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The Regulation of the Exit from Mitosis in *Saccharomyces cerevisiae*

by

Lena H. Hwang

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry

in the

GRADUATE DIVISION

of the

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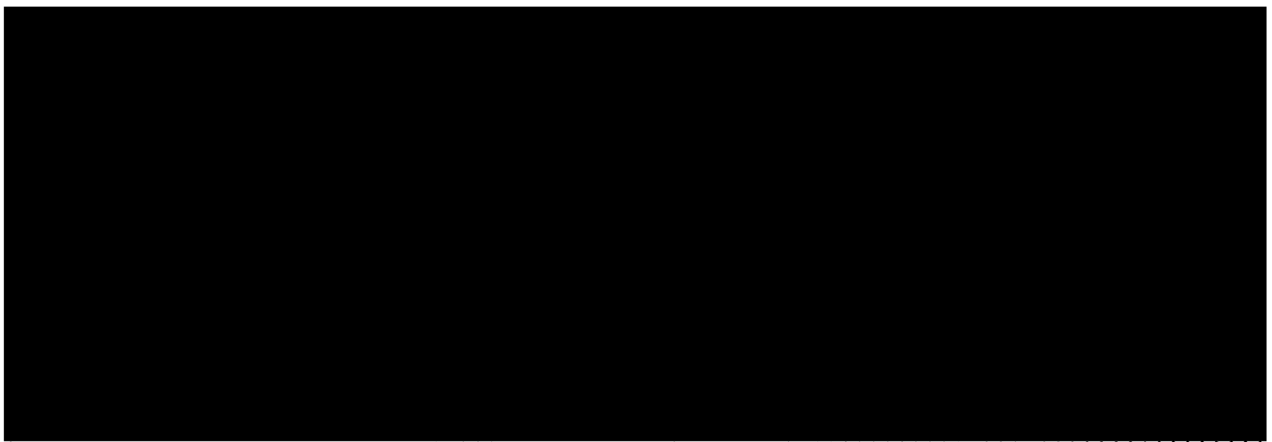

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This thesis is dedicated to my parents,

In Kil and Myung Hee Hwang

Acknowledgments

I have thoroughly enjoyed my time at UCSF, primarily due to the people I have had the pleasure of meeting and interacting with here. First and foremost, I would like to thank Andrew Murray. His guidance, support and friendship have made my time in graduate school a fun and fulfilling experience. I admire his scientific creativity and I appreciate the encouragement that he's given me throughout my graduate career.

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The Regulation of the Exit from Mitosis in *Saccharomyces cerevisiae*

Lena H. Hwang

Abstract

The exit from mitosis is tightly regulated to ensure that chromosomes are properly segregated to each daughter cell. To prevent mis-segregation, the spindle assembly checkpoint inhibits the exit from mitosis in the presence of a damaged spindle or unattached kinetochores. Once all of the chromosomes are properly aligned on the mitotic spindle, activation of the anaphase promoting complex (APC) initiates the exit from mitosis by targeting key mitotic regulatory proteins for destruction.

To identify genes that were involved in mitotic exit, we utilized a novel enrichment strategy to isolate mutants in *Saccharomyces cerevisiae* that were unable to exit from mitosis. Using this strategy, we identified a novel gene, *DOC1*, and a previously identified gene, *CDC26,* as components of the APC.

We then examined the regulation of the spindle assembly checkpoint on the activation of the APC. The spindle checkpoint had long been hypothesized to inhibit the destruction of mitotic regulatory proteins, however, the target of the checkpoint was unknown. We found that components of the spindle checkpoint associated with Cdc20, a substrate specific activator of the APC. We also identified dominant mutants in *CDC20* that were capable of

bypassing activation of the checkpoint, indicating that Cdc20 was the target of the spindle checkpoint.

To further study the regulation of the spindle assembly checkpoint on the activity of Cdc20 and the APC, we developed an *in vitro,* reconstituted assay for APC activity. The APC from a spindle checkpoint arrested extract is inactive in this assay, however, the assay appears to reflect Hct1/Cdh1 dependent APC activity, not Cdc20 dependent APC activity.

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Chapter 1

Introduction

Faithful transmission of genetic material is essential to the survival of the cell. This requires precise replication of the DNA and proper segregation of chromosomes into each daughter cell. Incomplete replication, mutation, or mis-segregation of DNA can result in cell death, aneuploidy, and tumorigenesis. The cell ensures the prevention of these defects with checkpoints that monitor the fidelity of replication and mitotic spindle assembly.

In G2, a DNA replication checkpoint detects incompletely replicated DNA. A DNA damage checkpoint senses damage to the DNA in both G1 and G2. Both of these defects, unreplicated DNA and damaged DNA, activate the checkpoint resulting in a cell cycle arrest until replication is completed and damage repaired. In mitosis, the spindle assembly checkpoint detects damage to the mitotic spindle and unattached kinetochores. Only when all chromosomes are properly attached to the mitotic spindle, does the checkpoint allow initiation of anaphase (reviewed in Rudner and Murray 1996, Wells 1996). These checkpoints ensure the accuracy of cell duplication.

The exact duplication of a parent cell into two daughter cells also requires that a single round of replication is followed by a single round of chromosome segregation. This alternation of replication and segregation is seen in all normal cell cycles. A few specialized cell cycles violate this rule, such as endoreduplication, with several successive rounds of replication without intervening mitoses; and meiosis, with a single round of DNA replication followed by two successive rounds of chromosome segregation.

Cyclin-dependent kinases (Cdks) drive progression through the phases of the cell cycle (reviewed in Morgan 1995). Their activity is

regulated by phosphorylation of the kinase subunit and by the cyclic accumulation and degradation of cyclins, the regulatory subunits of Cdks.

Mitotic events are driven by the expression and accumulation of B-type cyclins and their associated Cdk activity. The degradation of mitotic cyclins results in a rapid drop in mitotic Cdk activity, which is required for the exit from mitosis. This event both initiates exit from mitosis and formation of protein complexes on the DNA that will trigger initiation of DNA replication. Maintenance of mitotic cyclin degradation activity in G1 ensures that a second round of mitosis will not be initiated.

In this thesis I will discuss the identification of components of the machinery required for destruction of mitotic cyclins and other mitotic regulatory proteins and the role of the spindle assembly checkpoint in regulating that destruction.

Proteolysis in Mitosis

A short amino-terminal sequence, called the destruction box, targets the destruction of key mitotic regulatory proteins, including the mitotic cyclins and a factor involved in sister chromosome cohesion, Cut2 in fission yeast and Pds1 in budding yeast (Cohen-Fix, et al. 1996, Funabiki, et al. 1996, Glotzer, et al. 1991).

Deletion or mutation of the cyclin destruction box results in a non-degradable form of the cyclin. Yeast cells or frog extracts over expressing a non-degradable form of B-type cyclin are unable to exit mitosis and remain arrested in anaphase of the cell cycle (Holloway,

et al. 1993, Murray and Kirschner 1989, Surana, et al. 1993). Experiments with these non-degradable cyclins have shown that cyclin destruction is required for chromosome decondensation, nuclear envelope reformation, and cytokinesis, but is not required for sister chromosome separation. However, if fragments containing the destruction box of cyclin are present in excess in Xenopus egg extracts, cyclin degradation and sister chromosome segregation are both inhibited (Holloway, et al. 1993). This observation revealed the existence of an additional factor involved in sister chromosome cohesion that must be targeted for destruction by the same machinery that destroys cyclins.

