiment shows that Clb5 destruction is not affected in *CDC28-VF* cells, which is inconsistent with the model that the Cdc20-dependent APC is impaired in *CDC28-VF*. This experiment does raise some interesting questions about how Clb5 levels are regulated, and whether Clb5 can act as an independent oscillator in the absence of oscillations in bulk Cdc28-associated activity. This observation might help explain the controversial finding that APC mutants can re-replicate their DNA in certain conditions (Heichman and Roberts, 1996; Heichman and Roberts, 1998; Pichler et al., 1997).

Independent of my interest in the stability of Clb5 in *CDC28-VF*, I wondered if Clb5 only functions to inhibit the Hct1-dependent APC and mitotic progression, as has been proposed (Shirayama et al., 1999), or if it might have an earlier role in sister separation. I approached this problem by constructing a non-degradable Clb5 (*CLB5-*

Δ134). If Clb5 only functions late in mitosis, expression of a non-degradable Clb5 should arrest in anaphase, like a non-degradable Clb2. Expression of non-degradable Clb5, however, causes defects in sister separation (Figure 4). 65% of cells arrest with unseparated DNA masses, although, in 20% of cells, some spindle elongation ocurrs and the DNA segregates aberrantly. This phenotype is very different from cells expressing a non-degradable Clb2, in which 85% of cells have normal spindle elongation and DNA segregation. Cells expressing non-degradable Clb5 look much like what has been described for *esp1-1* mutants and cells expressing non-degradable Pds1 (Ciosk et al., 1998). I have not examined the half life of Clb5-Δ134, so it is possible its expression is somehow clogging up the APC, and thereby mimicking a nondegradable Pds1 arrest, though this seems unlikely. It is also possible that expression of Clb5-Δ134 causes defects in S-phase which activate the DNA damage or replication checkpoints. This seems unlikely as well, because checkpoint arrest usually causes a uniform large budded arrest with a short spindle and no aberrant DNA segregation.

Although preliminary, this result suggests that sister separation depends on loss of Clb5 activity. A similar phenotype, however, has been seen in flies and human cells expressing a non-degradable cyclin A, so this regulation may be conserved (Sigrist et al., 1995). Old experiments argued that a fall in Cdc28 or Cdc2 activity is not required for sister separation (Holloway et al., 1993; Surana et al., 1993), but these experiments examined the phenotypes of expressing non-degradable Clb2, not Clb5, in budding yeast, and non-degradable sea urchin cyclin B, not cyclin A, in frog egg extracts.

How might Clb5-associated kinase activity inhibit sister separation? The simplest explanation would be that Cdc28/Clb5 complexes inhibit Esp1, or activate Pds1. If expression of Clb5-Δ134 in *pds1Δ* cells causes a uniform anaphase arrest, then it is likely that Clb5 activates Pds1. No matter the mechanism, Cdc28/Clb5 complexes appear to inhibit the exit from mitosis at an early stage, and this contrasts with the results of Chapters 1 and 2, which show that Cdc28/Clb2 complexes activate the exit from mitosis.

In the absence of Pds1, Clb5 and Cdc20, sister chromatid separation occurs roughly on schedule, suggesting that either Esp1 activity or Scc1 sensitivity to Esp1 is regulated independently of these molecules (Nasmyth et al., 2000; Shirayama et al., 1999). An attractive model is that Esp1 activity is tied to the oscillation in Cdc28-Clb2 activity that occurs during mitosis. Such a model would propose that early in mitosis Clb5-associated kinase activity inhibits sister separation, but this inhibition is overcome as Clb2 is expressed and Cdc28/Clb2 complexes activate Esp1 and the APC.

Figure 1 Cdc20 accumulates to normal levels in *CDC28-VF*

All strain contain *CDC20-myc12*. *cdc15-2 CDC28-HA* (ADR1789), *cdc15-2 CDC28-VF* (ADR1794) and *clb2Δ CDC28-HA cdc15-2* (ADR1988) were grown overnight at 23°C in YPD to mid log phase, arrested in G1 with alpha factor (1μg/ml) and at t=0 the cells were released from the G1 arrest. At t=80, alpha factor (1.5μg/ml) was added back to the cultures to re-arrest the cells in the next G1. Samples were taken at the indicated times, lysed and immunoblotted for Cdc20-myc12, Clb2, Clb3 and Sic1.

Figure 1

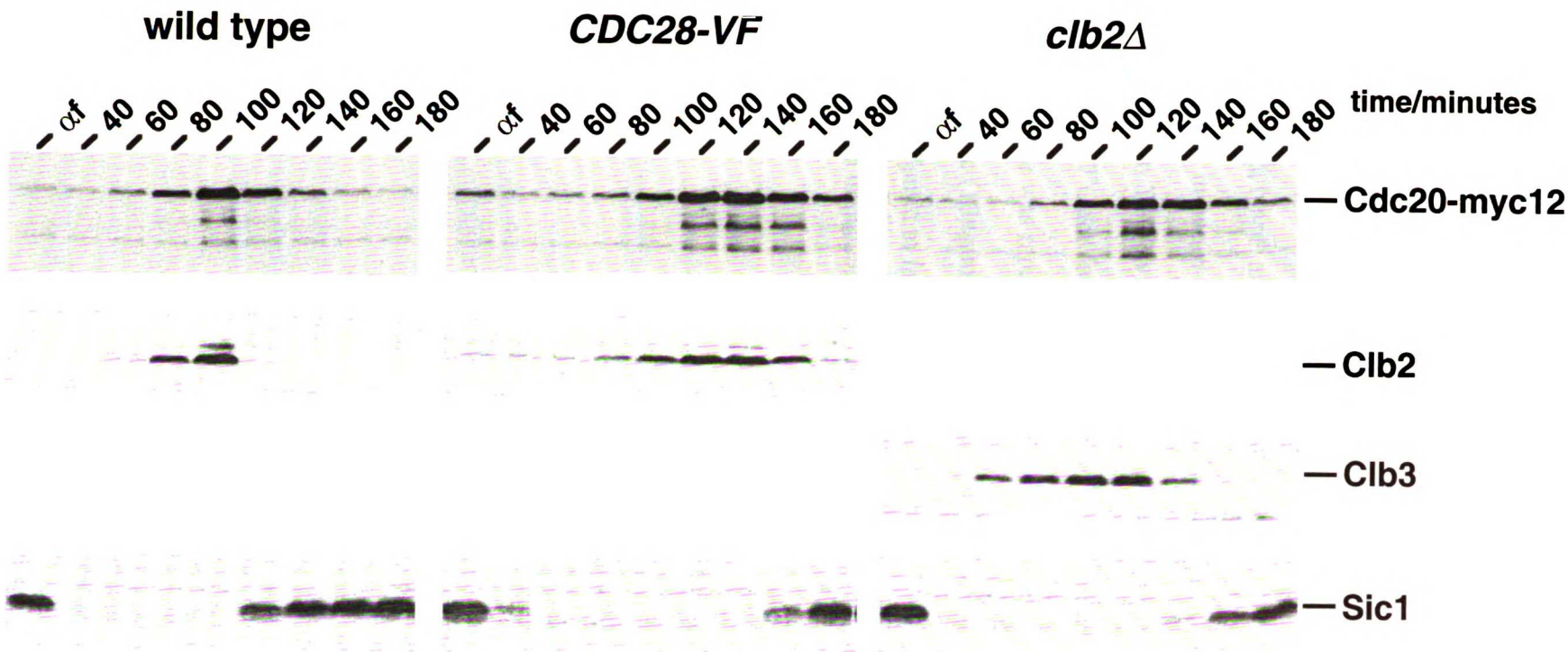

wild type
CDC28-VF
clb2Δ
αf
40
60
80
100
120
140
160
180
time/minutes
Cdc20-myc12
Clb2
Clb3
Sic1

Figure 2 Cdc20 may be unstable at the *cdc28-1N* arrest

(A) No Cdc20 is present at the *cdc28-1N* arrest. All strain contain *CDC20-myc12*. *cdc15-2 CDC28-HA* (ADR1790), *cdc15-2 CDC28-VF* (ADR1793) and *cdc28-1N* (ADR1796) were grown overnight at 23°C in YPD to mid log phase and the cultures were shifted to 37°C for 4 hours to arrest the cells in anaphase (*cdc15-2*) or in mitosis (*cdc28-1N*). Cells were harvested, lysed, and immunoblotted for Cdc20-myc12 and Cdc27.

(B) Cdc20 is destabilized in *cdc28-1N*. *cdc28-1N CDC20-myc12* (ADR1796) were were grown overnight at 23°C in YPD to mid log phase and the culture was split in two. One half (left) was shifted to 37°C for four hours, nocodazole was added to 15μg/ml for one hour, and at t=0 the culture was rapidly cooled to 23°C. The other half (right) was arrested in mitosis with nocodazole (10μg/ml), the cells were shifted to 37°C for one hour, and then at t=0 the cells were transferred to fresh media at 37°C. Cells were harvested at the indicated times, lysed, and immunoblotted for Cdc20-myc12 and Clb2.

Figure 2

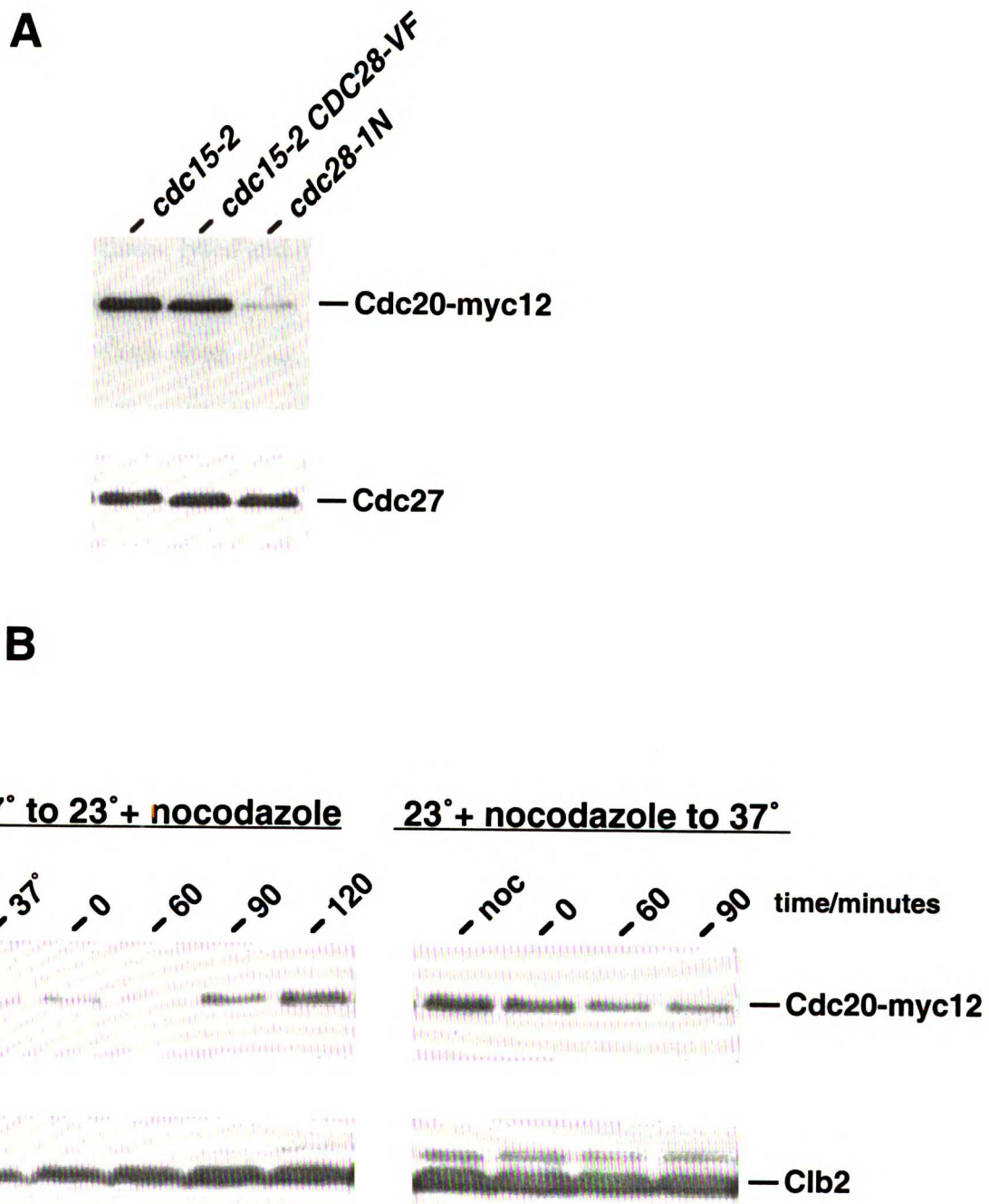

A
cdc15-2
cdc15-2 CDC28-VF
cdc28-1N
Cdc20-myc12
Cdc27
B
37˚ to 23˚+ nocodazole
23˚+ nocodazole to 37˚
37˚
0
60
90
120
noc
0
60
90
time/minutes
Cdc20-myc12
Clb2

Figure 3 Clb5 is not stabilized in *cdc23-1 CDC28-VF* cells

cdc23-1 GAL-CDC28 CLB5-3XHA (ADR2014) and *cdc23-1 GAL-CDC28-VF CLB5-3XHA* (ADR2015) were grown overnight at 23°C in YEP+2% raffinose to log phase, arrested in G1 with alpha factor (10μg/ml) for 3.5 hours and at t=0 released from the G1 arrest into fresh YEP+2% galactose. After cells had budded (t=2), alpha factor (15μg/ml) was added back to the cultures to re-arrest the cells in the next G1. Samples were taken at the indicated times, lysed and immunoblotted for Cdc16, Clb2, Clb5-3XHA and Sic1.