This factor was first identified in fission yeast as the product of the *cut2⁺* gene (Funabiki, et al. 1996). Cut2 protein is degraded as cells progress through anaphase, and this destruction is dependent on the presence of two destruction boxes at its amino-terminus. If the destruction boxes are deleted, Cut2 protein is now able to accumulate in G1. Destruction of Cut2 is dependent on the machinery responsible for cyclin destruction, and expression of a non-degradable version of Cut2 inhibits anaphase, although it does not block exit from mitosis.

Cut2's functional homologue in budding yeast is Pds1. Pds1 was originally identified in a screen for mutants that precociously dissociate their sister chromatids (Yamamoto, et al. 1996). Expression of a non-degradable form of Pds1 in budding yeast inhibits anaphase and also prevents exit from mitosis (Cohen-Fix, et al. 1996). Although *PDS1* is not essential, disruption of *PDS1* results

in cells unable to maintain cohesion of sister chromosomes (Yamamoto, et al. 1996).

Ubiquitin-Mediated Proteolysis

Cyclins, Cut2, Pds1, and other mitotic regulatory factors are targeted for destruction by ubiquitin-mediated proteolysis. In this process, multiple molecules of a small, 76 amino acid protein, ubiquitin, are covalently attached to a target protein. The multi-ubiquitin chain then targets the protein for destruction by the 26S proteosome (reviewed in Ciechanover 1994).

Ubiquitin-mediated proteolysis is a multi-step process that requires three to four factors (Figure 1-1). First, ubiquitin is activated by a ubiquitin-activating enzyme (E1). The E1 uses ATP to form a ubiquitin adenylate, followed by the formation of a thioester bond between the C-terminus of ubiquitin and an internal cysteine residue of the E1 enzyme. This activated ubiquitin is then transferred to a cysteine residue on one of several ubiquitin-conjugating enzymes (E2). For some substrates, the ubiquitin can then be transferred directly to the protein by the E2, however, many substrates require an additional factor, a ubiquitin-ligase enzyme (E3). The process of ubiquitin ligation varies for different E3 enzymes. E6-AP, the E3 that targets the destruction of p53, forms a thioester bond with ubiquitin before transferring the ubiquitin onto p53 (Scheffner, et al. 1995). On the other hand, Cdc4/Cdc53, the E3 for Sic1 appears to serve only as an accessory factor for substrate recognition.

Figure 1-1. Ubiquitin mediated proteolysis

Ubiquitin is activated by the E1 enzyme (ubiquitin-activating enzyme) through the hydrolysis of ATP. The ubiquitin is then transferred to an E2 enzyme (ubiquitin-conjugating enzyme), and then transferred to the substrate directly, or in conjugation with an E3 enzyme (ubiquitin ligase). This process is reiterated, resulting in a multi-ubiquitin chain on the substrate protein. The ubiquitin chain then targets the substrate for destruction by the 26S proteosome.

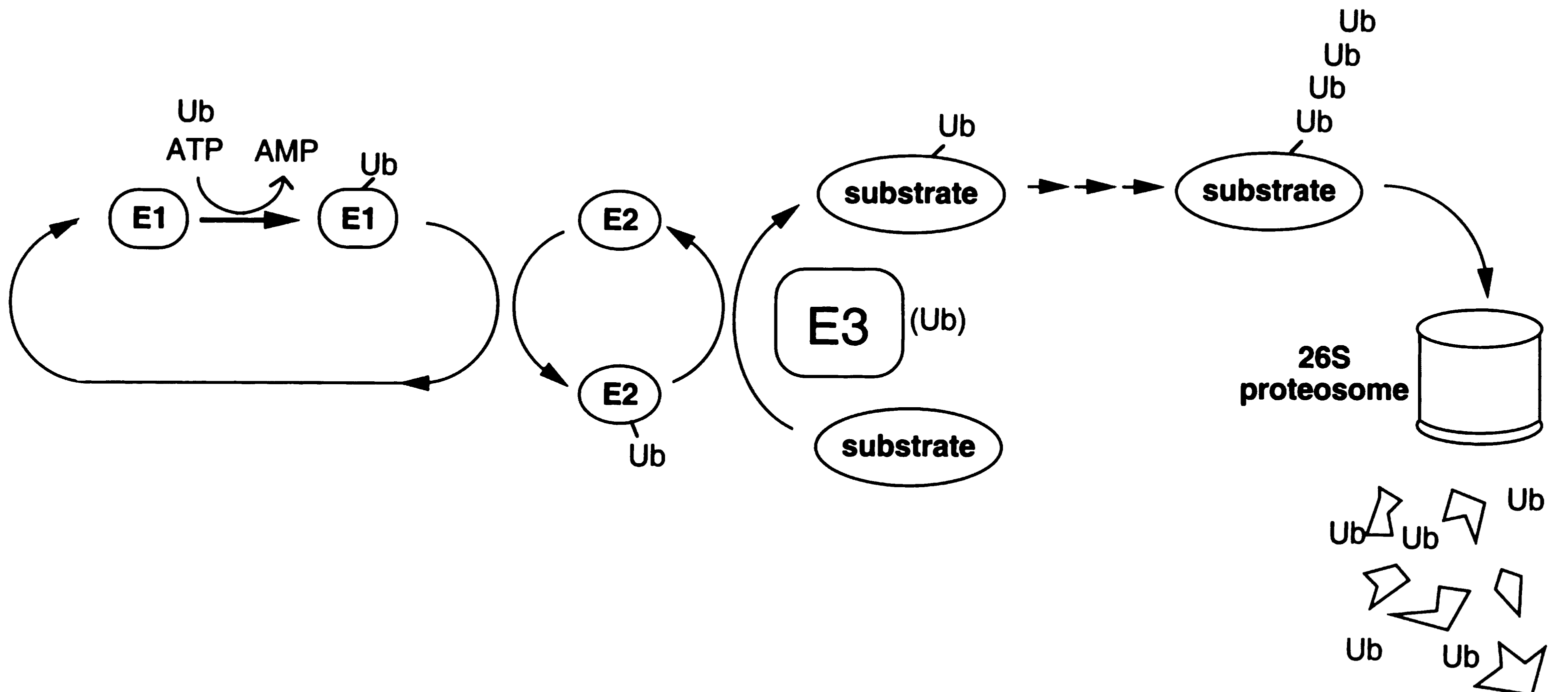

Figure 1-1

The C-terminal glycine residue of ubiquitin forms an isopeptide bond with a lysine in the target protein. Following the attachment of the first ubiquitin to the target protein, ubiquitin itself becomes a target for further ubiquitinations, leading to a multi-ubiquitin chain covalently attached to the substrate protein.

In budding yeast there is a single major El encoded by the *UBA1* gene (Dohmen, et al. 1995). There is a large family of E2 enzymes (*UBCs*). The E2 enzyme involved in cyclin destruction in yeast, however, has not been definitively identified. In clam egg extracts, E2-C functions as the E2 for cyclin ubiquitination *in vitro* (Sudakin, et al. 1995), and in frog egg extracts, a homologue of the budding yeast Ubc4 and a novel UBC, UBCX, are capable of functioning as E2's in a reconstituted cyclin ubiquitination assay (King, et al. 1995). The E3 for mitotic cyclins was first identified in clam and frog egg extracts as a 20S complex called the cyclosome or anaphase promoting complex (APC) (King, et al. 1995, Sudakin, et al. 1995).

Using both biochemistry and genetics, components of the APC/cyclosome have been identified in a number of systems. Purification of the APC from *Xenopus* egg extracts identified a large complex composed of eight subunits, including homologues of the budding yeast *CDC16, CDC23,* and *CDC27* gene products, as well as a homologue of *Aspergillus nidulans BIME* (King, et al. 1995, Peters, et al. 1996). The additional subunits were identified from human cDNA clones using peptide sequence obtained from the *Xenopus* APC subunits (Yu, et al. 1998). This identified four novel genes: *APC2, APC4, APC5*, and *APC7*. *APC2* shows homology to cullins, a family of

proteins involved in the ubiquitination of G1 cyclins and cyclin-dependent kinase inhibitors. *APC7* is a member of a family of tetratricopeptide repeat proteins, of which *CDC16*, *CDC23*, and *CDC27* are also members. *APC4* and *APC5* have no homology to proteins of known function.

Destruction of Clb2, the major mitotic cyclin, is initiated at the end of mitosis and continues into G1 until the reactivation of Cdc28 kinase activity by G1 cyclins. Irniger *et al.* utilized this property to isolate mutants that were defective in cyclin proteolysis, identifying *CDC16* and *CDC23*. Genetics in yeast identified *CDC16, CDC23, CDC26, CDC27,* and *APC1* (the *BIME* homologue) as genes required for the destruction of B-type cyclins (Hwang and Murray 1997, Irniger, et al. 1995, Lamb, et al. 1994, Zachariae and Nasmyth 1996, Zachariae, et al. 1996).

CDC16, CDC23, and *CDC27* were originally identified in the Hartwell cell cycle mutant screen (Hartwell, et al. 1970). Temperature sensitive mutants in these genes resulted in a cell cycle arrest in mitosis at the non-permissive temperature. These mutants arrest in metaphase with high levels of mitotic cyclins and unseparated sister chromosomes, in line with their role in proteolysis.

Biochemistry in yeast suggests that the *S. cerevisiae* APC contains twelve subunits. These include the five subunits identified through genetics, as well as five subunits identified through biochemical purification and mass spectrometry: Apc2, Apc4, Apc5, Apc9, and Apc11 (Zachariae, et al. 1998). Apc2 is homologous to

Table 1-1. APC subunits

	S. cerevisiae	*X. laevis*	*S. pombe*	*A. nidulans*	human	mouse	Motif/Family
APC1	*APC1*	APC1	*cut4*$^+$	BIME	APC1	Tsg24	
APC1	*APC2*	APC2			APC2		cullin
APC3	*CDC27*	CDC27	*nuc2*$^+$	BIMA	CDC27		TPR
APC4	*APC4*	APC4			APC4		
APC5	*APC5*	APC5			APC5		
APC6	*CDC16*	CDC16	*cut9*$^+$		CDC16		TPR
APC7	not present	APC7			APC7		TPR
APC8	*CDC23*	CDC23			CDC23		TPR
APC9	*APC9*						
APC10	*DOC1*		*apc10*$^+$				
APC11	*APC11*						RING-finger
APC12	*CDC26*		*hcn1*$^+$				

Cdc53, a cullin protein involved in the ubiquitination of Sic1 (Mathias, et al. 1996). Apc11 contains a RING-H2 domain, a variant of the RING finger, that binds two zinc ions and is believed to mediate protein-protein interactions. Apc4 and Apc5 are homologous to the human *APC4* and *APC5* and Apc9 has no homologues. Although there are some differences between the yeast and vertebrate APCs, this complex has been highly conserved from yeast to humans.

Regulation of the APC

The APC appears to be regulated by post-translational modification. CDC16 and CDC27 are phosphorylated in frog egg extracts during mitosis and phosphatase treatment inactivates both the frog and clam mitotic APC (King, et al. 1995, Sudakin, et al. 1995). The kinase that phosphorylates the APC has not been definitively identified. Candidates include Cdk1/Cyclin B, which is able to partially activate interphase clam APC after a lag phase (Sudakin, et al. 1995), and Polo-like kinase (Plk), which is capable of phosphorylating components of the APC *in vitro* (Kotani, et al. 1998).

In addition, APC activity is regulated by the association of the APC with specific regulatory proteins. Two highly related families of proteins containing seven WD-40 repeats, Cdc20 and Hct1/Cdh1 in *S. cerevisiae* (Schwab, et al. 1997, Visintin, et al. 1997), Fizzy and Fizzy-related in *Drosophila* (Sigrist, et al. 1995, Sigrist and Lehner 1997), Slp1 and Srw1 in *S. pombe* (Kim, et al. 1998), CDC20 and CDH1 in human (Fang, et al. 1998), and X-FZY and X-FZR in *Xenopus* (Lorca, et

al. 1998) are involved in the activation and perhaps substrate specificity of the APC.

In *Drosophila,* Fizzy is required for mitotic cyclin degradation and progression through mitosis (Sigrist, et al. 1995). Fizzy-related is required for maintenance of cyclin degradation during G1 after embryonic epidermal cell proliferation stops (Sigrist and Lehner 1997).

S. cerevisiae CDC20 was first identified in the original Hartwell screen as a temperature sensitive mutant that arrested in mitosis. There are some implications for its involvement in spindle dynamics (O'Toole, et al. 1997, Sethi, et al. 1991). Recently, however, *CDC20* and its relative, *HCT1* have been shown to be limiting, substrate specific activators of the APC (Schwab, et al. 1997, Visintin, et al. 1997). *HCT1* has also been identified in yeast as *CDH1*, however, I will refer to the yeast gene as *HCT1.*

In G1, temperature sensitive mutants in *CDC20* are defective in the proteolysis of Pds1, while mutants in *HCT1* are defective in Clb2 and Ase1 proteolysis. In addition, over-expression of *CDC20* in exponentially growing cells or cells arrested in S-phase destabilizes Pds1 but not Clb2 or Ase1. Over-expression of *HCT1* in these conditions destabilizes Clb2 and Ase1. However, in mitotically arrested cells, Clb2 and Pds1 are both destabilized by the over-expression of either *CDC20* or *HCT1* (Schwab, et al. 1997, Visintin, et al. 1997).

CDC20 and *HCT1* appear to give some substrate specificity to the APC, although that specificity is lost during mitosis. The story is further complicated by the fact that *cdc20-1* temperature sensitive

mutants arrest in metaphase of the cell cycle with unseparated sister chromosomes and high levels of mitotic cyclins (Sethi, et al. 1991, Shirayama, et al. 1998). In addition, *cdc20-1 pds1Δ* double mutants do not exit mitosis but arrest in anaphase with high levels of mitotic cyclins (Shirayama, et al. 1998, Yamamoto, et al. 1996). Therefore, *CDC20* must have some role in the initiation of the destruction of the mitotic cyclins. Cdc20 may itself initiate ubiquitination of the cyclins, or it may target the destruction of an inhibitor of *HCT1* dependent APC.

Recent evidence has shown that Hct1 is regulated by Cdc28 (Jaspersen in press Zachariae, et al. 1998). Phosphorylation of Hct1 by Cdc28 in mitosis inhibits its activity by blocking its interaction with the APC. It is possible that Cdc28 activates Cdc20 to initiate the destruction of mitotic cyclins, thereby allowing activation of Hct1 and eventual destruction of all mitotic cyclins.