Figure 3

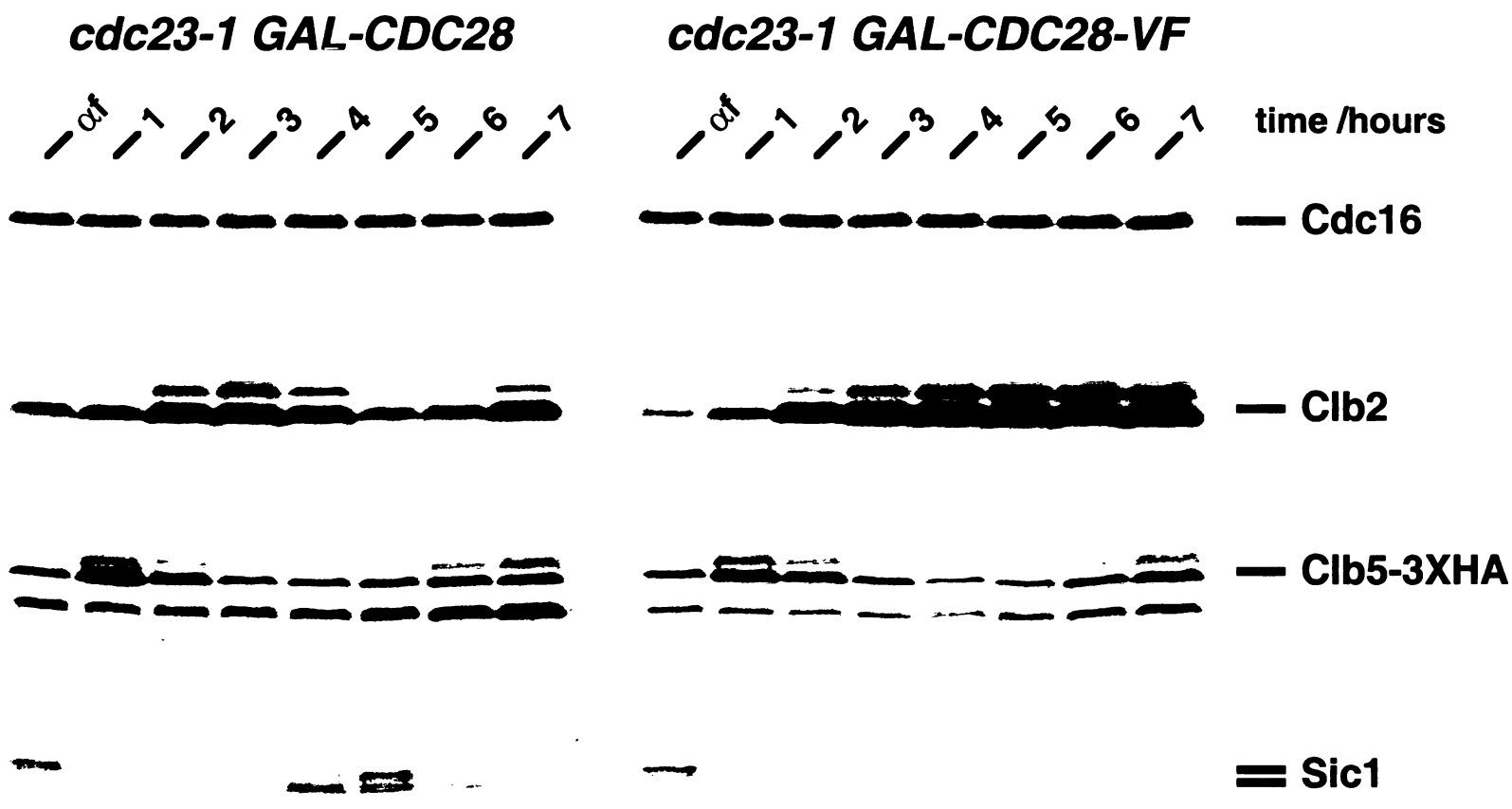
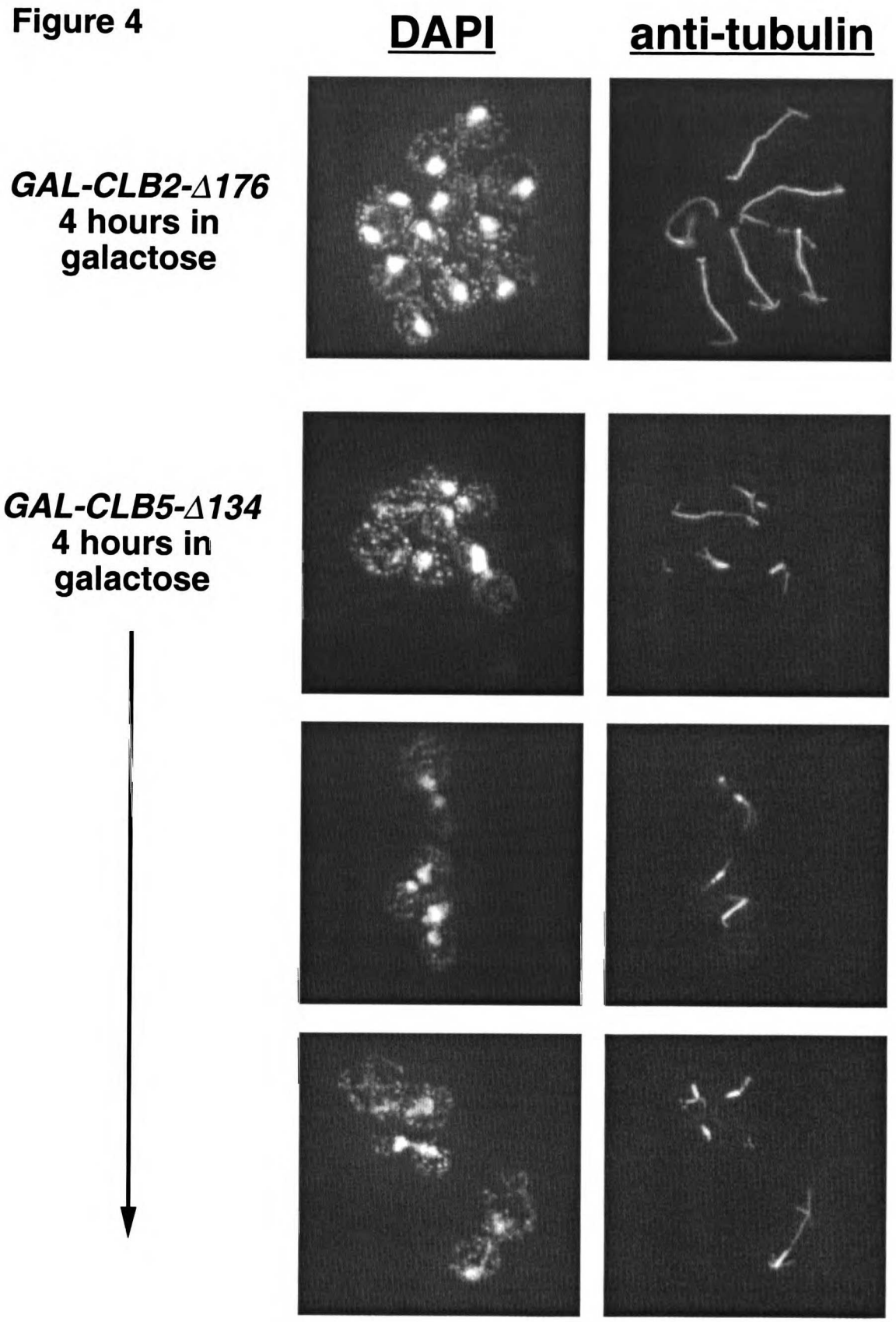

cdc23-1 GAL-CDC28
cdc23-1 GAL-CDC28-VF
0f
1
2
3
4
5
6
7
time /hours
Cdc16
Clb2
Clb5-3XHA
Sic1

Figure 4 Overexpression of *CLB5-Δ134* blocks DNA segregation

CLB5-Δ134 (ADR371) and *CLB2-Δ176* (ADR64) were grown overnight at 23°C in YEP+2% raffinose to log phase and then transferred to YEP+2% galactose for four hours. Cells were harvested, spindles and nuclei were visualized by indirect immunofluorescence of formaldehyde fixed cells probed with an anti-alpha tubulin antibody and stained with DAPI.

Figure 4


DAPI
anti-tubulin
GAL-CLB2-Δ176
4 hours in
galactose
GAL-CLB5-Δ134
4 hours in
galactose

Appendix 4

MAD genes inhibit the APC and sister separation

Introduction

A target of the spindle checkpoint is Cdc20, which when inhibited by checkpoint activation blocks sister separation and cell cycle progression (Hwang et al., 1998; Kim et al., 1998). The isolation of *cdc20* mutants that are checkpoint defective and are unable to bind Mad2 clearly demonstrated that Cdc20 is a target of the checkpoint. Other pieces of data, however, support the idea that the Mad pathway inhibits sister separation by inhibiting the APC. This appendix presents some of those experiments and a result suggesting that the spindle checkpoint can function in anaphase.

Results

cdc15-2 and *cdc23-1* mutants do not activate the checkpoint

The spindle checkpoint senses defects in chromosome attachment to the mitotic spindle. One of the ways the checkpoint has been studied has been to examine which mitotic mutants arrest because they activate the spindle checkpoint. Mutants in microtubule motors (*cin8Δ*), microtubule subunits (*tub2)* and spindle pole body components(*mps2-1*) all activate the checkpoint and arrest in mitosis (Hardwick et al., 1999). When combined with a *mad* mutant, these mutants no longer arrest in mitosis and die more rapidly than the single mutants. This rapid death is thought to be due to exiting mitosis in the presence of defects to the mitotic spindle. *cdc15-2*, which is defective in activating Cdc14 (Jaspersen et al., 1998), and *cdc23-1* a mutant in an APC subunit (Irniger et al., 1995; Zachariae and Nasmyth, 1996), do not activate the checkpoint, and double mutant combinations still arrest in mitosis (data not shown). *cdc15-2 mad* and *cdc23-1 mad* double mutants grown at 37°C die at the same rate as *cdc15-2* and *cdc23-1* which is consistent with the double mutants retaining their ability to arrest in mitosis (Figure 1). If anything, *mad1Δ* and *mad2Δ* partially suppress the death of the *cdc23-1* mutant (Figure 1, and see Figure 2 below). Now that detailed functions have been assigned to Cdc15 and Cdc23, this result seems uninteresting, but these results showed that Cdc15 and Cdc23 arrested in mitosis because they interfered with cell cycle progression, not because of any damage to machinery of mitosis.

The spindle checkpoint is active in every cycle

Although *cdc23 mad* doubles arrest and die in mitosis at the fully restrictive temperature, the maximum permissive temperature of these double mutants rise, similar to

what I have shown for *cdc55Δ cdc23-1* double mutants (see Chapter 3, Figure 6). All three *mad* mutants allow *cdc23-1* mutants to grow reasonably well at 30°C, and *mad3Δ* suppresses *cdc16-1*, another mutant in an APC subunit, at 34°C. This suppression does allow re-entry into the next cell cycle and degradation of Clb2 (data not shown). Formally this data shows that the *MAD* genes inhibit the APC, and suggest that the APC is the target of the checkpoint. This data also suggests that the Mad proteins might inhibit the APC during an unperturbed cell cycle, and when the *MAD* genes are deleted, the APC is relieved of this inhibition and has slightly increased activity. This idea is supported by the finding that *mad1Δ* and *mad2Δ* cells going through a synchronous cell cycle trigger Clb degradation and sister separation 10 minutes faster than wild type cells (data not found).

mad mutants separate sister inappropriately in nocodazole

In Chapter 3 I presented data showing that mad2Δ mutants inappropriately separate their sisters and degrade Clbs when treated with nocodazole. Sister separation in those experiments was visualized by integrating an array of 256 *lacO* repeats into the yeast genome, and expressing a *GFP-lacI* fusion protein that will bind to the *lacO* array (Straight et al., 1996). This technique has been used to perform genetic screens for mutants defective in sister separation, and has been used to analyze chromosome dynamics in real time. The first measurements of sister separation in *mad* mutants, however, were performed using fluorescent in situ hybridization (FISH). Hybridization with probes to the rDNA made it clear that sister chromatids separated prematurely in *mad* mutants treated with nocodazole (Minshull et al., 1996), and the use of cosmid probes allowed the quantification of the extent of sister chromatid separation in *mad1Δ* and *mad2Δ* mutants (Fig3 and data not shown). These measurements also allowed an independent verification of the GFP technique for visualizing sister chromatids (Figure 3). This was an issue,

because initially a tetramerizing form of *lacI* was fused to GFP and led to the incorrect conclusion that sister chromatids did not separate in *mad* mutants (Straight et al., 1996).

Activating the spindle checkpoint in anaphase

The primary consequence of spindle checkpoint activation is to block sister chromatid separation until all chromosomes are attached to the spindle. Once sister chromatids have separated, can the checkpoint still be activated? Teleologically there is no use to checkpoint activation in anaphase. If chromosomes have mis-segregated, activating the checkpoint in anaphase won't correct the mis-segregation. But if the checkpoint can be activated in anaphase it means that sister cohesion is not a prerequisite for checkpoint activation, and that there might be a function for this late activation. The checkpoint can be activated in anaphase in frog egg extracts (Alex Szidon and Andrew Murray, personal communication), but preliminary data in budding yeast suggested that it could not (Hwang, 1998). These experiments scored checkpoint activation either by Mad1 hyperphosphorylation or by the lack of re-budding following release from anaphase. Cdc20 function is required throughout anaphase, and monitoring Pds1 stability in anaphase is a sensitive way to directly measure Cdc20-dependent APC activity in vivo (Jaspersen et al., 1998). To investigate the checkpoint in anaphase I arrested *cdc15-2* cells in anaphase at 37°C, then induced expression of an epitope tagged Pds1 from the galactose inducible promoter *GAL1*. Expression was shut off by addition of glucose, and the Pds1 protein was rapidly degraded with a half life of approximately 15 minutes (Figure 4 and see Chapter 1, Figure 7). Addition of nocodazole to the culture, however, slowed the degradation of Pds1 significantly, suggesting that in this experiment the checkpoint can be activated in anaphase.

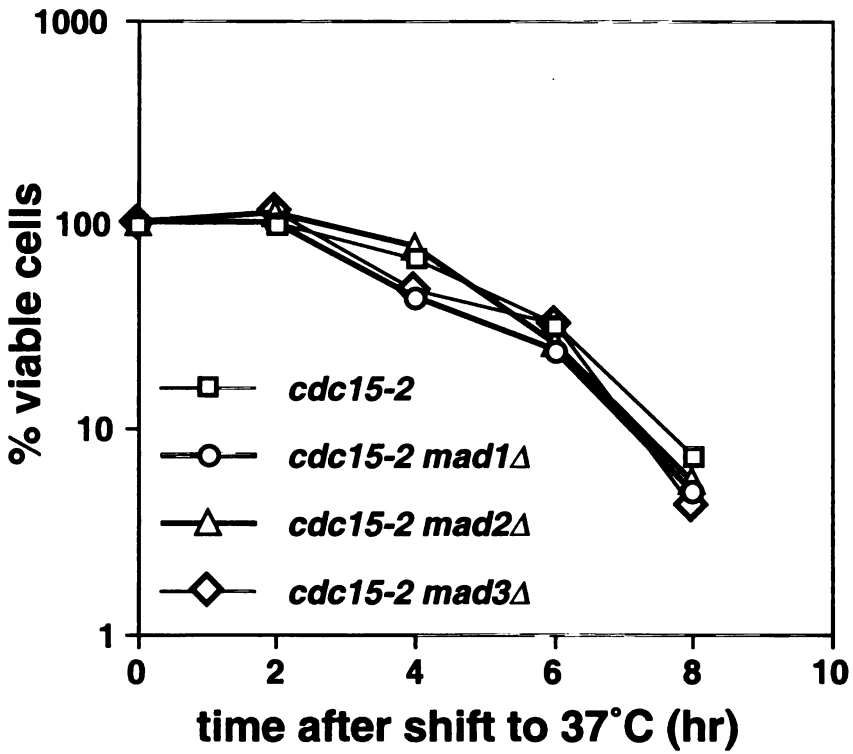
Why do *mad* mutants die?

A nagging question that remains in the study of the mad mutants is why they die after treatment with nocodazole. A simple explanation is they die because of gross chromosome mis-segregation which results from separating their sisters inappropriately after treatment with nocodazole. But is this true? Figure 7 in Chapter 3 suggests that this explanation may not be. Overexpression of Sic1-mut in nocodazole arrested cells causes the immediate inhibition of Clb2-associated kinase activity, activation of the APC, separation of sister chromatids, degradation of mitotic cyclins and entry into the next G1, all in the absence of a spindle. The exit from mitosis in these cells is quite similar to what is seen in *mad* or *mad bub2Δ* mutants treated with nocodazole (Alexandru et al., 1999; Minshull et al., 1996). However unlike *mad* mutants, these cells diploidize and maintain nearly 100% viability.

Figure 1 *cdc15-2* and *cdc23-1* do not activate the spindle checkpoint

(left) *cdc15-2* (—□—, K1993), *mad1Δ cdc15-2* (—○—, ADR1163), *mad2Δ cdc15-2* (—△—, PHD1) and *mad3Δ cdc15-2* (—◇—, PHD7), and (right) *cdc23-1* (—□—, ADR624), *mad1Δ cdc23-1* (—○—, ADR1135), *mad2Δ cdc23-1* (—△—, ADR1140), *mad3Δ cdc23-1* (—◇—, ADR1143) were arrested in G1 by nutrient starvation at 23°C and released into YPD at 37°C. amples were harvested at the indicated times, diluted and plated onto YPD plates. The number of colonies at each time was compared with the number immediately before nocodazole addition to determine the % viable cells. These experimetns were performed by Phoebe H. Dann.