Unlike *CDC20, HCT1* is a non-essential gene. *hct1Δ* mutants are able to exit from mitosis with high levels of mitotic cyclins (Schwab, et al. 1997, Visintin, et al. 1997). This exit appears to be dependent on *SIC1,* an inhibitor of Cdc28 kinase activity (Schwab, et al. 1997). Cells may use both proteolysis of cyclins and inhibition of the kinase to downregulate Cdc28 kinase activity during the exit from mitosis.

Human CDC20 and CDH1 have been shown to bind directly to the APC and are capable of activating both interphase and mitotic APCs when present in excess (Fang, et al. 1998). Interestingly, both CDC20 and CDH1 are also capable of activating a phosphatase treated APC, indicating that phosphorylation of the APC may not be a critical step in its activation. Human CDC20 confers a strict requirement for a

destruction box for targeting by the APC, whereas hCDH1 appears to have a much more relaxed specificity for the destruction box (Fang, et al. 1998).

In both humans and yeast, CDC20 protein levels are regulated through the cell cycle (Fang, et al. 1998, Prinz, et al. 1998). The protein levels accumulate starting in G2, peak in mitosis and then drop rapidly during the exit from mitosis. In fact, Cdc20 in yeast has been shown to be a substrate for the APC (Prinz, et al. 1998, Shirayama, et al. 1998). CDH1 protein, on the other hand, remains stable throughout the cell cycle. It has been postulated that CDH1's primary role is as an activator of the APC during G1.

CDC20 and CDH1 are important for the activation of the APC and progression through the cell cycle. Work described in this thesis has identified a role for CDC20 in the spindle assembly checkpoint, linking the cell cycle machinery to the checkpoint.

The Spindle Assembly Checkpoint

In mitosis, the spindle assembly checkpoint monitors the integrity of the mitotic spindle and the proper attachment of each of the sister chromosomes to the spindle (reviewed in Rudner and Murray 1996, Wells 1996). In fact, a single unattached chromosome can activate the checkpoint.

Components of this spindle assembly checkpoint were first identified in budding yeast. A number of genes have been identified, including *MAD1-3, BUB1-3, MPS1,* and *CDC55,* (Hoyt, et al. 1991, Li and Murray 1991, Minshull, et al. 1996, Weiss and Winey 1996) and

homologues of a subset of these genes have been identified in fission yeast, *Xenopus,* humans, flies and worms (Chen, et al. 1996, Jin, et al. 1998, Kim, et al. 1998, Li and Benezra 1996, Taylor, et al. 1998, Taylor and McKeon 1997). Mutants in these genes are unable to respond to damage to the spindle or mis-attached chromosomes, leading to aneuploidy and death upon exit from mitosis in the presence of these defects. Mutations in checkpoint genes have been implicated in human cancers indicating a conserved function through evolution.

The regulation of this pathway is still unclear. Mad1 appears to be post-translationally modified by hyper-phosphorylation upon activation of the checkpoint by addition of spindle depolymerizing drugs or the over-expression of one of the checkpoint components, *MPS1* (Hardwick and Murray 1995, Hardwick, et al. 1996). A rough ordering of the pathway, using phosphorylation of Mad1 as an indicator has placed *MAD2, BUB1, BUB3,* and *MPS1* upstream of *MAD1*, and *MAD3* and *BUB2* downstream. However, it is unclear if hyper-phosphorylation is essential for the activation of Mad1 in the spindle assembly checkpoint. Recent evidence, with a *BUB1* dominant mutant, indicates that hyper-phosphorylation of Mad1 is not essential (Farr and Hoyt 1998).

Biochemical experiments, as well as two-hybrid screens have shown that Mad1 and Mad2 associate in a complex in yeast and *Xenopus* (R.H. Chen, K. Hardwick, personal communication). In addition, Bub1 and Bub3 appear to associate in both yeast and humans (Roberts, et al. 1994, Taylor, et al. 1998). There is some evidence in two-hybrid screens that several of the spindle assembly

checkpoint components may associate in a multi-subunit complex (D. Smith, personal communication).

Indications as to how the spindle assembly checkpoint may be sensing defects have come from localization studies in *Xenopus* and human cells. *Xenopus* and human MAD2 localize to unattached kinetochores. XMAD2 and HsMAD2 associate with kinetochores during prometaphase and appear to dissociate once kinetochores attach to microtubules (Chen, et al. 1996, Li and Benezra 1996). MAD2 protein also associates with kinetochores after treatment with nocodazole, which depolymerizes microtubules. Interestingly, unlike the 3F3/2 phosphoepitope which becomes dephosphorylated in response to tension at the kinetochore, MAD2 binding appears to depend on microtubule attachment, instead of tension (Waters, et al. 1998). Human BUB1 and BUB3 also localize to unattached kinetochores, and BUB1's localization is dependent on its association with BUB3 (Taylor, et al. 1998, Taylor and McKeon 1997). A number of checkpoint components appear to localize specifically to unattached kinetochores, perhaps in a large signaling complex.

We now have some clues as to how the checkpoint may detect unattached chromosomes, but how does the spindle assembly checkpoint cause an arrest in the cell cycle? It had long been hypothesized that the checkpoint worked by inhibiting the destruction of key mitotic regulatory proteins, including the mitotic cyclins. Activation of the checkpoint results in a metaphase arrest with sisters held together and high mitotic cyclin levels. I will present evidence in this thesis that the APC is not active in

checkpoint arrested cells and that the spindle assembly checkpoint acts through *CDC20*.

Linking the Spindle Assembly Checkpoint to the Cell Cycle Machinery

CDC20's involvement in checkpoints was first identified when it was shown that overexpression of *CDC20* allows cells to bypass the DNA damage checkpoint induced by a *cdc13* mutant (Lim and Surana 1996). I will show in this thesis that Cdc20 is directly involved in the spindle assembly checkpoint and associates with components of the checkpoint (Hwang, et al. 1998). Cdc20's role in the checkpoint has also been shown in fission yeast. Slp1, the *S. pombe* homologue of Cdc20, was shown through two-hybrid to bind Mad2, and mutants in Slp1 that are no longer capable of binding Mad2 are now able to bypass activation of the spindle assembly checkpoint (Kim, et al. 1998).

It was later shown that human CDC20 (p55CDC) forms a complex with hMAD2 and the APC, and that hCDC20 is required for the association of hMAD2 with the APC (Kallio, et al. 1998, Li, et al. 1997). In addition, hMAD2 is capable of inhibiting the activation of the APC by hCDC20 (Fang, et al. 1998). CDC20 appears to be a direct, physical link between the spindle assembly checkpoint and the APC, and is likely the target of the checkpoint.

Figure 1-2. Regulation of the progression through mitosis

Checkpoint complexes at the kinetochores of unattached chromosomes activate the spindle assembly checkpoint to inhibit progression through mitosis. Activation of the spindle assembly checkpoint inhibits the activity of Cdc20, a regulator of the APC. Once all chromosomes are properly attached to the spindle, activation of Cdc20 initiates anaphase through the destruction of Pds1. Hct1 then targets the APC for the destruction of Clb2 and Ase1, leading to the exit from anaphase. Interestingly, Cdc20 appears to be required for the activation of Hct1. This activation is likely to be indirect and may be due to the destruction of an inhibitor of Hct1 by Cdc20 dependent APC.