Figure 1


1000
100
10
1
% viable cells
cdc15-2
cdc15-2 mad1Δ
cdc15-2 mad2Δ
cdc15-2 mad3Δ
0
2
4
6
8
10
time after shift to 37˚C (hr)

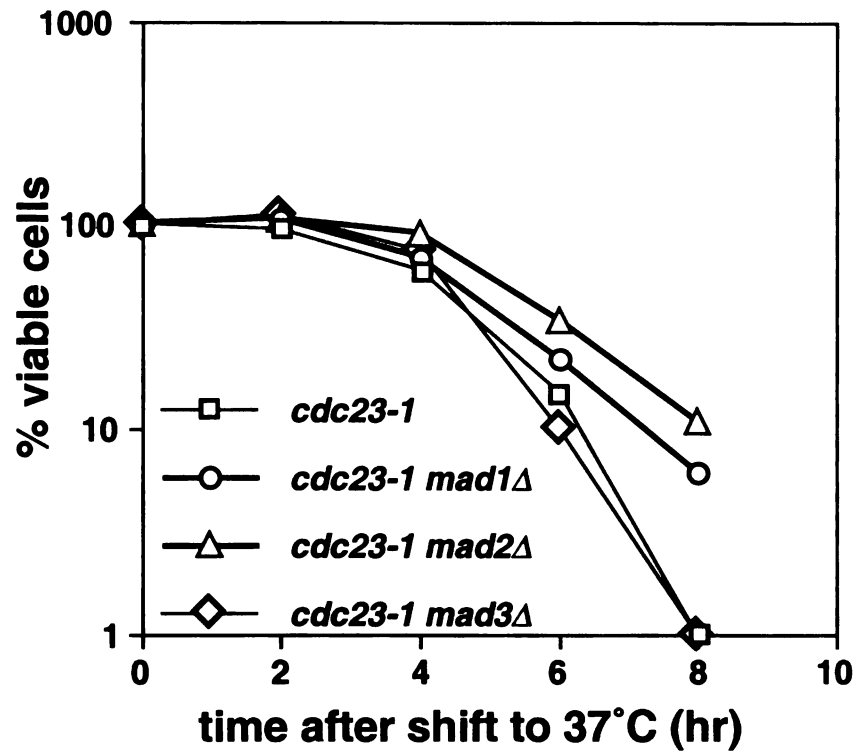

1000
100
10
1
% viable cells
cdc23-1
cdc23-1 mad1Δ
cdc23-1 mad2Δ
cdc23-1 mad3Δ
0
2
4
6
8
10
time after shift to 37˚C (hr)

Figure 2 *mad* mutants suppress *cdc23-1* and *cdc16-1*

(A) *cdc23-1* (ADR624) was crossed to *mad1Δ* (KH124), *mad2Δ* (ADR852) and *mad3Δ* (KH176), and (B) *cdc16-1* (ADR1147) was crossed to *mad3Δ* (KH176), diploids were sporulated and tetrads dissected. 3 representative tetrads were grown to saturation for two days in YEP+ 2% glucose at 23°C, diluted ten-fold and four-fold serial dilutions were prepared in a multi-well dish and spotted onto a YEP+2% glucose plate which was grown at 30°C or 34°C (for *mad3Δ cdc16-1*) for 3 days. Double mutants (for example: 2C-*mad1Δ cdc23-1* (ADR1135), 1A-*mad2Δ cdc23-1* (ADR1140) and 3D-*mad3Δ cdc23-1* (ADR1143), and 1B-*mad3Δ cdc16-1* (ADR1160)) are rescued for growth at 30°C.

Figure 2

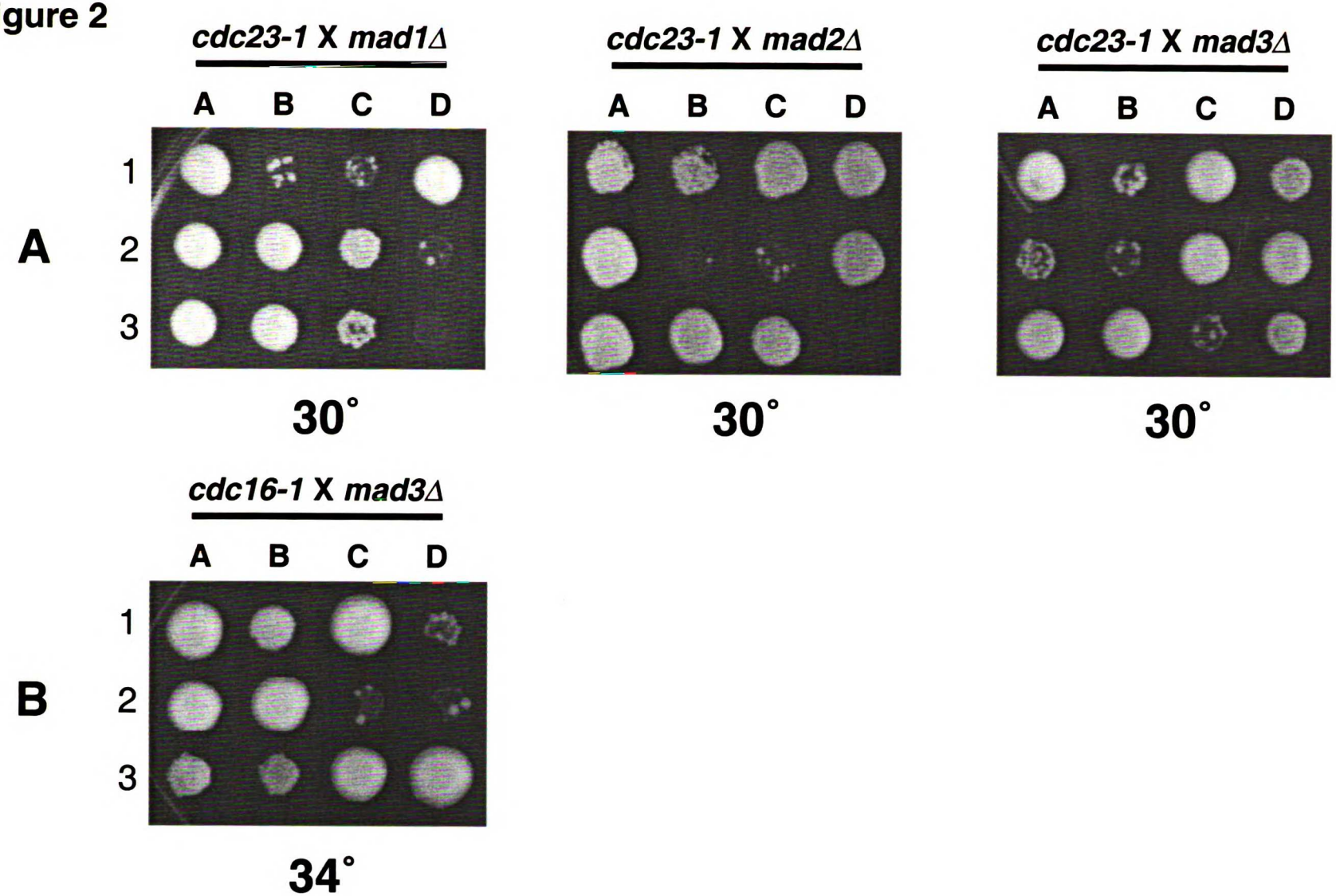

cdc23-1 X mad1Δ
cdc23-1 X mad2Δ
cdc23-1 X mad3Δ
A B C D
A
1
2
3
30°
cdc16-1 X mad3Δ
B
34°

Figure 3 *mad2Δ* mutants separate sisters inappropriately when grown in nocodazole.

Wild type (JM478) and *mad2Δ* (JM479) were grown overnight at 23°C in YPD to mid log phase, the cells were transferred to CSM-his+2% dextrose containing 10mM 3-aminotriazole for 30 minutes to induce expression of *pHIS3-GFP-lacI*, and then the cultures were transferred into fresh YPD containing 10μg/ml nocodazole. Cells were harvested at the indicated times,washed, fixed and sister chromatid separation was scored by counting the number of fluorescent spots (one or two) in the nucleus. Sister chromatids were visualized either by GFP-lacI bound to 256 tandem repeats of *lacO* (wild type —□—, *mad2Δ* —○—) or by fluorescent in situ hybridization (FISH) using a cosmid probe to chromosome III (wild type —△—, *mad2Δ* —◇—).

Figure 3

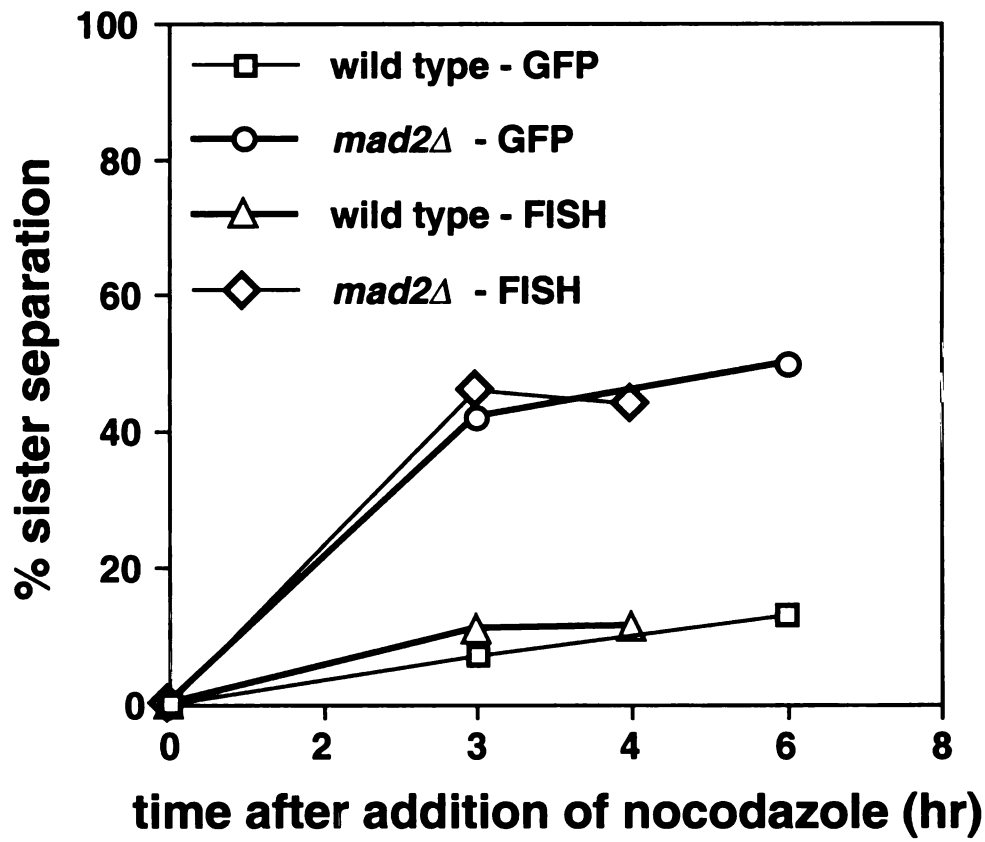

100
80
60
40
20
0
% sister separation
wild type - GFP
mad2Δ - GFP
wild type - FISH
mad2Δ - FISH
0
2
3
4
6
8
time after addition of nocodazole (hr)

Figure 4 Activation of the spindle checkpoint in anaphase

cdc15-2 CDC28-HA pGAL-PDS1-HA (ADR1742) was grown overnight at 23°C in YEP+2% raffinose to log phase and shifted to 37°C to arrest the cells in anaphase (raf). When greater then 90% of the cells had reached anaphase (after 4 hours, as judged by nuclear division which was scored by DAPI staining) the culture was split and nocodazole (10μg/ml) was added to one of the cultures. Pds1-HA expression was induced in both cultures for 1 hour by the addition of galactose (to 2%), and at t=0 its expression was terminated by the addition of glucose (to 2%). Samples were harvested at the indicated times, lysed and immunoblotted for Pds1-HA, Clb2 and Cdc28-HA. Cdc28-HA is shown as a loading control.

Figure 4


– nocodazole
+ nocodazole
raf
0
15
30
45
60
75
90
time/minutes
Pds1-HA
Clb2
Cdc28-HA

Appendix 5

tub1-177 is hypersensitive to spindle depolymerization

Introduction

Many of the proteins required for sister chromatid cohesion have been isolated in three screens that looked for mutants that were defective in maintaining the linkage between sisters (Biggins et al., 1999; Michaelis et al., 1997; Yamamoto et al., 1996a; Yamamoto et al., 1996b). The first screen in involved isolating a collection of temperature sensitive mutants, screening through them for ones which were hyper-sensitive to nocodazole treatment, and then finally screening them by FISH and DAPI staining for mutants which separated sister chromatids prematurely or had defects in DNA segregation. Years ago, I undertook a similar screen for mutants that were defective in sister chromatid cohesion using an existing bank of cold sensitive mutants (Moir et al., 1982). This appendix briefly describes the one interesting mutant I isolated.

Results

The primary screen for mutants defective in sister chromatid cohesion was a screen for benomyl sensitive mutants. The rationale for this screening was that an existing mutant in sister cohesion, *pds1*, is hyper-sensitive to nocodazole and, like *mad* mutants, separates its sister chromatids after nocodazole treatment and dies (Yamamoto et al., 1996a). 10 mutants were isolated that were sensitive to benomyl on plates, and after backcrossing, only one, mutant 177, died very rapidly when treated with nocodazole in liquid cultures, but unlike *mad* mutants and like *pds1Δ* mutants, did not re-bud when treated with nocodazole (Figure 1A and 1B). 177 was also an extremely tight cold sensitive allele.

Initially I screened 177 by FISH and thought it displayed a sister chromatid cohesion defect (data not shown). I backcrossed 177 further, and the nocodazole sensitivity and the cold sensitivity segregated together, but rescreeening of the sister separation defect both with FISH and using *GFP-lacI* bound to integrated *lacO* arrays revealed that this mutant did not have a strong defect in sister chromatid cohesion (Figure 2A). In addition 177 was able to maintain high levels of Clb2 during a nocodazole arrest (Figure 2B).

While testing for complementation with known nocodazole sensitive mutants, I discovered that 177 was tightly linked to *TUB1*, the major alpha tubulin in budding yeast. Although I have not yet determined the precise mutation in 177, hereafter I will refer to 177 as *tub1-177*, *tub1-177* might be a weak dominant negative because although *TUB1* on a *CEN* plasmid complements the cold sensitivity of *tub1-177* well, it only weakly complements its nocodazole sensitivity. *tub1-177* is an unusual allele of *TUB1* because of its very strong nocodazole sensitivity. Although other alleles of *TUB1* are as sensitive on benomyl plates, they do not die rapidly when exposed to nocodazole (Wells, 1995). The only other allele of *TUB1* that behaves similarly is the *tub1-171* mutation, which has been shown to not be a mutation in tubulin, but in a neighboring gene (Marion Shonn and

Andrew Murray, personal communication). The experience with the former *tub1-171* suggests that I have been too hasty in naming *tub1-177*, and rescue and sequencing of the mutation are required.

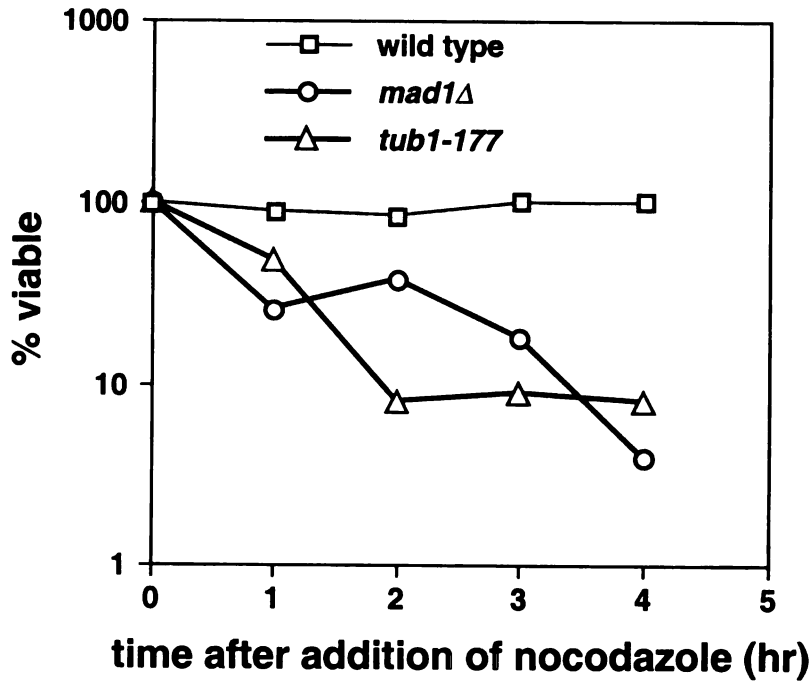
Figure 1 *tub1-177* is hypersensitive to spindle depolymerization

(A) *tub1-177* dies rapidly in nocodazole. Wild type (—□—, ADR21) and *tub1-177* (—△—, ADR551) and *mad1Δ* (—○—, ADR54) were grown overnight at 23°C in YPD to mid log phase, and the cultures were transferred into fresh YPD containing 10μg/ml nocodazole. Samples were harvested at the indicated times, diluted and plated onto YPD plates. The number of colonies at each time was compared with the number immediately before nocodazole addition to determine the % viable cells.

(B) Rebudding in *tub1-177*. The same strains as in (B) and *cin1Δ* (—◇—, ADR12) were tgrown as in (B). Samples were harvested at the indicated times and rebudding was scored. A rebudded cell is one which after sonication has three buds.

Figure 1

A


1000
100
10
1
% viable
wild type
mad1Δ
tub1-177
0
1
2
3
4
5
time after addition of nocodazole (hr)

B

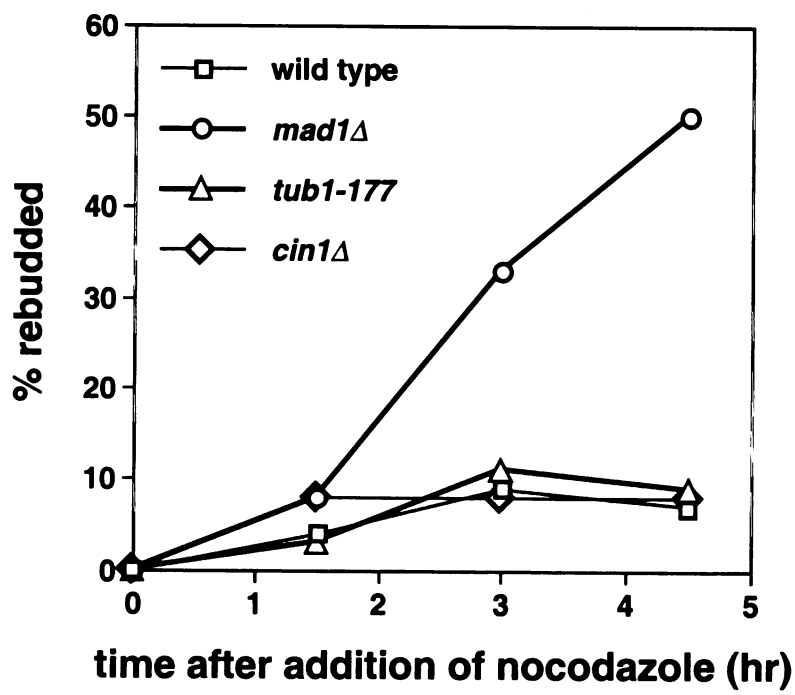
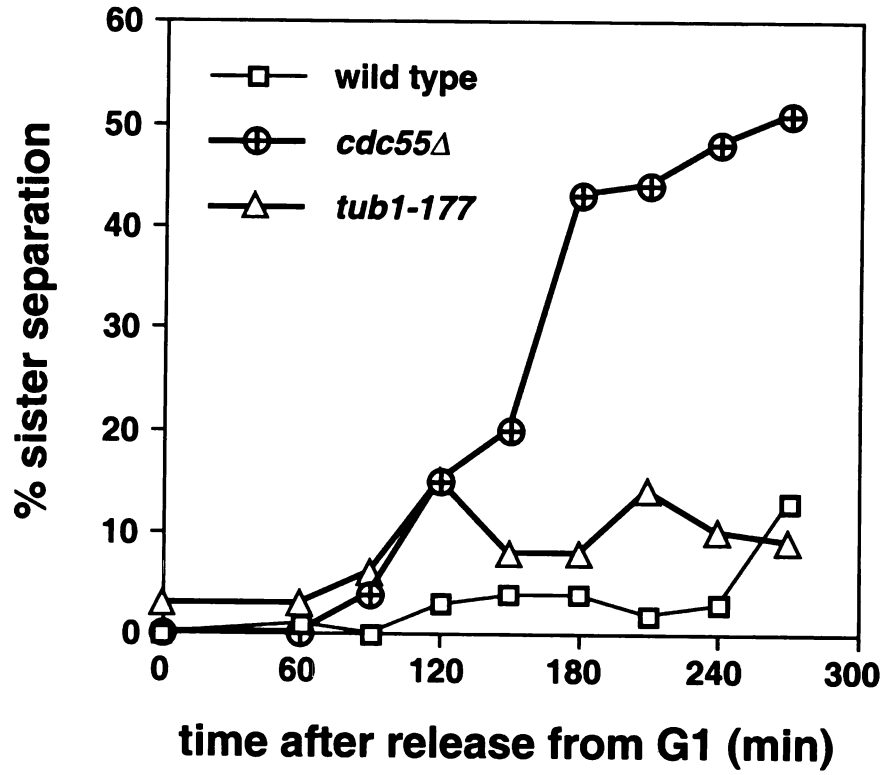

60
50
40
30
20
10
0
% rebudded
wild type
mad1Δ
tub1-177
cin1Δ
0
1
2
3
4
5
time after addition of nocodazole (hr)

Figure 2 *tub1*-177 maintains sister cohesion in nocodazole

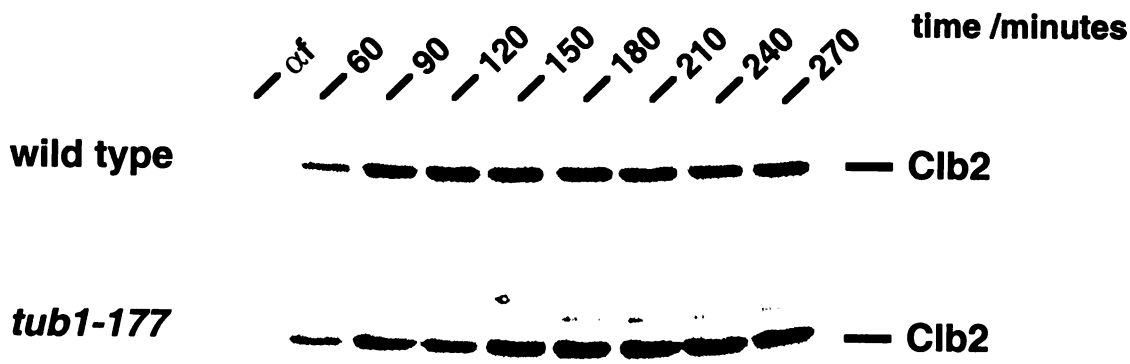
tub1-177 arrests in nocodazole. Wild type (—□—, JM478), *tub1-177* (—△—, ADR570) and *cdc55Δ* (JM480) were grown overnight at 23°C in YPD to mid log phase, arrested in G1 with alpha factor (10μg/ml) for 2.5 hours and at t=0 the cells were released from the G1 arrest. For the last 30 minutes of the alpha factor arrest, cells were transferred to CSM-his+2% dextrose containing 10mM 3-aminotriazole and alpha factor (10μg/ml) to induce expression of *pHIS3-GFP-lacI*. At t=90, alpha factor (15μg/ml) was added back to the cultures to prevent re-accumulation of Clb2 proteins in cells that had exited from mitosis. Duplicate samples were taken at the indicated times and one set was lysed and immunoblotted for Clb2 (A). The other set was washed, fixed and sister chromatid separation was scored by counting the number of fluorescent spots (one or two) in the nucleus (B). Sister chromatids were visualized either by GFP-lacI bound to 256 tandem repeats of *lacO*. *cdc55Δ* was used as a control that separates its sisters in nocodazole.

Figure 2

A


60
50
40
30
20
10
0
% sister separation
wild type
cdc55Δ
tub1-177
0
60
120
180
240
300
time after release from G1 (min)

B


time /minutes
αf
60
90
120
150
180
210
240
270
wild type
Clb2
tub1-177
Clb2

Materials and Methods

Strain and plasmid construction

Table 1 lists the strains used in this work. All strains are derivatives of the W303 strain background (W303-1a, Rodney Rothstein, Columbia University, NY). Standard genetic techniques were used to manipulate yeast strains (Sherman et al., 1974) and standard protocols were used for DNA manipulation (Maniatis et al., 1982). All deletions were confirmed by polymerase chain reaction (PCR) or by mutant phenotype. The sequences of all oligonucleotide primers used in this study are available upon request. The strains TG1 and DH5α were used for all bacterial manipulations.

The strains used for the crosses in Chapter 1, Table 1were: JM434 (*CDC28-VF*), JM469 (*CDC28-V*), JM467 (*CDC28-F*), ADR1541 (*CDC28-AF*), ADR2035 (*CDC28-F88G*), KH208 (*swe1Δ*), ADR484 (*cdc28-1N*), ADR840 (*cdc28-4*), ADR314 (*clb2Δ*), ADR719 (*cdc23-1*), ADR1147 (*cdc16-1*), LH226 (*doc1-1*), LH125 (*cdc20-1*), ADR1435 (*hct1Δ*), JC126 (*cdc5-1*), ADR1298 (*cdc13-1*), K1993 (*cdc15-2*). For all crosses at least 22 tetrads were analyzed.

BAR1 was deleted using pJGsst1 (a gift of Jeremy Thorner, UC Berkeley, CA). *PDS1-myc18* strains were made by crossing K6445 (Shirayama et al., 1998) to the appropriate strains. *pCUP-GFP12-lacI12, lacO:TRP1* and *lacO:LEU2* were integrated using pSB116 (Biggins et al., 1999), pAFS52 and pAFS59 (Straight et al., 1996) respectively. *GAL-MPS1* strains were made with pAFS120 (Hardwick et al., 1996). *MAD3* was deleted using pKH181 (Hardwick et al., 1998). *swe1Δ* strains were made by crossing JM449 (a gift of Jeremy Minshull, Maxygen, CA) to the appropriate strains. *MIH1* was deleted using pIP33 (a gift of Peter Sorger, MIT, MA). *CDC55* was deleted using pJM6 (Minshull et al., 1996). *clb2Δ* strains were made by crossing K1890 (Surana et al., 1991) to the appropriate strains. *CDC28-F88G* strains were made by crossing JAU02 (a gift of Jeff Ubersax, UC San Francisco, CA) to the appropriate strains. The *2μ-CDC28* plasmid is pEF190 (a gift of Eric Foss, Seattle, WA) and is the *CDC28* gene

cloned into pRS425 (Sikorski and Hieter, 1989). *pHIS3-GFP-TUB1* was integrated using pAFS71 (a gift of Aaron Straight, Harvard Med, MA). *cdc20-3* strains were made by crossing K8029 (Shirayama et al., 1998) to the appropriate strains. *pGAL-PDS1-HA* strains were made by crossing RTK43 (Jaspersen et al., 1998) to the appropriate strains.*CDC27-MBP* strains were made by crossing JC35 (a gift of Julia Charles, UC San Francisco, CA) to the appropriate strains. *cdc26Δ* strains were described previously (Hwang and Murray, 1997). *clb2Δ* strains were made by crossing K1890 (a gift of Kim Nasmyth, IMP, Vienna, Austria) to the appropriate strains. *APC9* was tagged by the PCR-targeting method. Cells were transformed with a cassette containing the bacterial KAN^R gene which confers G418 resistance in W303. The cassette was amplified by PCR from pFA6a-3HA-kanMX6 (Longtine et al., 1998) with primers that containing the sequences that flank the stop codon of *APC9* .

Mutants in *CDC28-VF-HA*, *CDC28-F-HA*, *CDC28-V-HA* and *CDC28-HA* were made as described previously (Booher et al., 1993). *CDC28-AF-HA* was made by cloning the HindIII/XhoI fragment from pSF35 (Peter Sorger) into pRD96 (a gift of Ray Deshaies, California Institute of Technology, CA) cut with HindIII/XhoI to create pAR155 which was used to create a *CDC28-AF-HA* strain as previously described for pRD96 (Booher et al., 1993). The *pGAL-CDC28* plasmids were made as follows: A full length *CDC28-HA* (pAR106) was made by cutting out the 1.1 kb XhoI-HindIII fragment of *CDC28-VF-HA* from pRD96, and replacing it with the same fragment from pRD47. The BstBI-BamHI fragment of pRD96 or pAR106 was then cloned into pDK20 (a gift of Doug Kellogg, UC Santa Cruz) cut with SmaI and BamHI, to create pAR109 (yIP-*pGAL-CDC28:URA3*) and pAR108 (yIP-*pGAL-CDC28-VF:URA3*). The plasmids were cut with StuI and integrated at the *URA3* locus.

CLB2-Δ176 strains were made with pAR39. A EcoRI/BamHI fragment of *CLB2* that lacks the first 176 amino acids was amplified by PCR and cloned into pDK20 cut with

EcoRI and BamHI to create pAR39. The plasmid was cut with StuI for integration at the *URA3* locus.

cks1-38 strains are *cks1Δ::KAN^R* covered by *cks1-38* integrated at *TRP1* (pAR183). *CKS1* was deleted by the PCR-targeting method. Diploid cells were transformed with a cassette containing the bacterial *KAN^R* gene which confers G418 resistance in W303. The cassette was amplified by PCR from pFA6a-kanMX6 (Longtine et al., 1998) with primers that containing the sequences that flank the *CKS1* ORF. pAR183 (*cks1-38:TRP)* was constructed by cloning an BamHI/SphI fragment from pSE271 (Tang and Reed, 1993) into YIplac204 (Gietz and Sugino, 1988), the resulting plasmid was cut with EcoRV and integrated into the *CKS1/cks1Δ::KAN^R* heterozygote at the *TRP1* locus. The diploid was sporulated, and a resulting spore, ADR1767, which is temperature sensitive, *TRP$^+$* and *KAN^R* was used to create the strains used in this study.

HCT1 was deleted using pAR127. An EcoRI/HindIII fragment of the *HCT1* locus was amplified by PCR and cloned into pSK(-) (Stratagene) to create pAR125. A XbaI/SmaI fragment of the *HIS3* gene was then cloned into pAR125 cut with SpeI/XmnI, to create pAR127, which replaces the entire *HCT1* ORF with *HIS3*. The EcoRI/NotI fragment of pAR127 was used to transform yeast.

CDC20 was tagged at the N-terminus. A cassette containing 12 myc tags (pLH71) was inserted into pCM4 (Hwang et al., 1998) cut with BsteII, to create pLH83. The resultant plasmid was cut with EcoNI and BglII, removing the *CEN-ARS* sequences, blunted and religated, to create pLH92. This plasmid was cut with BspEI for integration at the *CDC20* locus, creating a duplication of *CDC20* and *CDC20-12myc*, marked with the *URA3* gene. This strain was grown on 5-FOA to select against the *URA3* gene and the resulting colonies were screened by western blot for the presence of *CDC20-12myc* to create LHH371. This strain was crossed to the appropriate strains to create those used in this study.

Alanine substituted mutants in *CDC16*, *CDC23*, and *CDC27* were made using site directed mutagenesis (Kunkel, 1985). Mutations were confirmed by the introduction of new restriction enzyme sites and by sequencing (ABI). For *CDC16*, The EcoR1/Xho1 fragment of pWAM10 (Lamb et al., 1994) was cloned into KS (-) (Stratagene) to create pAR290. pAR290 was mutagenized to create pAR293 which contains all six serine/threonine to alanine substitutions. pAR294 was cut with EcoRI and NotI, and ligated to a EcoRI/PstI PCR fragment that contains the 3'end of *CDC16*, a PstI/SpeI PCR fragment that contains the *TRP1* gene, and a SpeI/NotI PCR fragment that contains the 3'UTR of the *CDC16* gene. The resultant plasmid, pAR303, was cut with XhoI and NotI, and integrated at the *CDC16* locus. The *TRP*$^+$ transformants were screened by PCR for the presence of all mutations. For *CDC23*, the BamHI/NotI fragment of pRS239 (Lamb et al., 1994) was cloned into KS (-) to create pAR228. pAR228 was mutagenized to create pAR240 which contains the single serine to alanine substitution in *CDC23*. pAR228 was cut with BamHI and NotI, transformed into *cdc23-1* cells (ADR1285) and selected for growth at 37°C. Transformants were screened by western for the HA tag present at the 3' end of the gene, and by PCR for the presence of the alanine substitution. For *CDC27*, the PstI/NotI fragment of pJL25 (Lamb et al., 1994) was cloned into KS (-) to create pAR201. pAR201 was mutagenized to create pAR203, which contains all five serine/threonine to alanine substitutions in *CDC27*. pAR203 was cut with NdeI and NotI, and ligated to a NdeI/XbaI PCR fragment that contains the *KAN*^{R} gene and a XbaI/NotI PCR fragment containing the 3'UTR of *CDC27*. The resultant plasmid, pAR271, was cut with KpnI and NotI, and integrated at the *CDC27* locus. Transformants were screened by PCR for the presence of all mutations.

Physiology

Stock solutions of inhibitors were: 10mg/ml alpha factor (Biosynthesis), 10mg/ml nocodazole (Sigma), Hydroxyurea (HU, Sigma) was added directly to media at a final concentration of 200mM. All stocks were stored at -20°C in DMSO.

For microcolony assays, cells were grown to mid-log phase in YEP+2% raffinose, spotted onto a YEP+2% galactose plate and unbudded cells were picked out into a grid with a dissection needle. The number of cells in each microcolony was counted at different times after incubation at 30°C. Each bud is counted as a cell and the original cells which did not bud are not included in the analysis.

For serial dilution and spotting, cells were prepared in a multi-well dish or a micrometer plate, and using a multi-prong applicator, approximately 10μl of each strain and its dilutions were spotted onto various plates and incubated at either 23°C or 30°C.