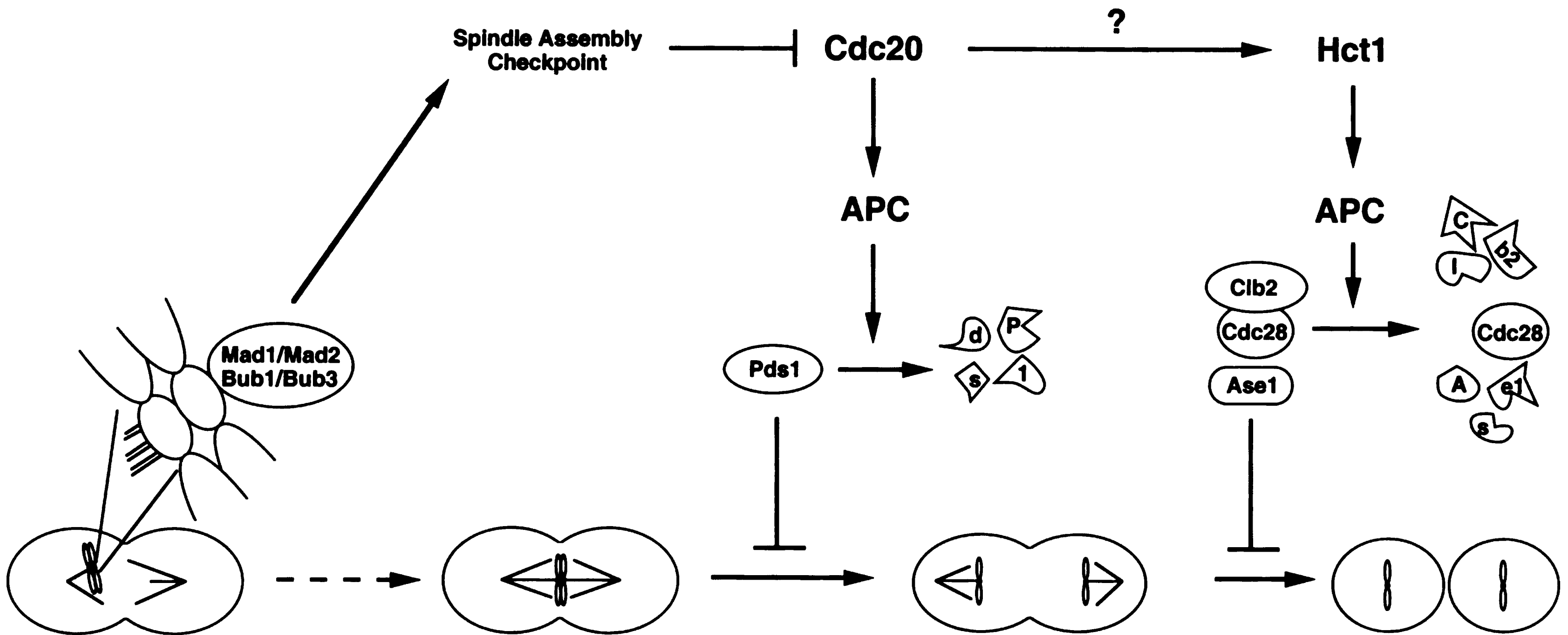

Figure 1-2

Conclusion

The entry into and exit from mitosis requires the coordinated activity of cyclin dependent kinases as well as ubiquitin-mediated proteolysis. Cdk activity drives entry into mitosis, presumably by phosphorylation of proteins important for the events required during mitosis, such as chromosome condensation and spindle assembly. Cdk activity also drives exit from mitosis by inducing the APC to ubiquitinate mitotic proteins and target them for destruction, including mitotic cyclins, the regulatory subunit of the Cdk. This process appears to be regulated in two complementary ways: a timing mechanism to ensure that all necessary early mitotic events are completed before anaphase is initiated, and a checkpoint that monitors proper formation of a bipolar mitotic spindle.

In the second chapter of this thesis, I will describe the identification of components of the APC using a novel enrichment strategy to isolate mutants that cause a mitotic arrest. The third chapter identifies Cdc20, an activator of the APC, as the target of the spindle assembly checkpoint. Finally, the fourth chapter describes the development of an *in vitro* reconstituted assay that measures APC activity from *S. cerevisiae*.

References

Chen, R.-H., Waters, J. C., Salmon, E. D. and Murray, A. W. 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

Cohen-Fix, O., Peters, J. M., Kirschner, M. W. and Koshland, D. 1996. Anaphase initiation in Saccharomyces cerevisiae is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. *Genes Dev* 10: 3081-93

Dohmen, R. J., Stappen, R., McGrath, J. P., Forrova, H., Kolarov, J., Goffeau, A. and Varshavsky, A. 1995. An essential yeast gene encoding a homolog of ubiquitin-activiating enzyme. *J. Biol. Chem.* 270: 18099-109

Fang, G., Yu, H. and Kirschner, M. W. 1998. 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., Yu, H. and Kirschner, M. W. 1998. Direct Binding of CDC20 Protein Family Members Activates the Anaphase-Promoting Complex in Mitosis and G1. *Molecular Cell* 2: 163-171

Farr, K. A. and Hoyt, M. A. 1998. Bub1p kinase activates the Saccharomyces cerevisiae spindle assembly checkpoint. *Mol Cell Biol* 18: 2738-47

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

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

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

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

Hartwell, L. H., Culotti, J. and Reid, B. 1970. Genetic control of cell division in yeast, I. Detection of mutants. *Proc. Natl. Acad. Sci. USA* 66: 352-359

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

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

Hwang, L. H., Lau, L. F., Smith, D. L., Mistrot, C. A., Hardwick, K. G., Hwang, E. S., Amon, A. and Murray, A. W. 1998. Budding yeast Cdc20: a target of the spindle checkpoint. *Science* 279: 1041-4

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

Jin, D. Y., Spencer, F. and Jeang, K. T. 1998. Human T cell leukemia virus type 1 oncoprotein Tax targets the human mitotic checkpoint protein MAD1. *Cell* 93: 81-91

Kallio, M., Weinstein, J., Daum, J. R., Burke, D. J. and Gorbsky, G. J. 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

Kim, S. H., Lin, D. P., Matsumoto, S., Kitazano, A. and Matsumoto, T. 1998. Fission yeast slp1+ encodes the effector of the Mad2-dependent spindle checkpoint. *Science* 279: 1045-7

King, R. W., Peters, J. M., Tugendreich, S., Rolfe, M., Hieter, P. and Kirschner, M. W. 1995. A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. *Cell* 81: 279-88

Kotani, S., Tugendreich, S., Fujii, M., Jorgensen, P.-M., Watanabe, N., Hoog, C., Hieter, P. and Todokoro, K. 1998. PKA and MPF-Activated Polo-like Kinase Regulate Anaphase-Promoting Complex Activity and Mitosis Progression. *Mol. Cell* 1: 371-380

Lamb, J. R., Michaud, W. A., Sikorski, R. S. and Hieter, P. A. 1994. Cdc16p, Cdc23p and Cdc27p form a complex essential for mitosis. *Embo J* 13: 4321-8

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

Li, Y. and Benezra, R. 1996. Identification of a human mitotic checkpoint gene: hsMAD2. *Science* 274: 246-8

Li, Y., Gorbea, C., Mahaffey, D., Rechsteiner, M. and Benezra, R. 1997. MAD2 associates with the cyclosome/anaphase-promoting complex and inhibits its activity. *Proc. Natl. Acd. Sci.* 94: 12431-12436

Lim, H. H. and Surana, U. 1996. Cdc20, a beta-transducin homologue, links RAD9-mediated G2/M checkpoint control to mitosis in Saccharomyces cerevisiae. *Mol Gen Genet* 253: 138-48