Sister chromatid separation was visualized using a fusion of green flourescent protein (GFP) and lacI bound to repeats of *lacO* which had been integrated at specific locations on yeast chromosomes (Straight et al., 1996). Small samples of cells were harvested at the indicated times and either scored live, or fixed for 10 minutes in 3.7% paraformaldehyde at 4°C, and then washed two times in 0.1M KPO_4 pH 7.4. The cells were then mounted on slides and viewed by fluorescence microscopy (Nikon). In all experiments a minimum of 200 cells were counted per time point.

The anaphase arrest of *pGAL-CLB2-Δ176* was visualized using *pHIS3-GFP-TUB1*. GFP-Tub1 was induced by transferring cells to CSM-His +2% galactose + 10mM 3-aminotriazole for the first 30 minutes after alpha factor removal. Cells were then transferred into YEP+ 2% galactose. Spindle length was scored in living cells by fluorescence microscopy. The anaphase arrest of *cdc15-2* was scored by fixing cells for 5 minutes in 70% ethanol, washing in 50mM Tris-Cl pH7.5 and then resuspending in 50mM

Tris-Cl pH7.4 + 1μg/ml DAPI (Molecular Probes). Cells were then mounted on slides and viewed by fluorescence microscopy.

Cells were fixed for indirect immunofluorescence in 3.7% formaldehyde for one hour. The spindles were visualized by anti-alpha-tubulin (Harlan Sera-Lab) immunofluorescence as described previously (Hardwick and Murray, 1995) except that the blocking reagent used was 2% BSA, PBS. Short spindles are bipolar spindles less than 2 μm long.

Immunoprecipitation and western blots

In experiments where only western blots are shown, yeast extracts were prepared by bead beating (multi-tube bead beater; Biospec) frozen cell pellets in 1X SDS sample buffer (2% SDS, 80mM Tris-Cl pH6.8, 10% Glycerol, 10mM EDTA, 0.02% Bromophenol Blue, 1mM Na_3VO_4, 1mM PMSF and 5mM NaF) and an equal volume of acid washed glass beads (Biospec) for one pulse of ninety seconds. Samples were then normalized based on OD_{600} readings of the original yeast samples taken during the time course. We have found that this method allows even loading of the samples and works as well as other techniques.

Yeast extracts for immunoprecipitation and western blots were made by bead beating frozen cell pellets in lysis buffer (100mM NaCl, 50mM Tris-Cl pH7.5, 50mM NaF, 50mM Na-ß-glycerophosphate pH7.4, 2mM EGTA, 2mM EDTA, 0.1% TritonX-100, 1mM Na_3VO_4, 1mM PMSF, Leupeptin, Pepstatin and Chymostatin all at 1μg/ml) and an excess of acid washed glass beads for two pulses of ninety seconds, incubating on ice for five minutes between each pulse. The resulting lysate was separated from the glass beads and centrifuged in a microfuge at 14,000 rpm for five minutes to remove insoluble material. Protein concentration of each lysate was determined using Bradford reagent (0.04% Coomassie Blue G-250 dissolved in 4.75% Ethanol, then mixed with 8.5% o-

Phosphoric Acid and H_2O) and samples were normalized based on these measurements. A portion of the lysate was mixed with an equal volume of 2X SDS sample buffer (4% SDS, 160mM Tris-Cl pH6.8, 20% Glycerol, 20mM EDTA, 0.04% Bromophenol Blue, and 1mM PMSF).

Standard methods were used for polyacrylamide gel electrophoresis and protein transfer to nitrocellulose (Schleicher and Schuell) (Minshull et al., 1996). Blots were stained with Ponceau S to confirm transfer and equal loading of samples, and then blocked for 30 minutes in antibody specific blocking buffer (see below). All antibodies were incubated overnight at 4°C or 2 hours at 25°C. After washing in PBST (PBS+ 0.1% tween-20) (Maniatis et al., 1982) the blots were then incubated in HRP-conjugated anti-rabbit or anti-mouse antibodies (Amersham) at a 1:5000 dilution in PBST for thirty minutes at 25°C, washed again, incubated in Amersham ECL detection reagents (Amersham) or Renaissance reagents (NEN), using the manufacturer's instructions, and then exposed to X-OMAT film (Kodak).

The following antibodies were used in western blots: 9E10 ascites (BabCO) was used at a dilution of 1:1000 in PBST + 0.02% NaN_3 after blocking in 4% non-fat dried milk (Carnation) in PBST. Affinity-purified rabbit polyclonal anti-Clb2 and anti-Clb3 antibodies (Kellogg and Murray, 1995) were used at a dilution of 1:1200 in blocking buffer (2% BSA, PBST, 0.5M NaCl, 0.02% NaN_3). Rabbit polyclonal anti-Sic1 serum (a gift of Mike Mendenhall, University of Kentucky, KY) was used at 1:1000 in blocking buffer (4% non-fat dried milk, 2% BSA, PBST, 0.02% NaN_3, 10μg/ml cell lysate made from *sic1Δ* cells (JM408, made with MDMp203 (Mike Mendenhall))). 12CA5 ascites (BabCO) was used at 1:1000, rabbit polyclonal anti-Cdc16 and anti-Cdc23(Lamb et al., 1994) were used at 1:2000, and anti-Cdc27 (Phil Hieter) was used at 1:2500. These four antibodies were all diluted in blocking buffer (4% non-fat dried milk, PBST, 0.02% NaN_3).

The remaining lysate was used for immunoprecipitation. 2-20 mg of lysate per sample was used depending on the experiment. 0.33-3 μg of antibody was added to the

lysate and incubated on ice for 20 minutes. Samples were then centrifuged in a microfuge at 14,000 rpm for five minutes at 4°C and transferred to 10-15μl of ProteinA CL-4B sepharose beads (Sigma) which had been equilibrated in lysis buffer. The beads were rotated at 4°C for one to two hours. The beads were manipulated as described below.

To resolve the phosphorylated forms of Cdc27 by western blot, samples were electrophoresed on a 12.5% polyacrylamide gel containing 0.025% bisacrylamide. The phosphorylated forms of Cdc16 were resolved by western blot on a 10% polyacrylamide gel containing 0.13% bisacrylamide.

Histone H1 kinase assays

For histone H1 kinase reactions, 0.33μg of anti-Clb2 or anti-Clb3 antibody were used for immunoprecipitation in 1-5 mg of cell lysate. After immunoprecipitation the beads were washed three times in kinase bead buffer (500mM NaCl, 50mM Tris-Cl pH7.4, 50mM NaF, 5mM EGTA, 5mM EDTA, 0.1% Triton-X-100) (transferring the beads to fresh tubes after the second wash) and two times in kinase buffer (80mM Na-ß-glycerophosphate pH7.4, 15mM $MgCl_2$, 20mM EGTA). All washes were performed on ice. Kinase reactions were performed in 15μl of kinase buffer containing 1mM DTT, 25μM ATP, 2.5 μg histone H1 (Upstate Biotechnology) and 1μCi of [γ-^{32}P] ATP (Amersham) and were incubated for fifteen minutes at 25°C. Reactions were stopped by adding 15μl of 2X SDS sample buffer and heating samples to 99°C for five minutes. Samples were run on a 15% polyacrylamide gel, stained and dried. Kinase gels were quantified using a Molecular Dynamics phosphoimager and ImageQuant software.

Phosphotyrosine detection

For anti-phosphotyrosine blots, 2 μg of 12CA5 antibody was used to immunoprecipitate Cdc28-HA or Cdc28-VF-HA from 15-20 mg of cell lysate. The beads were washed three times in kinase bead buffer and two times in PBS. Standard methods were used for polyacrylamide gel electrophoresis and protein transfer to nitrocellulose. The blot was then blocked in P-Tyr blocking buffer (1% BSA, 10mM Tris-Cl pH 7.5, 100mM NaCl, 0.1% Tween 20) and incubated overnight at 4°C in blocking buffer containing an anti-P-Tyr Fab fragment conjugated to HRP, RC20H (Transduction Laboratories), diluted to 1:2500. The blot was washed five times in TBST (10mM Tris-Cl pH7.5, 100mM NaCl, 0.1% Tween 20), incubated in SuperSignal chemiluminescent substrate (Pierce), using the manufacturer's instructions, and then exposed to X-ray film. After exposure, the blot was stripped in an SDS containing buffer, and reprobed with the 12CA5 antibody to see if the same amount of Cdc28 had been immunoprecipitated.

APC assay

For APC assay, 1μg of rabbit polyclonal anti-Cdc26 antibody (Hwang and Murray, 1997) was used to immunoprecipitate the APC from 5mg of cell lysate. The APC assay was conducted as previously described (Charles et al., 1998) except that cells were lysed in the lysis buffer described above, the beads were washed in APC bead buffer (250mM NaCl, 50mM Tris-Cl pH7.4, 50mM NaF, 5mM EGTA, 5mM EDTA, 0.1% NP-40, 1mM DTT) three times, and then two times in QA+NaCl buffer (20mMTris-Cl pH 7.6, 100mM NaCl, 1mM $MgCl_2$). 25nM of purified Hct1 (Jaspersen et al., 1999) was added to some samples during the ubiquitination reaction.

Cdc20 binding to the APC

For Cdc20 binding experiments, 3μg of anti-Cdc26 antibody was used to immunoprecipitate 10-20mg of cell lysate. After immunoprecipitation, the beads were washed three times in Cdc20 bead buffer (200mM NaCl, 50mM Tris-Cl pH7.4, 50mM NaF, 5mM EGTA, 5mM EDTA, 0.1% NP-40, 1mM DTT), and then two times in low salt kinase buffer (10mM NaCl, 20mM Hepes-KOH pH 7.4, 5mM $MgCl_2$). The immunoprecipitates were then processed for western blots as described above.

In vivo labeling of the APC

Cells were grown as described in figure legends. Once the cells were arrested at the indicated stage of the cell cycle, 50ml of OD_{600} 0.8 cells were harvested by centrifugation, washed twice in H_2O, and resuspended in 1ml phosphate-free CSM (Rothblatt and Schekman, 1989) containing 0.5-1 mCi $^{32}PO_4$ (Amersham). Cells were labeled for 1 hour, harvested by centrifugation, washed once in H_2O, and then frozen in screw-cap tubes (Sarstedt). These tubes were used throughout the procedure to prevent radioactive contamination. The frozen yeast pellets were processed for immunoprecipitation as described in the accompanying paper with the following modifications. 2-3 μg anti-Cdc26 antibody was bound to 20μl protein-A beads for 20 minutes on ice. These beads were then incubated with 10-20 mg of unlabeled cell lysate made from *cdc26Δ* cells for 1-2 hours. After incubation, the beads were washed 2 times in lysis buffer. At the same time, the labeled cell lysate (typically 10mg) was pre-cleared in 75μl protein-A CL-4B sepharose beads (Sigma) for 1 hour, and then centrifuged at 14,000 rpm for 5 minutes at 4°C. The labeled lysate was then added to the antibody bound protein-A beads and incubated with rotation for 1-2 hours. The beads were washed four times with kinase bead buffer (transferring the beads to fresh tubes after the fourth wash), and then two times with 50mM

Tris-Cl pH7.5. The beads were then rotated in 50mM Tris-Cl pH7.5 containing 0.5mg/ml RNAse A for 30 minutes at 4°C, washed an additional two times in kinase bead buffer (transferring the beads to fresh tubes after the second wash), and then a final wash in 50mM Tris-Cl pH7.5.

In vitro phosphorylation of the APC

Cells were arrested in G1 by alpha factor as described in the figure legends. Cells were harvested by centrifugation, frozen and processed for immunoprecipitation. 10-15 mg of cell lysate was pre-cleared in 50μl protein-A beads, and then the APC was immunoprecipitated with 2μg anti-Cdc26 antibodies which were pre-bound to protein-A beads as described above. After immunoprecipitation, the beads were washed three times in kinase bead buffer (transferring the beads to fresh tubes after the second wash), and then two times in low salt kinase buffer (10mM NaCl, 20mM Hepes-KOH pH 7.4, 5mM $MgCl_2$, 1mM DTT). 5ng of purified Cdc28-His_6, 50ng purified Clb2-MBP (gifts of Jeff Ubersax, UC San Francisco, CA), and 100ng purified Cks1 (see below), in 2μl of kinase dilution buffer (300mM NaCl, 25mM Hepes-KOH pH7.4, 10% glycerol, 0.1mg/ml BSA) were added to a 13μl of low salt kinase buffer containing 10μm ATP, 2μCi [γ-^{32}P] ATP (Amersham), and 10μm okadaic acid (Calbiochem). This reaction mix was added to the immunoprecipitated APC and incubated at 25°C for 20 minutes. The beads were then washed three times in kinase bead buffer containing 1μm okadaic acid (transferring the beads to fresh tubes after the second wash), and then two times in low salt kinase buffer containing 1μm okadaic acid. These washes remove Clb2-MBP and proteolytic fragments of Clb2-MBP, which are well phosphorylated and obscure APC phosphorylation. Cdc5 phosphorylation was performed by adding the following to immunoprecipitated APC: purified His_6-HA-Cdc5 (a gift of Julia Charles, UC San Francisco, CA) in 5μl of Cdc5 storage buffer (250mM KCl, 20mM Hepes-KOH pH7.4, 10% glycerol, 5mM NaF,

0.1mg/ml BSA) added to 15μl of Cdc5 kinase reaction buffer (20mM KCl, 20mM Hepes-KOH pH7.4, 2mM $MgCl_2$, 2mM $MnCl_2$ (final concentrations in 20μl reaction)) containing 10μm ATP, 2μCi [γ-^{32}P] ATP and 10μm okadaic acid.

Cks1 bead pulldowns

Cks1 protein was made as described previously (Booher et al., 1993) using pCKS1-1. After the ammonium sulfate precipitation, the pellet was resuspended in lysis buffer (50mM Tris-Cl pH8.0, 2mM EDTA, 10% glycerol) and then desalted on a PD-10 column (Pharmacia) which had been equilibrated in CnBr coupling buffer (500mM NaCl, 100mM Na_2CO_3 pH8.3). Cks1 was then coupled to CnBr-activated Sepharose 6MB or 4B (Pharmacia) according to the manufacturer's instructions. Beads were washed and stored in lysis buffer (100mM NaCl, 50mM Tris-Cl pH7.5, 50mM NaF, 50mM Na-ß-glycerophosphate pH7.4, 2mM EGTA, 2mM EDTA, 0.1% TritonX-100, 0.02% NaN_3). 3-5 mg of cell lysate was incubated with 10μl Cks1-coupled beads for 1-2 hours, washed three times in kinase bead buffer (500mM NaCl, 50mM Tris-Cl pH7.4, 50mM NaF, 5mM EGTA, 5mM EDTA, 0.1% Triton-X-100) (transferring the beads to fresh tubes after the second wash), and then two times in low salt kinase buffer. Phosphatase treatment of Cks1-bead pulldowns was performed as previously described (Hardwick and Murray, 1995) using lambda phosphatase (NEBL).

Bibliography

Ahn, S.H., A. Acurio, and S.J. Kron. 1999. Regulation of G2/M progression by the STE mitogen-activated protein kinase pathway in budding yeast filamentous growth. *Mol Biol Cell.* 10:3301-3316.

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

Amon, A. 1997. Regulation of B-type cyclin proteolysis by Cdc28-associated kinases in budding yeast. *Embo J.* 16:2693-2702.

Amon, A. 1999. The spindle checkpoint. *Curr Opin Genet Dev.* 9:69-75.

Amon, A., S. Irniger, and K. Nasmyth. 1994. Closing the cell cycle circle in yeast: G2 cyclin proteolysis initiated at mitosis persists until the activation of G1 cyclins in the next cycle. *Cell.* 77:1037-1050.

Amon, A., U. Surana, I. Muroff, and K. Nasmyth. 1992a. Regulation of p34^{CDC28} tyrosine phosphorylation is not required for entry into mitosis in *S. cerevisiae. Nature.* 355:368-371.

Amon, A., U. Surana, I. Muroff, and K. Nasmyth. 1992b. Regulation of p34^{CDC28} tyrosine phosphorylation is not required for entry into mitosis in *S. cervisiae. Nature.* 355:368-371.

Amon, A., M. Tyers, B. Futcher, and K. Nasmyth. 1993. Mechanisms that help the yeast cell cycle clock tick: G2 cyclins transcriptionally activate G2 cyclins and repress G1 cyclins. *Cell.* 74:993-1007.

Andreassen, P.R., and R.K. Margolis. 1994. Induction of partial mitosis in BHK cells by 2-aminopurine. *J. Cell Biol.* 127:789-802.

Baber-Furnari, B.A., N. Rhind, M.N. Boddy, P. Shanahan, A. Lopez-Girona, and P. Russell. 2000. Regulation of mitotic inhibitor Mik1 helps to enforce the DNA damage checkpoint. *Mol Biol Cell.* 11:1-11.

Bahler, J., A.B. Steever, S. Wheatley, Y. Wang, J.R. Pringle, K.L. Gould, and D. McCollum. 1998. Role of polo kinase and Mid1p in determining the site of cell division in fission yeast. *J Cell Biol.* 143:1603-1616.

Balasubramanian, M.K., D. McCollum, and U. Surana. 2000. Tying the knot: linking cytokinesis to the nuclear cycle. *J Cell Sci.* 113:1503-1513.

Basu, J., E. Logarinho, S. Herrmann, H. Bousbaa, Z. Li, G.K.T. Chan, T.J. Yen, C.E. Sunkel, and M.L. Goldberg. 1998. Localization of the drosophila checkpoint control protein bub3 to the kinetochore requires bub1 but not Zw10 or Rod [In Process Citation]. *Chromosoma.* 107:376-385.

Belmont, L.D., A.A. Hyman, K.E. Sawin, and T.J. Mitchison. 1990. Real-time visualization of cell cycle-dependent changes in microtubule dynamics in cytoplasmic extracts. *Cell.* 62:579-589.

Beltraminelli, N., M. Murone, and V. Simanis. 1999. The S. pombe zfs1 gene is required to prevent septation if mitotic progression is inhibited. *J Cell Sci.* 112 Pt 18:3103-3114.

Berlin, V., C.A. Styles, and G.R. Fink. 1990. BIK1, a protein required for microtubule function during mating and mitosis in Saccharomyces cerevisiae, colocalizes with tubulin. *J Cell Biol.* 111:2573-2586.

Biggins, S., F.F. Severin, N. Bhalla, I. Sassoon, A.A. Hyman, and A.W. Murray. 1999. The conserved protein kinase Ipl1 regulates microtubule binding to kinetochores in budding yeast. *Genes Dev.* 13:532-544.

Booher, R.N., R.J. Deshaies, and M.W. Kirschner. 1993. Properties of *Saccharomyces cerevisiae* wee1 and its differential regulation of $p34^{CDC28}$ in response to G_1 and G_2 cyclins. *EMBO J.* 12:3417-3426.

Brady, D.M., and K.G. Hardwick. 2000. Complex formation between mad1p, bub1p and bub3p is crucial for spindle checkpoint function [In Process Citation]. *Curr Biol.* 10:675-678.

Brown, N.R., M.E. Noble, J.A. Endicott, and L.N. Johnson. 1999. The structural basis for specificity of sustrate and recruitment peptides for cyclin-dependent kinases. *Nat Cell Biol.* 1:438-443.

Cahill, D.P., C. Lengauer, J. Yu, G.J. Riggins, J.K. Willson, S.D. Markowitz, K.W. Kinzler, and B. Vogelstein. 1998. Mutations of mitotic checkpoint genes in human cancers. *Nature.* 392:300-303.

Cerutti, L., and V. Simanis. 1999. Asymmetry of the spindle pole bodies and spg1p GAP segregation during mitosis in fission yeast. *J Cell Sci.* 112:2313-2321.
Cerutti, L., and V. Simanis. 2000. Controlling the end of the cell cycle. *Curr Opin Genet Dev.* 10:65-69.

Chan, G.K., B.T. Schaar, and T.J. Yen. 1998. Characterization of the kinetochore binding domain of CENP-E reveals interactions with the kinetochore proteins CENP-F and hBUBR1. *J Cell Biol.* 143:49-63.

Charles, J., S.L. Jaspersen, R.L. Tinker-Kulberg, L. Hwang, A. Szidon, and D.O. Morgan. 1998. The Polo-related kinase Cdc5 activates and is destroyed by the mitotic cyclin destruction machinery in *S. cerevisiae. Curr. Biol.* 9:497-507.

Chen, R.-H., A. Shevchenko, M. Mann, and A.W. Murray. 1998. Metaphase arrest induced by an excess of the spindle checkpoint protein Xmad2 is independent of Xmad1. *J. Cell Biol.* 143:283-295.

Chen, R.-H., J.C. Waters, E.D. Salmon, and A.W. Murray. 1996. Association of spindle assembly checkpoint component XMAD2 with unattached kinetochores. *Science.* 274:242-246.

Ciechanover, A. 1994. The ubiquitin-proteasome proteolytic pathway. *Cell.* 79:13-21.

Ciosk, R., W. Zachariae, C. Michaelis, A. Shevchenko, M. Mann, and K. Nasmyth. 1998. An ESP1/PDS1 complex regulates loss of sister chromatid metaphase to anaphase transition in yeast. *Cell.* 93:1067-1076.

Clarke, P.R., I. Hoffmann, G. Draetta, and E. Karsenti. 1993. Dephosphorylation of cdc25-C by a type-2A protein phosphatase: specific regulation during the cell cycle in Xenopus egg extracts. *Mol Biol Cell.* 4:397-411.

Cohen-Fix, O., and D. Koshalnd. 1997. The anaphase inhibitor of *Saccharomyces cerevisiae* Pds1p is a target of the DNA damage checkpoint pathway. *Proc. Natl. Acad. Sci. U. S. A.* 94:14361-14366.

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

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

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

Dahmann, C., J.F. Diffley, and K.A. Nasmyth. 1995. S-phase-promoting cyclin-dependent kinases prevent re-replication by inhibiting the transition of replication origins to a pre-replicative state. *Curr Biol.* 5:1257-1269.

De Bondt, H.L., J. Rosenblatt, J. Jancarik, H.D. Jones, D.O. Morgan, and S.H. Kim. 1993. Crystal structure of cyclin-dependent kinase 2. *Nature.* 363:595-602.

Descombes, P., and E.A. Nigg. 1998. The polo-like kinase Plx1 is required for M phase exit and destruction of mitotic regulators in Xenopus egg extracts. *Embo J.* 17:1328-1335.

Detwiler, P.B., and M.P. Gray-Keller. 1996. The mechanisms of vertebrate light adaptation: speeded recovery versus slowed activation. *Curr. Opin. Neurobiol.* 6:440-444.

Donovan, J.D., J.H. Toyn, A.L. Johnson, and L.H. Johnston. 1994. p40SDB25, a putative CDK inhibitor, has a role in the M/G1 transition in *Saccharomyces cerevisiae. Genes Dev.* 8:1640-1653.

Dunphy, W.G. 1994. The decision to enter mitosis. *Trends Cell Biol.* 4:202-207.

Enoch, T., and P. Nurse. 1990. Mutation of fission yeast cell cycle control genes abolishes dependence of mitosis on DNA replication. *Cell.* 60:665-673.

Epstein, C.B., and F.R. Cross. 1992. CLB5: a novel B cyclin from budding yeast with a role in S phase. *Genes Dev.* 6:1695-1706.

Fang, G., H. Yu, and M.W. Kirschner. 1998a. The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation. *Genes Dev.* 12:1871-1883.

Fang, G., H. Yu, and M.W. Kirschner. 1998b. Direct binding of CDC20 protein family members activates the anaphase- promoting complex in mitosis and G1. *Mol Cell.* 2:163-171.

Fankhauser, C., J. Marks, A. Reymond, and V. Simanis. 1993. The S. pombe cdc16 gene is required both for maintenance of p34cdc2 kinase activity and regulation of septum formation: a link between mitosis and cytokinesis? *Embo J.* 12:2697-2704.

Farruggio, D.C., F.M. Townsley, and J.V. Ruderman. 1999. Cdc20 associates with the kinase aurora2/Aik. *Proc Natl Acad Sci U S A.* 96:7306-7311.

Featherstone, C., and P. Russell. 1991. Fission yeast p107^{wee1} mitotic inhibitor is a tyrosine/serine kinase. *Nature.* 349:808-811.

Felix, M.A., J.C. Labbe, M. Doree, T. Hunt, and E. Karsenti. 1990. Triggering of cyclin degradation in interphase extracts of amphibian eggs by cdc2 kinase. *Nature.* 346:379-382.

Fesquet, D., P.J. Fitzpatrick, A.L. Johnson, K.M. Kramer, J.H. Toyn, and L.H. Johnston. 1999. A Bub2p-dependent spindle checkpoint pathway regulates the Dbf2p kinase in budding yeast. *Embo J.* 18:2424-2434.

Fitch, I., C. Dahmann, U. Surana, A. Amon, K. Nasmyth, L. Goetsch, B. Byers, and B. Futcher. 1992. Characterization of Four B-Type Cyclin Genes of the Budding Yeast *Saccharomyces cerevisiae. Molecular Biology of the Cell.* 3:805-818.

Fraschini, R., E. Formenti, G. Lucchini, and S. Piatti. 1999. Budding yeast Bub2 is localized at spindle pole bodies and activates the mitotic checkpoint via a different pathway from Mad2. *J Cell Biol.* 145:979-991.

Funabiki, H., K. Kumada, and M. Yanagida. 1996a. Fission yeast Cut1 and Cut2 are essential for sister chromatid separation, concentrate along the metaphase spindle and form large complexes. *Embo J.* 15:6617-6628.

Funabiki, H., H. Yamano, K. Kumada, K. Nagao, T. Hunt, and M. Yanagida. 1996b. Cut2 proteolysis required for sister-chromatid separation in fission yeast. *Nature*. 381:438-441.

Funabiki, H., H. Yamano, K. Nagao, H. Tanaka, H. Yasuda, T. Hunt, and M. Yanagida. 1997. Fission yeast Cut2 required for anaphase has two destruction boxes. *Embo J.* 16:5977-5987.

Furnari, B., A. Blasina, M.N. Boddy, C.H. McGowan, and P. Russell. 1999. Cdc25 inhibited in vivo and in vitro by checkpoint kinases Cds1 and Chk1. *Mol Biol Cell.* 10:833-845.

Furnari, B., N. Rhind, and P. Russell. 1997. Cdc25 mitotic inducer targeted by chk1 DNA damage checkpoint kinase. *Science*. 277:1495-1497.

Gardner, R., C.W. Putnam, and T. Weinert. 1999. RAD53, DUN1 and PDS1 define two parallel G2/M checkpoint pathways in budding yeast. *Embo J.* 18:3173-3185.

Ghiara, J.B., H.E. Richardson, K. Sugimoto, M. Henze, D.J. Lew, C. Witenberg, and S.I. Reed. 1991. A cyclin B homolog in *S. cerevisiae*: chronic activation of the Cdc28 protein kinase by cyclin prevents exit from mitosis. *Cell*. 65:163-174.

Gietz, R.D., and A. Sugino. 1988. New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene*. 74:527-534.

Glotzer, M., A.W. Murray, and M.W. Kirschner. 1991. Cyclin is degraded by the ubiquitin pathway. *Nature*. 349:132-138.

Gorbsky, G.J., R.-H. Chen, and A.W. Murray. 1998. Microinjection of antibody to Mad2 protein into mammalian cells in mitosis induces premature anaphase. *J Cell Biol.* 141:1193-1205.

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

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

Grandin, N., and S.I. Reed. 1993. Differential function and expression of *Saccharomyces cerevisiae* B-type cyclins in mitosis and meiosis. *Mol Cell Biol.* 13:2113-2125.

Hadwiger, J.A., C. Wittenberg, M.D. Mendenhall, and S.I. Reed. 1989. The *Saccharomyces cerevisiae CKS1* gene, a homolog of the *Schizosaccharomyces pombe suc1+* gene, encodes a subunit of the Cdc28 protein kinase complex. *Mol Cell Biol.* 9:2034-2041.

Hagan, I., J. Hayles, and P. Nurse. 1988. Cloning and sequencing of the cyclin-related *cdc13*$^{+}$ gene and a cytological study of its role in fission yeast mitosis. *J. Cell Sci.* 91:587-595.

Hardwick, K., and A.W. Murray. 1995. Mad1p, a phosphoprotein component of the spindle assembly checkpoint in budding yeast. *J. Cell Biol.* 131:709-720.

Hardwick, K.G. 1998. The spindle checkpoint. *Trends Genet.* 14:1-4.

Hardwick, K.G., R. Li, C. Mistrot, R.-H. Chen, P. Dann, A. Rudner, and A.W. Murray. 1999. Lesions in many different spindle components activate the spindle checkpoint in the budding yeast Saccharomyces cerevisiae. *Genetics.* 152:509-518.

Hardwick, K.G., D. Smith, R. Johnston, and A.W. Murray. 1998. *MAD3* encodes a novel component of the spindle checkpoint which interacts with both Bub3p and Cdc20p. *J. Cell Biol.* submitted.

Hardwick, K.G., E. Weiss, F.C. Luca, M. Winey, and A.W. Murray. 1996. Activation of the budding yeast spindle assembly checkpoint without mitotic spindle disruption. *Science.* 273:953-956.

Hardy, C.F., and A. Pautz. 1996. A novel role for Cdc5p in DNA replication. *Mol Cell Biol.* 16:6775-6782.

Hayles, J., and P. Nurse. 1995. A pre-start checkpoint preventing mitosis in fission yeast acts independently of p34cdc2 tyrosine phosphorylation. *Embo J.* 14:2760-2771.

Heald, R., and F. McKeon. 1990. Mutations of phosphorylation sites in lamin A that prevent nuclear lamina disassembly in mitosis. *Cell.* 61:579-589.

Healy, A.M., S. Zolnierowicz, A.E. Stapleton, M. Goebl, A.A. DePaoli-Roach, and J.R. Pringle. 1991. *CDC55*, a *Saccharomyces cerevisiae* gene involved in cellular

morphogenesis: identification, characterization and homology to the B subunit of mammalian type 2A protein phosphatase. *Mol. Cell Biol.* 11:5767-5780.

Heichman, K.A., and J.M. Roberts. 1996. The yeast CDC16 and CDC27 genes restrict DNA replication to once per cell cycle. *Cell.* 85:39-48.

Heichman, K.A., and J.M. Roberts. 1998. CDC16 controls initiation at chromosome replication origins. *Mol Cell.* 1:457-463.

Hershko, A., D. Ganoth, V. Sudakin, A. Dahan, L.H. Cohen, F.C. Luca, J.V. Ruderman, and E. Eytan. 1994. Components of a system that ligates cylin to ubiquitin and their regulation by protein kinase cdc2. *J. Biol. Chem.* 269:4940-4946.

Hirano, T., R. Kobayashi, and M. Hirano. 1997. Condensins, chromosome condensation protein complexes containing XCAP-C, XCAP-E and a Xenopus homolog of the Drosophila Barren. *Cell.* 89:511-521.

Hirano, T., T.J. Mitchison, and J.R. Swedlow. 1995. The SMC family: from chromosome condensation to dosage compensation. *Curr Opin Cell Biol.* 7:329-336.

Holloway, S.L., M. Glotzer, R.W. King, and A.W. Murray. 1993. Anaphase is initiated by proteolysis rather than by the inactivation of MPF. *Cell.* 73:1393-1402.

Hoyt, M.A., L. He, K.K. Loo, and W.S. Saunders. 1992. Two *Saccharomyces cerevisiae* kinesin-related gene products required for mitotic spindle assembly. *J. Cell Biol.* 118:109-120.

Hoyt, M.A., T. Stearns, and D. Botstein. 1990. Chromosome instability mutants of *Saccharomyces cerevisiae* that are defective in microtubule-mediated processes. *Mol. Cell. Biol.* 10:223-234.

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

Hwang, L. 1998. PhD dissertation, UCSF. .

Hwang, L.H., L.F. Lau, D.L. Smith, C.A. Mistrot, K.G. Hardwick, E.S. Hwang, A. Amon, and A.W. Murray. 1998. Budding yeast Cdc20: a target of the spindle checkpoint. *Science.* 279:1041-1044.

Hwang, L.H., and A.W. Murray. 1997. A novel yeast screen for mitotic arrest mutants identifies *DOC1*, a new gene involved in cyclin proteolysis. *Mol. Biol. Cell.* 8:1877-1887.

Irniger, S., S. Piatti, C. Michaelis, and K. Nasmyth. 1995. Genes involved in sister chromatid separation are needed for B-type cyclin proteolysis in budding yeast. *Cell.* 77:1037-1050.

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

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

Jaspersen, S.L., and D.O. Morgan. 2000. Cdc14 activates cdc15 to promote mitotic exit in budding yeast [In Process Citation]. *Curr Biol.* 10:615-618.