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

Mathias, N., Johnson, S. L., Winey, M., Adams, A. E., Goetsch, L., Pringle, J. R., Byers, B. and Goebl, M. G. 1996. Cdc53p actis in concert with Cdc4p and Cdc34p to control the G1-to-S-phase transition and identifies a conserved family of proteins. *Mol. Cell. Biol.* 16: 6634-6643

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

Morgan, D. O. 1995. Principles of CDK regulation. *Nature* 374: 131-134

Murray, A. W. and Kirschner, M. W. 1989. Dominoes and Clocks: the union of two views of cell cycle regulation. *Science* 246: 614-621

O'Toole, E. T., Mastronarde, D. N., Giddings, T. H., Jr., Winey, M., Burke, D. J. and McIntosh, J. R. 1997. Three-dimensional analysis and ultrastructural design of mitotic spindles from the cdc20 mutant of Saccharomyces cerevisiae. *Mol Biol Cell* 8: 1-11

Peters, J. M., King, R. W., Hoog, C. and Kirschner, M. W. 1996. Identification of BIME as a Subunit of the Anaphase-Promoting Complex. *Science* 274: 1199-1201

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

Roberts, R. T., Farr, K. A. and Hoyt, M. A. 1994. The *Saccharomyces cerevisiae* checkpoint gene *BUB1* encodes a novel protein kinase. *Molec. Cell. Biol.* 14: 8282-8291

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

Scheffner, M., Nuber, U. and Huibregtse, J. M. 1995. Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. *Nature* 373: 81-83

Schwab, M., Lutum, A. S. and Seufert, W. 1997. Yeast Hct1 is a regulator of Clb2 cyclin proteolysis. *Cell* 90: 683-93

Sethi, N., Monteagudo, M. C., Koshland, D., Hogan, E. and Burke, D. J. 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

Shirayama, M., Zachariae, W., Ciosk, R. and Nasmyth, K. 1998. The Polo-like kinase Cdc5p and the WD-repeat protein Cdc20/fizzy are regulators and substrates of the anaphase promoting complex in *Saccharomyces cerevisiae. EMBO* 17:
Sigrist, S., Jacobs, H., Stratmann, R. and Lehner, C. F. 1995. Exit from mitosis is regulated by Drosophila fizzy and the sequential destruction of cyclins A, B and B3. *Embo J* 14: 4827-38

Sigrist, S. J. and Lehner, C. F. 1997. Drosophila fizzy-related down-regulates mitotic cyclins and is required for cell proliferation arrest and entry into endocycles. *Cell* 90: 671-81

Sudakin, V., Ganoth, D., Dahan, A., Heller, H., Hersko, J., Luca, F., Ruderman, J. V. and Hershko, A. 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

Surana, U., Amon, A., Dowzer, C., McGrew, J., Byers, B. and Nasmyth, K. 1993. Destruction of the CDC28/CLB mitotic kinase is not required for

the metaphase to anaphase transition in budding yeast. *EMBO J.* 12: 1969-1978

Taylor, S. S., Ha, E. and McKeon, F. 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 McKeon, F. 1997. Kinetochore localization of murine Bub1 is required for normal mitotic timing and checkpoint response to spindle damage. *Cell* 89: 727-35

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

Waters, J. C., Chen, R.-H., Murray, A. W. and Salmon, E. D. 1998. Localization of Mad2 to Kinetochores Depends on Microtubule Attachment, Not Tension. *J. Cell Biol.* 141: 1181-1191

Weiss, E. and Winey, M. 1996. The S. cerevisiae SPB duplication gene *MPS1* is part of a mitotic checkpoint. *J. Cell Biol.* 132: 111-123

Wells, W. A. E. 1996. The spindle-assembly checkpoint: aiming for a perfect mitosis, every time. *TICB* 6: 228-234

Yamamoto, A., Guacci, V. and Koshland, D. 1996. Pds1p is required for faithful execution of anaphase in the yeast, Saccharomyces cerevisiae. *J Cell Biol* 133: 85-97

Yamamoto, Y., A., Guacci, V. and Koshland, D. 1996. 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

Yu, H., Peters, J. M., King, R. W., Page, A. M., Hieter, P. and Kirschner, M. W. 1998. Identification of a Cullin Homology Region in a Subunit of the Anaphase-Promoting Complex. *Science* 279: 1219-1222

Zachariae, W. and Nasmyth, K. 1996. TPR proteins required for anaphase progression mediate ubiquitination of mitotic B type cyclins in yeast. *Mol. Biol. Cell* 7: 791-801

Zachariae, W., Schwab, M., Nasmyth, K. and Seufert, W. 1998. Control of Cyclin Ubiquitination by CDK-Regulated Binding of Hct1 to the Anaphase Promoting Complex. *Science* 282: 1721-1724

Zachariae, W., Shevchenko, A., Andrews, P. D., Ciosk, R., Galova, M., Stark, M. J. R., Mann, M. and Nasmyth, K. 1998. Mass Spectrometric Analysis of the Anaphase-Promoting Complex from Yeast: Identification of a Subunit Related to Cullins. *Science* 279: 1216-1219

Zachariae, W., Shin, T. H., Galova, M., Obermaier, B. and Nasmyth, K. 1996. Identification of subunits of the anaphase-promoting complex of Saccharomyces cerevisiae. *Science* 274: 1201-4

Chapter 2

A novel yeast screen for mitotic arrest mutants identifies *DOC1*, a new gene involved in cyclin proteolysis.

A novel yeast screen for mitotic arrest mutants identifies *DOC1*, a new gene involved in cyclin proteolysis.

Lena H. Hwang and Andrew W. Murray

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Abstract

B-type cyclins are rapidly degraded at the transition between metaphase and anaphase and their ubiquitin-mediated proteolysis is required for cells to exit mitosis. We used a novel enrichment to isolate new budding mutants that arrest the cell cycle in mitosis. Most of these mutants lie in the *CDC16, CDC23,* and *CDC27* genes, which have already been shown to play a role in cyclin proteolysis and encode components of a 20S complex (called the cyclosome or anaphase promoting complex (APC)) that ubiquitinates mitotic cyclins. We show that mutations in *CDC26* and a novel gene, *DOC1*, also prevent mitotic cyclin proteolysis. Mutants in either gene arrest as large budded cells with high levels of the major mitotic cyclin (Clb2) protein at 37°C, and cannot degrade Clb2 in G1-arrested cells. Cdc26 associates *in vivo* with Doc1, Cdc16, Cdc23, and Cdc27. In addition, a population of Doc1 co-sediments at 20S with Cdc27 in a sucrose gradient, indicating that Cdc26 and Doc1 are components of the APC.

Introduction

Oscillations in the activity of cyclin dependent kinases (Cdk) control the eukaryotic cell cycle (Morgan 1995). Multicellular eukaryotes express a number of Cdks with different activities through the cell cycle. Yeasts, however, express a single Cdk, called Cdc28 in budding yeast (*Saccharomyces cerevisiae*) and Cdc2 in fission yeast (*Schizosaccharomyces pombe*). The oscillation of Cdk activity in all eukaryotes depends on phosphorylations of the kinase subunit and the cyclic expression and degradation of cyclins, the regulatory subunits of Cdks. Entry into mitosis requires the expression of B-type (mitotic) cyclins, and exit from mitosis requires their degradation (Murray and Kirschner 1989, Nurse 1990). The Cdk activity associated with B-type cyclins is required for chromosome condensation and proper formation of the mitotic spindle. The degradation of B-type cyclins, which leads to inactivation of the mitotic Cdk, results in chromosome decondensation, breakdown of the mitotic spindle, and cell division.