Jin, P., Y. Gu, and D.O. Morgan. 1996. Role of inhibitory CDC2 phosphorylation in radiation-induced G2 arrest in human cells. *J Cell Biol.* 134:963-970.

Johnston, G.C., J.R. Pringle, and L.H. Hartwell. 1977. Coordination of growth cell division in the yeast *Saccharomyces cervisiae. Exp. Cell. res.* 105:79-98.

Juang, Y.L., J. Huang, J.M. Peters, M.E. McLaughlin, C.Y. Tai, and D. Pellman. 1997. APC-mediated proteolysis of Ase1 and the morphogenesis of the mitotic spindle. *Science.* 275:1311-1314.

Kaiser, P., V. Moncollin, D.J. Clarke, M.H. Watson, B.L. Bertolaet, S.I. Reed, and E. Bailly. 1999. Cyclin-dependent kinase and Cks/Suc1 interact with the proteasome in yeast to control proteolysis of M-phase targets. *Genes Dev.* 13:1190-1202.

Kallio, M., J. Weinstein, J.R. Daum, D.J. Burke, and G.J. Gorbsky. 1998. Mammalian p55CDC mediates association of the spindle checkpoint protein Mad2 with the cyclosome/anaphase-promoting complex, and is involved in regulating anaphase onset and late mitotic events. *J Cell Biol.* 141:1393-1406.

Karaiskou, A., C. Jessus, T. Brassac, and R. Ozon. 1999. Phosphatase 2A and polo kinase, two antagonistic regulators of cdc25 activation and MPF auto-amplification. *J Cell Sci.* 112:3747-3756.

Kellogg, D.R., and A.W. Murray. 1995. NAP1 acts with Clb2 to perform mitotic functions and suppress polar bud growth in budding yeast. *J. Cell Biol.* 130:675-685.

Kim, S.H., D.P. Lin, S. Matsumoto, A. Kitazano, and T. Matsumoto. 1998. Fission yeast *slp1+* encodes the effector of the Mad2-dependent spindle checkpoint. *Science.* 279:1045-1047.

Kimura, K., M. Hirano, R. Kobayashi, and T. Hirano. 1998. Phosphorylation and activation of 13S condesin by Cdc2 in vitro. *Science.* 282:487-490.

King, R.W., J.M. Peters, S. Tugendreich, M. Rolfe, P. Hieter, and M.W. Kirschner. 1995. A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. *Cell.* 81:279-288.

Kinoshita, N., H. Yamano, H. Niwa, T. Yoshida, and M. Yanagida. 1993. Negative regulation of mitosis by the fission yeast protein phosphatase ppa2. *Genes Dev.* 7:1059-1071.

Kitada, K., A.L. Johnson, L.H. Johnston, and A. Sugino. 1993. A multicopy suppressor gene of the *Saccharomyces cerevisiae* G1 cell cycle mutant gene *dbf4* encodes a protein kinase and is identified as *CDC5. Mol. Cell Biol.* 13:4445-4457.

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

Korinek, W.S., M.J. Copeland, A. Chaudhuri, and J. Chant. 2000. Molecular linkage underlying microtubule orientation toward cortical sites in yeast. *Science.* 287:2257-2259.

Kotani, S., H. Tanaka, H. Yasuda, and K. Todokoro. 1999. Regulation of APC activity by phosphorylation and regulatory factors. *J Cell Biol.* 146:791-800.

Kotani, S., S. Tugendreich, M. Fujii, P.M. Jorgensen, N. Watanabe, C. Hoog, P. Hieter, and K. Todokoro. 1998. PKA and MPF-activated polo-like kinase regulate anaphase-promoting complex activity and mitosis progression. *Mol Cell.* 1:371-380.

Kramer, E.R., C. Gieffers, G. Holzl, M. Hengstschlager, and J.M. Peters. 1998. Activation of the human anaphase-promoting complex by proteins of the CDC20/Fizzy family. *Curr Biol.* 8:1207-1210.

Kramer, E.R., N. Scheuringer, V. Podtelejnikov, M. Mann, and J.M. Peters. 2000. Mitotic regulation of the APC activator proteins CDC20 and CDH1. *Molecular Biology of the Cell.* 11:1555-1569.

Kumada, K., T. Nakamura, K. Nagao, H. Funabiki, T. Nakagawa, and M. Yanagida. 1998. Cut1 is loaded onto the spindle by binding to Cut2 and promotes anaphase spindle movement upon Cut2 proteolysis. *Curr Biol.* 8:633-641.

Kumagai, A., and W.G. Dunphy. 1996. Purification and molecular cloning of Plx1, a Cdc25-regulatory kinase from Xenopus egg extracts. *Science.* 273:1377-1380.

Kumagai, A., Z. Guo, K.H. Emami, S.X. Wang, and W.G. Dunphy. 1998. The Xenopus Chk1 protein kinase mediates a caffeine-sensitive pathway of checkpoint control in cell-free extracts. *J Cell Biol.* 142:1559-1569.

Kung, A.L., S.W. Sherwood, and R.T. Schimke. 1990a. Cell line-specific differences in the control of cell cycle progression in the absence of mitosis. *Proc. Natl. Acad. Sci. USA.* 87:9553-9557.

Kung, A.L., A. Zetterburg, S.W. Sherwood, and R.T. Schimke. 1990b. Cytotoxic effects of cell cycle phase specific agents: result of cell cycle perturbation. *Cancer Res.* 50:7307-7317.

Kunkel, T.A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc Natl Acad Sci U S A.* 82:488-492.

Lahav-Baratz, S., V. Sudakin, J.V. Ruderman, and A. Hershko. 1995. Reversible phosphorylation controls the activity of cyclosome- associated cyclin-ubiquitin ligase. *Proc Natl Acad Sci U S A.* 92:9303-9307.

Lamb, J.R., W.A. Michaud, R.S. Sikorski, and P.A. Hieter. 1994. Cdc16p, Cdc23p and Cdc27p form a complex essential for mitosis. *Embo J.* 13:4321-4328.

Lee, L., J.S. Tirnauer, J. Li, S.C. Schuyler, J.Y. Liu, and D. Pellman. 2000. Positioning of the mitotic spindle by a cortical-microtubule capture mechanism. *Science.* 287:2260-2262.

Lee, T.H., C. Turck, and M.W. Kirschner. 1994. Inhibition of cdc2 activation by INH/PP2A. *Mol. Biol. Cell.* 5:323-338.

Lehner, C.F., and P.H. O'Farrell. 1990. The roles of Drosophila cyclins A and B in mitotic control. *Cell.* 61:535-547.

Leu, J.Y., and G.S. Roeder. 1999. The pachytene checkpoint in S. cerevisiae depends on Swe1-mediated phosphorylation of the cyclin-dependent kinase Cdc28. *Mol Cell.* 4:805-814.

Lew, D.J. 2000. Cell-cycle checkpoints that ensure coordination between nuclear and cytoplasmic events in Saccharomyces cerevisiae. *Curr Opin Genet Dev.* 10:47-53.

Lew, D.J., and S.I. Reed. 1993. Morphogenesis in the yeast cell cycle: regulation by Cdc28 and cyclins. *J Cell Biol.* 120:1305-1320.

Lew, D.J., and S.I. Reed. 1995. A cell cycle checkpoint monitors cell morphogenesis in budding yeast. *J Cell Biol.* 129:739-749.

Li, J., A.N. Meyer, and D.J. Donoghue. 1995. Requirement for phosphorylation of cyclin B1 for *Xenopus* oocyte maturation. *Mol Biol Cell.* 6:1111-1124.

Li, R. 1999. Bifurcation of the mitotic checkpoint pathway in budding yeast. *Proc Natl Acad Sci U S A.* 96:4989-4994.

Li, R., and A.W. Murray. 1991. Feedback control of mitosis in budding yeast. *Cell.* 66:519-531.

Li, X., and M. Cai. 1997. Inactivation of the cyclin-dependent kinase Cdc28 abrogates cell cycle arrest induced by DNA damage and disassembly of mitotic spindles in *Saccharomyces cerevisiae. Mol Cell Biol.* 17:2723-2734.

Lim, H.H., P.Y. Goh, and U. Surana. 1996. Spindle pole body separation in Saccharomyces cerevisiae requires dephosphorylation of the tyrosine 19 residue of Cdc28. *Mol Cell Biol.* 16:6385-6397.

Lim, H.H., P.Y. Goh, and U. Surana. 1998. Cdc20 is essential for the cyclosome-mediated proteolysis of both Pds1 and Clb2 during M phase in budding yeast. *Curr Biol.* 8:231-234.

Lim, H.H., and U. Surana. 1996. Cdc20, a beta-transducin homologue, links *RAD9*-mediated G2/M checkpoint control to mitosis in *Saccharomyces cerevisiae. Mol Gen Genet.* 253:138-148.

Lin, F.C., and K.T. Arndt. 1995. The role of *Saccharomyces cerevisiae* type 2A phosphatase in the actin cytoskeleton and in entry into mitosis. *Embo J.* 14:2745-2759.

Loeb, J.D., G. Schlenstedt, D. Pellman, D. Kornitzer, P.A. Silver, and G.R. Fink. 1995. The yeast nuclear import receptor is required for mitosis. *Proc Natl Acad Sci U S A.* 92:7647-7651.

Longtine, M.S., A. McKenzie, 3rd, D.J. Demarini, N.G. Shah, A. Wach, A. Brachat, P. Philippsen, and J.R. Pringle. 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae. Yeast.* 14:953-961.

Lorca, T., A. Castro, A.M. Martinez, S. Vigneron, N. Morin, S. Sigrist, C. Lehner, M. Doree, and J.C. Labbe. 1998. Fizzy is required for activation of the APC/cyclosome in *Xenopus* egg extracts. *Embo J.* 17:3565-3575.

Lowe, M., C. Rabouille, N. Nakamura, R. Watson, M. Jackman, E. Jamsa, D. Rahman, D.J. Pappin, and G. Warren. 1998. Cdc2 kinase directly phosphorylates the cis-Golgi matrix protein GM130 and is required for Golgi fragmentation in mitosis. *Cell.* 94:783-793.

Loy, C.J., D. Lydall, and U. Surana. 1999. NDD1, a high-dosage suppressor of cdc28-1N, is essential for expression of a subset of late-S-phase-specific genes in Saccharomyces cerevisiae. *Mol Cell Biol.* 19:3312-3327.

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

Lundgren, K., N. Walworth, R. Booher, M. Dembski, M. Kirschner, and D. Beach. 1991. mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2. *Cell.* 64:1111-1122.

Lydall, D., Y. Nikolsky, D.K. Bishop, and T. Weinert. 1996. A meiotic recombination checkpoint controlled by mitotic checkpoint genes. *Nature.* 383:840-843.

Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: a Laboratory Manual. Cold Spring Harbour Laboratory, NY)., Cold Spring Harbour, New York.

McMillan, J.N., R.A. Sia, E.S. Bardes, and D.J. Lew. 1999. Phosphorylation-independent inhibition of Cdc28p by the tyrosine kinase Swe1p in the morphogenesis checkpoint. *Mol Cell Biol.* 19:5981-5990.

McMillan, J.N., R.A.L. Sia, and D.J. Lew. 1998. A morphogenesis checkpoint monitors the actin cytoskeleton in yeast. *J Cell Biol.* 142:1487-1499.

Mendenhall, M.D. 1993. An inhibitor of $p34^{CDC28}$ protein kinase activity from *Saccharomyces cerevisiae*. *Science.* 259:216-219.

Michaelis, C., R. Ciosk, and K. Nasmyth. 1997. Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. *Cell.* 91:35-45.

Miller, R.K., and M.D. Rose. 1998. Kar9p is a novel cortical protein required for cytoplasmic microtubule orientation in yeast. *J Cell Biol.* 140:377-390.

Minn, A.J., L.H. Boise, and C.B. Thompson. 1996. Expression of Bcl-xL and loss of p53 can cooperate to overcome a cell cycle checkpoint induced by mitotic spindle damage. *Genes Dev.* 10:2621-2631.

Minshull, J., A. Straight, A. Rudner, A. Dernburg, A. Belmont, and A.W. Murray. 1996. Protein Phosphatase 2A Regulates MPF Activity and Sister Chromatid Cohesion in Budding Yeast. *Curr. Biol.* 6:1609-1620.

Minshull, J., H. Sun, N.K. Tonks, and A.W. Murray. 1994. MAP-kinase dependent mitotic feedback arrest in *Xenopus* egg extracts. *Cell.* 79:475-486.

Moir, D., S.E. Stewart, B.C. Osmond, and D. Botstein. 1982. Cold-sensitive cell-division-cycle mutants of yeast: isolation, properties, and pseudoreversion studies. *Genetics.* 100:547-563.

Moore, J.D., J. Yang, R. Truant, and S. Kornbluth. 1999. Nuclear import of Cdk/cyclin complexes: identification of distinct mechanisms for import of Cdk2/cyclin E and Cdc2/cyclin B1. *J Cell Biol.* 144:213-224.

Moreno, S., J. Hayles, and P. Nurse. 1989. Regulation of p34cdc2 protein kinase during mitosis. *Cell.* 58:361-372.

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

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

Muhua, L., N.R. Adames, M.D. Murphy, C.R. Shields, and J.A. Cooper. 1998. A cytokinesis checkpoint requiring the yeast homologue of an APC-binding protein. *Nature*. 393:487-491.

Muhua, L., T.S. Karpova, and J.A. Cooper. 1994. A yeast actin-related protein homologous to that in vertebrate dynactin complex is important for spindle orientation and nuclear migration. *Cell*. 78:669-679.

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

Murray, A.W., and M.W. Kirschner. 1989. Cyclin synthesis drives the early embryonic cell cycle. *Nature*. 339:275-280.

Murray, A.W., M.J. Solomon, and M.W. Kirschner. 1989. The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. *Nature*. 339:280-286.

Nasmyth, K., J.M. Peters, and F. Uhlmann. 2000. Splitting the chromosome: cutting the ties that bind sister chromatids [In Process Citation]. *Science*. 288:1379-1385.

Nicklas, R.B., S.C. Ward, and G.J. Gorbsky. 1995. Kinetochore chemistry is sensitive to tension and may link mitotic forces to a cell cycle checkpoint. *J. Cell Biol.* 130:929-939.

Norbury, C., J. Blow, and P. Nurse. 1991. Regulatory phosphorylation of the $p34^{cdc2}$ protein kinase in vertebrates. *EMBO J.* 10:3321-3329.

Nugroho, T.T., and M.D. Mendenhall. 1994. An inhibitor of yeast cyclin-dependent protein kinase plays an important role in ensuring the genomic integrity of daughter cells. *Mol Cell Biol.* 14:3320-3328.

Nurse, P. 1975. Genetic control of cell size at cell division in yeast. *Nature*. 256:547-551.

O'Farrell, P.H., B.A. Edgar, D. Lakich, and C.F. Lehner. 1989. Directing cell division during development. *Science*. 246:635-640.

Pangilinan, F., and F. Spencer. 1996. Abnormal kinetochore structure activates the spindle assembly checkpoint in budding yeast. *Mol. Biol. Cell.* 7:1195-1208.

Parkinson, J.S. 1993. Signal transduction schemes of bacteria. *Cell.* 73:857-871.

Patra, D., and W.G. Dunphy. 1996. Xe-p9, a *Xenopus* SUC1/CKS homolog, has multiple essential roles in cell cycle control. *Genes Dev.* 10:1503-1515.

Patra, D., and W.G. Dunphy. 1998. Xe-p9, a Xenopus Suc1/Cks protein, is essential for the Cdc2-dependent phosphorylation of the anaphase- promoting complex at mitosis. *Genes Dev.* 12:2549-2559.

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

Pellman, D., M. Bagget, H. Tu, and G.R. Fink. 1995. Two microtubule-associated proteins required for anaphase spindle movement in Saccharomyces cerevisiae. *J Cell Biol.* 130:1373-1385.

Peter, M., J. Nakagawa, M. Dorée, J.C. Labbé, and E.A. Nigg. 1990. In vitro disassembly of the nuclear lamina and M-phase-specific phosphorylation of lamins by cdc2 kinase. *Cell.* 61:591-602.

Peters, J.M., R.W. King, C. Hoog, and M.W. Kirschner. 1996. Identification of BIME as a subunit of the anaphase-promoting complex. *Science.* 274:1199-1201.

Pichler, S., S. Piatti, and K. Nasmyth. 1997. Is the yeast anaphase promoting complex needed to prevent re-replication during G2 and M phases? *Embo J.* 16:5988-5997.

Piggott, J.R., R. Rai, and B.L.A. Carter. 1982. A bifunctional gene involved in two phases of the yeast cell cycle. *Nature.* 298:391-394.