A short N-terminal sequence found in A and B-type cyclins, termed the destruction box, is required for their proteolysis (Glotzer, et al. 1991). Deletion or mutation of this sequence produces a non-degradable cyclin, and cells or extracts expressing these non-degradable B-type cyclins are unable to exit mitosis and remain arrested in anaphase (Holloway, et al. 1993, Murray, et al. 1989, Sigrist, et al. 1995, Surana, et al. 1993). In contrast, inhibiting the machinery that is required for cyclin proteolysis arrests cells in metaphase with unseparated sister chromatids (Holloway, et al.

1993). This observation suggests that additional proteins, whose destruction is required for the separation of sister chromatids, must also be targets of the machinery that degrades cyclins. Candidates for these regulators of sister cohesion have recently been identified (Funabiki, et al. 1996, Yamamoto, et al. 1996).

The degradation of cyclin is regulated by the assembly of multi-ubiquitin chains onto the cyclin substrate which target it for proteolysis by the proteasome. In frog egg extracts, mutations in cyclins that block their degradation also block ubiquitination (Glotzer, et al. 1991, King, et al. 1995), and methylated ubiquitin, an inhibitor of multi-ubiquitin chain formation, inhibits the degradation of cyclins in clam egg extracts (Hershko, et al. 1991). In yeast, a number of mutants that block cyclin degradation also block ubiquitination (Zachariae and Nasmyth 1996).

Ubiquitination of proteins begins with the activation of ubiquitin by a ubiquitin-activating enzyme (E1). The E1 forms a thiol ester with ubiquitin and then transfers that ubiquitin onto a ubiquitin-carrier protein (E2). Some substrates are directly ubiquitinated by an E2. Other substrates, however, also require a ubiquitin-protein ligase (E3), which appears to provide a higher level of specificity to the degradation system. Finally, the 26S proteasome recognizes multi-ubiquitinated substrates and degrades them (reviewed in (Ciechanover 1994)).

In budding yeast, clam and frog, a non-specific E1 is capable of activating ubiquitin. A large family of E2's (*UBC*'s) exist in yeast, and the E2 required for Clb degradation has not yet been definitively identified. E2-C (clam), homologs of the budding yeast UBC4, and a

novel UBC, UBCX (frog) are capable of acting as E2s for cyclin B in reconstituted cyclin ubiquitination systems derived from clam and frog egg extracts (King, et al. 1995, Sudakin, et al. 1995). The E3 for B-type cyclins was first identified as a 20S complex in clam and frog egg extracts, known as the cyclosome or anaphase promoting complex (APC) (King, et al. 1995, Sudakin, et al. 1995). In frog egg extracts, yeast homologs of *CDC16* and *CDC27* were identified as components of this E3 complex, which appears to be active only in mitosis in both clams and frogs.

CDC16, CDC23, and *CDC27* are all members of a family of tetratricopeptide repeat (TPR) proteins, and form a complex in yeast that is required for the ubiquitination and degradation of Clb2 (the major mitotic cyclin) (Irniger, et al. 1995, Lamb, et al. 1994, Zachariae and Nasmyth 1996). Temperature sensitive mutants in any of these genes cause a metaphase arrest with high levels of Clb2 and unseparated sister chromosomes. Another gene, *CSE1* has also been implicated in cyclin ubiquitination (Irniger, et al. 1995). Mutations in *CDC16, CDC23,* and *CSE1* all result in increased chromosome loss, suggesting that proper regulation of the cyclin proteolysis machinery is required for faithful chromosome segregation (Hartwell and Smith 1985, Xiao, et al. 1993).

We developed an enrichment for cell cycle arrest mutants in budding yeast and used this to screen for new mitotic arrest mutants. We isolated a large number of alleles of previously identified *cdc* (cell division cycle) mutants, and identified a novel mutant, *doc1* (destruction of cyclin B). *CDC26* and *DOC1* are involved in the degradation of Clb2 and the products of both genes associate

Materials and Methods

Yeast Strains, Media, and Genetic Methods

All yeast strains are derivatives of W303, except those used for complementation analysis. All strains used in this work are listed in Table 1. Yeast media and genetic manipulations were performed as described (Sherman, et al. 1974). LH103 (*mec1-1*) was made by backcrossing AFS85 (*MATa, mec1-1, GAL-CLN3::URA3::cln3Δ, ssd1-v1::LEU2, leu2-3,112, ura3, his3-11,15, ade2-1, trp1-1*) 4 times into W303 and subsequently crossing in *mad1Δ::HIS3* (from KH123, (Hardwick and Murray 1995)) and *bar1* (from AFS92, kindly provided by Aaron Straight). BglII cut pLH17 (*CLB2-LacZ)* was integrated at *CLB2*, recombination and loss of the *URA3* marker were selected for on 5-FOA media, and retention of *CLB2-LacZ* was screened for using β-gal plate assays. Strains containing *GAL-CLB2* were made by integrating pDK27 (kindly provided by Doug Kellogg) at *URA3*.

Hydroxyurea (Sigma, St. Louis, MO) was used at 10mg/ml final concentration in media. α-factor (Bio-synthesis, Lewisville, TX) was used at 1μg/ml for *bar1-* strains and 10μg/ml for *BAR1* strains, from a stock solution at 10mg/ml in DMSO (Aldrich, Milwaukee, WI). Nocodazole (Sigma, St. Louis, MO) was used at 15μg/ml from a stock solution of 10mg/ml in DMSO. Cycloheximide (Sigma, St. Louis, MO) was used at 10μg/ml from a stock solution of 10mg/ml. Nocodazole

treatments were carried out at 23°C. The other treatments were performed at various temperatures.

Plasmid Constructions

For *CLB2-LacZ* (pLH17), *CLB2* (including the ORF and 301bp upstream) was amplified by PCR using oligomers containing BamHI and SalI sites at 5' and 3' ends respectively. This was ligated into pRS304 (Sikorski and Hieter 1989) then cut with SalI and KpnI. The KpnI site was blunted using T4 polymerase. *LacZ* was cut from pAFS35 (kindly provided by Aaron Straight) using SalI and BamHI. The BamHI site was blunted using the Klenow fragment of DNA polymerase I. The *LacZ* fragment was then ligated into the *CLB2* construct. A second BamHI site was created 3' of LacZ by ligation of the blunted BamHI and KpnI sites. A 270bp fragment of sequence immediately 3' of the *CLB2* ORF was amplified by PCR with BamHI and SalI sites 5' and 3' respectively. This was ligated into pRS306 (Sikorski and Hieter 1989). The BamHI fragment containing *CLB2-LacZ* was then ligated into the pRS306 construct containing the 3' flanking region of *CLB2*.. The Clb2-LacZ protein is functional and capable of acting as the sole mitotic cyclin since pLH17 rescues the temperature sensitivity of a strain deleted for *CLB1, CLB3,* and *CLB4* with *CLB2* replaced with a temperature sensitive allele, (K3080) (Amon, et al. 1993).

pLH25 was made by PCR amplifying the open reading frame of *DOC1* with 508 base pairs upstream with a SalI site 5' and an NcoI site 3'

to the ORF. The PCR product was ligated into pKH511 (kindly provided by Kevin Hardwick), to place a single myc tag at the C-terminus of the protein. To make pLH23, pLH25 was cut with EcoRV and BamHI and the 1.0 kb fragment was ligated into pDK20 (kindly provided by Doug Kellogg) cut with SmaI and BamHI, to place the *DOC1* open reading frame behind the GAL1-10 promoter. pLH24 was made by cutting pLH25 with SalI and BamHI and ligating the 1.5 kb fragment into YIplac211, cut with the same enzymes. pLH 26-1B was made by cutting T1 (the original rescuing plasmid from the YCp50 library) with Nde1 and blunting with Klenow. For pLH26-1A, *URA3* was cut from pLH3 with XhoI and XbaI and also blunted with Klenow. The *URA3* fragment was ligated into the blunted NdeI site in T1.