Prinz, S., E.S. Hwang, R. Visintin, and A. Amon. 1998. The regulation of Cdc20 proteolysis reveals a role for APC components Cdc23 and Cdc27 during S phase and early mitosis. *Curr Biol.* 8:750-760.

Reed, S.I. 1980. The selection of *S. Cerevisiae* mutants defective in the start event of cell division. *Genetics.* 95:561-577.

Reed, S.I. 1991. Pheromone signaling pathways in yeast. *Curr. Opin. Genet. Dev.* 1:391-396.

Rhind, N., B. Furnari, and P. Russell. 1997. Cdc2 tyrosine phosphorylation is required for the DNA damage checkpoint in fission yeast. *Genes Dev.* 11:504-511.

Rhind, N., and P. Russell. 1998. Tyrosine phosphorylation of cdc2 is required for the replication checkpoint in Schizosaccharomyces pombe. *Mol Cell Biol.* 18:3782-3787.

Rieder, C.L., R.W. Cole, A. Khodjakov, and G. Sluder. 1995. The checkpoint delaying anaphase in response to chromosome monoorientation is mediated by an inhibitory signal produced by unattached kinetochores. *J. Cell Biol.* 130:941-948.

Rieder, C.L., A. Khodjakov, L.V. Paliulis, T.M. Fortier, R.W. Cole, and G. Sluder. 1997. Mitosis in vertebrate somatic cells with two spindles: implications for the metaphase/anaphase transition checkpoint and cleavage [see comments]. *Proc Natl Acad Sci U S A.* 94:5107-5112.

Rieder, C.L., and R.E. Palazzo. 1992. Colcemid and the mitotic cell cycle. *J. Cell Sci.* 102:387-392.

Roof, D.M., P.B. Meluh, and M.D. Rose. 1992. Kinesin-related proteins required for assembly of the mitotic spindle. *J. Cell Biol.* 118:95-108.

Rothblatt, J., and R. Schekman. 1989. A hitchhiker's guide to analysis of the secretory pathway in yeast. *Methods Cell Biol.* 32:3-36.

Rudner, A.D., and A.W. Murray. 1996. The spindle assembly checkpoint. *Curr. Opin. Cell Biol.* 8:773-780.

Russell, P., S. Moreno, and S.I. Reed. 1989. Conservation of mitotic controls in fission and budding yeasts. *Cell.* 57:295-303.

Russell, P., and P. Nurse. 1986a. cdc25+ functions as an inducer in the mitotic control of fission yeast. *Cell.* 45:145-153.

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

Russell, P., and P. Nurse. 1987. Negative regulation of mitosis by wee1+, a gene encoding a protein kinase homolog. *Cell.* 49:559-567.

Sanchez, Y., J. Bachant, H. Wang, F. Hu, D. Liu, M. Tetzlaff, and S.J. Elledge. 1999. Control of the DNA damage checkpoint by chk1 and rad53 protein kinases through distinct mechanisms. *Science*. 286:1166-1171.

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

Schwob, E., T. Bohm, M.D. Mendenhall, and K. Nasmyth. 1994. The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in *S. cerevisiae*. *Cell*. 79:233-244.

Schwob, E., and K. Nasmyth. 1993. CLB5 and CLB6, a new pair of B cyclins involved in DNA replication in *Saccharomyces cerevisae*. *Genes and Dev.* 7:1160-1175.

Sethi, N., M.C. Monteagudo, D. Koshland, E. Hogan, and D.J. Burke. 1991. The *CDC20* gene product of *Saccharomyces cerevisiae*, a β-transducin homolog,is required for a subset of microtubule-dependent cellular processes. *Mol Cell Biol.* 11:5592-5602.

Sherman, F., G. Fink, and C. Lawrence. 1974. Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

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

Shirayama, M., A. Toth, M. Galova, and K. Nasmyth. 1999. APC^{Cdc20} promotes exit from mitosis by destroying the anaphase inhibitor Pds1 and cyclin Clb5. *Nature*. 402:203-207.

Shirayama, M., W. Zachariae, R. Ciosk, and K. Nasmyth. 1998. The Polo-like kinase Cdc5p and the WD-repeat protein Cdc20p/fizzy are regulators and substrates of the anaphase promoting complex in *Saccharomyces cerevisiae*. *Embo J.* 17:1336-1349.

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

Shteinberg, M., and A. Hershko. 1999. Role of Suc1 in the activation of the cyclosome by protein kinase Cdk1/cyclin B. *Biochem Biophys Res Commun*. 257:12-18.

Shteinberg, M., Y. Protopopov, T. Listovsky, M. Brandeis, and A. Hershko. 1999. Phosphorylation of the cyclosome is required for its stimulation by Fizzy/cdc20. *Biochem Biophys Res Commun.* 260:193-198.

Shu, Y., H. Yang, E. Hallberg, and R. Hallberg. 1997. Molecular genetic analysis of Rts1p, a B' regulatory subunit of Saccharomyces cerevisiae protein phosphatase 2A. *Mol Cell Biol.* 17:3242-3253.

Sia, R.A., E.S. Bardes, and D.J. Lew. 1998. Control of Swe1p degradation by the morphogenesis checkpoint. *Embo J.* 17:6678-6688.

Sigrist, S., H. Jacobs, R. Stratmann, and C.F. Lehner. 1995. Exit from mitosis is regulated by *Drosophila* fizzy and the sequential destruction of cyclins A, B and B3. *Embo J.* 14:4827-4838.

Sigrist, S.J., and C.F. Lehner. 1997. *Drosophila* fizzy-related down-regulates mitotic cyclins and is required for cell proliferation arrest and entry into endocycles. *Cell.* 90:671-681.

Sikorski, R.S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae. Genetics.* 122:19-27.

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

Solsbacher, J., P. Maurer, F.R. Bischoff, and G. Schlenstedt. 1998. Cse1p is involved in export of yeast importin alpha from the nucleus. *Mol Cell Biol.* 18:6805-6815.

Sorger, P.K., and A.W. Murray. 1992. S-phase feedback control in budding yeast independent of tyrosine phosphorylation of p34^{CDC28}. *Nature.* 355:365-368.

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

Stearns, T., M. Hoyt, A., and D. Botstein. 1990. Yeast mutants sensitive to antimicrotubule drugs define three genes that affect microtubule function. *Genetics.* 124:251-262.

Stern, B., and P. Nurse. 1996. A quantitative model for the cdc2 control of S phase and mitosis in fission yeast. *Trends Genet.* 12:345-350.

Straight, A.F., A.S. Belmont, C.C. Robinett, and A.W. Murray. 1996. GFP tagging of budding yeast chromosomes reveals that protein-protein interactions can mediate sister chromatid cohesion. *Curr. Biol.* 6:1599-1608.

Straight, A.F., W. Shou, G.J. Dowd, C.W. Turck, R.J. Deshaies, A.D. Johnson, and D. Moazed. 1999. Net1, a Sir2-associated nucleolar protein required for rDNA silencing and nucleolar integrity. *Cell.* 97:245-256.

Stueland, C.S., D.J. Lew, M.J. Cismowski, and S.I. Reed. 1993. Full activation of $p34^{CDC28}$ histone H1 kinase activity is unable to promote entry into mitosis in checkpoint-arrested cells of the yeast *Saccharomyces cerevisiae*. *Mol Cell Biol.* 13:3744-3755.

Sudakin, V., D. Ganoth, A. Dahan, H. Heller, J. Hersko, F. Luca, J.V. Ruderman, and A. Hershko. 1995. The cyclosome, a large complex containing cyclin-selective ubiquitination ligase activity, targets cyclins for destruction at the end of mitosis. *Mol. Biol. Cell.* 6:185-198.

Sudakin, V., M. Shteinberg, D. Ganoth, J. Hershko, and A. Hershko. 1997. Binding of activated cyclosome to p13(suc1). Use for affinity purification. *J Biol Chem.* 272:18051-18059.

Sullivan, D.S., and T.C. Huffaker. 1992. Astral microtubules are not required for anaphase B in Saccharomyces cerevisiae. *J Cell Biol.* 119:379-388.

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

Surana, U., H. Robitsch, C. Price, T. Schuster, I. Fitch, A.B. Futcher, and K. Nasmyth. 1991. The role of CDC28 and cyclins during mitosis in the budding yeast *S. cerevisiae*. *Cell.* 65:145-161.

Takizawa, C.G., K. Weis, and D.O. Morgan. 1999. Ran-independent nuclear import of cyclin B1-Cdc2 by importin beta. *Proc Natl Acad Sci U S A.* 96:7938-7943.

Tang, Y., and S.I. Reed. 1993. The Cdk-associated protein Cks1 functions both in G1 and G2 in *Saccharomyces cerevisiae*. *Genes Dev.* 7:822-832.

Tavormina, P.A., and D.J. Burke. 1998. Cell cycle arrest in cdc20 mutants of Saccharomyces cerevisiae is independent of Ndc10p and kinetochore function but requires a subset of spindle checkpoint genes. *Genetics.* 148:1701-1713.

Taylor, S.S., E. Ha, and F. McKeon. 1998. The human homologue of Bub3 is required for kinetochore localization of Bub1 and a Mad3/Bub1-related protein kinase. *J Cell Biol.* 142:1-11.

Taylor, S.S., and F. McKeon. 1997. Kinetochore localization of murine Bub1 is required for normal mitotic timing and checkpoint response to spindle damage. *Cell.* 89:727-735.

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

Toczyski, D.P., D.J. Galgoczy, and L.H. Hartwell. 1997. CDC5 and CKII control adaptation to the yeast DNA damage checkpoint. *Cell.* 90:1097-1106.

Torre, V., J.F. Ashmore, T.D. Lamb, and A. Menini. 1995. Transduction and adaptation in sensory receptor cells. *J. Neurosci.* 15:7757-7768.

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

Toyn, J.H., and L.H. Johnston. 1994. The Dbf2 and Dbf20 protein kinases of budding yeast are activated after the metaphase to anaphase cell cycle transition. *EMBO.* 13:1103-1113.

Uhlmann, F., F. Lottspeich, and K. Nasmyth. 1999. Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1. *Nature.* 400:37-42.

Uhlmann, F., and K. Nasmyth. 1998. Cohesion between sister chromatids must be established during DNA replication. *Curr Biol.* 8:1095-1101.

Verde, F., J.-C. Labbe, M. Doree, and E. Karsenti. 1990. Regulation of microtubule dynamics by cdc2 protein kinase in cell-free extracts of Xenopus eggs. *Nature.* 343:233-238.

Verma, R., R.S. Annan, M.J. Huddleston, S.A. Carr, G. Reynard, and R.J. Deshaies. 1997. Phosphorylation of Sic1p by G1 Cdk required for its degradation and entry into S phase. *Science*. 278:455-460.

Visintin, R., and A. Amon. 2000. The nucleolus: the magician's hat for cell cycle tricks. *Curr Opin Cell Biol*. 12:372-377.

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

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

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

Vorlaufer, E., and J.M. Peters. 1998. Regulation of the cyclin B degradation system by an inhibitor of mitotic proteolysis. *Mol Biol Cell*. 9:1817-1831.

Wang, Y., and D.J. Burke. 1995. Checkpoint genes required to delay cell division in response to nocodazole respond to impaired kinetochore function in the yeast *Saccharomyces cerevisiae* . *Mol Cell Biol*. 15:6838-6844.

Wang, Y., and D.J. Burke. 1997. Cdc55p, the B-type regulatory subunit of protein phosphatase 2A, has multiple functions in mitosis and is required for the kinetochore/spindle checkpoint in *Saccharomyces cerevisiae*. *Mol Cell Biol*. 17:620-626.

Waters, J.C., R.H. Chen, A.W. Murray, G.J. Gorbsky, E.D. Salmon, and R.B. Nicklas. 1999. Mad2 binding by phosphorylated kinetochores links error detection and checkpoint action in mitosis. *Curr Biol*. 9:649-652.

Weinstein, J. 1997. Cell cycle-regulated expression, phosphorylation, and degradation of p55Cdc. A mammalian homolog of CDC20/Fizzy/slp1. *J Biol Chem*. 272:28501-28511.

Weinstein, J., F.W. Jacobsen, J. Hsu-Chen, T. Wu, and L.G. Baum. 1994. A novel mammalian protein, p55CDC, present in dividing cells is associated with protein kinase activity and has homology to the Saccharomyces cerevisiae cell division cycle proteins Cdc20 and Cdc4. *Mol Cell Biol*. 14:3350-3363.

Weiss, E., and M. Winey. 1996. The *S. cerevisiae* SPB duplication gene *MPS1* is part of a mitotic checkpoint. *J. Cell Biol.* 132:111-123.

Wells, W.A.E. 1995. PhD dissertation, UCSF. .

Wells, W.A.E., and A.W. Murray. 1996. Aberrantly segregating centromeres activate the spindle assembly checkpoint in budding yeast. *J. Cell Biol.* 133:75-84.

Westendorf, J.M., P.N. Rao, and L. Gerace. 1994. Cloning of cDNAs for M-phase phosphoproteins recognized by the MPM2 monoclonal antibody and determination of the phosphorylated epitope. *Proc Natl Acad Sci U S A.* 91:714-718.

Winey, M., L. Goetsch, P. Baum, and B. Byers. 1991. MPS1 and MPS2: Novel Yeast genes defining distinct steps of spindle pole body duplication. *J. Cell Biol.* 114:745-754.

Yamada, H., K. Kumada, and M. Yanagida. 1997. Distinct subunit functions and cell cycle regulated phosphorylation of 20S APC/cyclosome required for anaphase in fission yeast. *J Cell Sci.* 110:1793-1804.

Yamaguchi, S., H. Murakami, and H. Okayama. 1997. A WD Repeat Protein Controls the Cell Cycle and Differentiation by Negatively Regulating Cdc2/B-Type Cyclin Complexes. *Mol Biol Cell.* 8:2475-2486.

Yamamoto, A., V. Guacci, and D. Koshland. 1996a. Pds1p is required for faithful execution of anaphase in the yeast, Saccharomyces cerevisiae. *J Cell Biol.* 133:85-97.

Yamamoto, A., V. Guacci, and D. Koshland. 1996b. Pds1p, an inhibitor of anaphase in budding yeast, plays a critical role in the APC and checkpoint pathway(s). *J Cell Biol.* 133:99-110.

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

Yamashita, Y.M., Y. Nakaseko, I. Samejima, K. Kumada, H. Yamada, D. Michaelson, and M. Yanagida. 1996. 20S cyclosome complex formation and proteolytic activity inhibited by the cAMP/PKA pathway. *Nature.* 384:276-279.

Yanagida, M., Y.M. Yamashita, H. Tatebe, K. Ishii, K. Kumada, and Y. Nakaseko. 1999. Control of metaphase-anaphase progression by proteolysis: cyclosome function

regulated by the protein kinase A pathway, ubiquitination and localization. *Philos Trans R Soc Lond B Biol Sci.* 354:1559-1569; discussion 1569-1570.

Yang, J., and S. Kornbluth. 1999. All aboard the cyclin train: subcellular trafficking of cyclins and their CDK partners. *Trends Cell Biol.* 9:207-210.

Yang, S.S., E. Yeh, E.D. Salmon, and K. Bloom. 1997. Identification of a mid-anaphase checkpoint in budding yeast. *J Cell Biol.* 136:345-354.

Yeh, E., R.V. Skibbens, J.W. Cheng, E.D. Salmon, and K. Bloom. 1995. Spindle dynamics and cell cycle regulation of dynein in the budding yeast, Saccharomyces cerevisiae. *J Cell Biol.* 130:687-700.

Yeong, M.F., H.H. Lim, P. C.G., and U. Surana. 2000. Exit from mitosis in budding yeast: biphasic inactivation of the Cdc28-Clb2 mitotic kinase and the role of Cdc20. *Mol Cell.* 5:501-511.

Zachariae, W., and K. Nasmyth. 1996. TPR proteins required for anaphase progression mediate ubiquitination of mitotic B type cyclins in yeast. *Mol. Biol. Cell.* 7:791-801.

Zachariae, W., and K. Nasmyth. 1999. Whose end is destruction: cell division and the anaphase-promoting complex. *Genes Dev.* 13:2039-2058.

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

Zachariae, W., T.H. Shin, M. Galova, B. Obermaier, and K. Nasmyth. 1996. Identification of subunits of the anaphase-promoting complex of *Saccharomyces cerevisiae.* *Science.* 274:1201-1204.

Zou, H., T.J. McGarry, T. Bernal, and M.W. Kirschner. 1999. Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. *Science.* 285:418-422.