To make pLH32, the open reading frame of *DOC1* with 508 base pairs upstream was amplified by PCR with a SalI site 5' and a KpnI 3' to the ORF. The KpnI site was designed so that *DOC1*'s stop codon was excluded and the 3' end of *DOC1* would be in frame with a triple hemagglutinin (3XHA) tag in YCplac111-3XHA. The *DOC1* PCR product was cut with SalI, blunted with Klenow, and then cut with KpnI. YCplac111-3XHA was cut with EcoRI, blunted with Klenow, and then cut with KpnI. The PCR fragment was then ligated into YCplac111-3XHA. All enzymes were from New England Biolabs (Beverly, MA) and used according to the manufacturer's specifications.

Plasmids expressing Ub-R-βgal and Ub^{V76}-V-$e^{\Delta K}$-βgal were kindly provided by Dr. Erica Johnson (Rockefeller University, NY). Plasmids containing hemagglutinin tagged *CDC16* (pWAM10), *CDC23* (pRS239), and *CDC27* (pRS248) were kindly provided by Dr. Phillip Heiter (Johns Hopkins University, MD).

Mutant Isolation

Strain LH103 (8 x 10^6 cells) was mutagenized with EMS (Sigma) to 50% killing. The mutagenized cells were diluted 1:25 and allowed to recover for 12 hours at room temperature in YPD. They were shifted to 37°C, to prearrest potential G1 and mitotic arrest mutants, for 2 hours, then hydroxyurea was added to 10mg/ml and cells were incubated at 37°C for an additional 5 hours. This culture was then plated on YPD plates and incubated at 23°C. Surviving colonies were replica-plated onto YPD plates in duplicate with one set at 23°C and the other at 37°C to test for temperature sensitivity.

Clb2-LacZ and Visual Screen

Temperature sensitive mutants were patched onto YPD plates and allowed to grow overnight at 23°C. They were then replica plated to YPD and placed at 37°C for 4 hours and replica plated again to Whatman filters (VWR, San Francisco, CA) on YPD plates that contained 1mg/ml α-factor and returned to 37°C for 5 hours. The filters were assayed for β-gal activity by freezing them in liquid nitrogen, thawing them and incubating them on Whatmann paper

soaked in Z buffer plus X-gal(5-Bromo-4-chloro-3-indolyl-β-D-galactoside, US Biological, Swampscott, MA) (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$, 0.03% X-gal) at 30°C overnight.

Strains that produced bright blue patches were further analyzed by microscopy. The strains were grown in liquid media to log phase at 23°C and then shifted to 37°C. Cells were taken and fixed in 3.7% formaldehyde (Fisher, Santa Clara, CA) after 3 and 6 hours at 37°C. They were stained with DAPI (1μg/ml) and anti-tubulin (YOL1/34, Accurate Chemical & Scientific Corpn, Westbury, NY) diluted 1:200, and their arrest phenotype was determined by light microscopy.

Mitotic arrest mutants were tested for complementation against a collection of known mitotic mutants. Only two mutants were not identified as previously isolated mutants. One was cloned and named *DOC1*, and the other (2 alleles) has not been cloned and appears to arrest in mitosis with pleiotropic defects. One allele of *cdc14* was also isolated, however, it was isolated as a double mutant with a *cdc16* mutant. *CDC14* codes for a phosphatase, and mutants in this gene arrest in anaphase of the cell cycle (Wan, et al. 1992).

Cloning of DOC1

A YCp50 library (described in (Hardwick and Murray 1995)) was transformed into *doc1-1* by lithium acetate transformation and plated on -Ura media at 37°C. Plasmids that were capable of rescuing the temperature sensitivity were recovered and the inserts were

sequenced at both 5' and 3' ends. These sequences were used to probe the yeast genome database.

Preparation of Antibodies Against Cdc26, Western Blot Analysis, and Immunoprecipitations

The open reading frame of *CDC26* was amplified by PCR with oligomers containing BamHI and EcoRI sites 5' and 3' to the ORF respectively. This fragment was cloned into pGEX-1 cut with BamHI and EcoRI (Smith and Johnson 1988), forming a glutathione-S-transferase (GST) fusion construct (pLH29). pLH29 was transformed into *E. coli* strain TG1 (Maniatis, et al. 1982) and induced for expression with 0.1mM IPTG (US Biologicals, Swampscott, MA) for 2 hours at 37°C. Cells were pelleted and washed with PBS (140mM Na_2HPO_4, 1.8mM KH_2PO_4, 138mM NaCl, 2.7mM KCl, pH 7.2), pelleted again and frozen in liquid nitrogen. The frozen pellet was resuspended in five volumes PBS containing 1mM EDTA, 1mM EGTA, 1mM PMSF, 200mg/ml lysozyme, and then briefly sonicated. Triton X-100 was added to 0.5% and DTT to 15mM and cells were sonicated again. The lysate was spun at 15,000 rpm in an SS34 rotor (Sorvall, Burbank, CA) for 30 minutes then the supernatant was transferred to a new tube and spun again for 20 minutes. The cleared supernatant was then loaded onto a 5ml glutathione agarose column (Sigma Chemical Co., St. Louis, MO) which was then washed with PBS containing 0.5M NaCl, 1mM EDTA, and 1mM EGTA. The Cdc26-GST fusion protein was eluted with 5mM reduced glutathione (Sigma, St. Louis, MO) in 50mM Tris, pH 8.1. The peak fractions were pooled

and dialyzed into 50mM Hepes pH 7.6, 50mM KCl, 30% glycerol. The protein was sent to Berkeley Antibody Company (Berkeley, CA) where it was used to immunize a rabbit. The rabbit serum was passed over a 50 ml column of GST protein coupled to Affigel 10 (Bio-Rad Labs, Hercules, CA) to remove anti-GST antibodies, then affinity purified using a 5 ml column of the Cdc26-GST fusion protein coupled to Affigel 10 (Bio-rad, Richmond, CA).

Yeast extracts for immunoblotting and immunoprecipitations were made by bead beating cells 2 times 90 seconds in lysis buffer A (50mM Hepes pH 7.6, 75mM KCl, 1mM $MgCl_2$, 1mM EGTA, 0.1% NP-40, 50mM NaF, 100mM Na Vanadate, 1mM PMSF and LPC (10mg/ml leupeptin, pepstatin, and chymostatin), Boehringer, Indianapolis, IN). The lysates were spun briefly to separate beads from the lysate and then cleared by centrifugation for 5 minutes in an Eppendorf micro-centrifuge at 4°C. This step was repeated 3 times. Protein concentrations of extracts were determined with the Bradford assay using bovine serum albumin as a standard (Bradford 1976). For immunoblotting, the extracts were adjusted to the same protein concentration and diluted with 2X SDS sample buffer (1X SDS sample buffer: 80mM Tris, pH 6.8, 2% SDS, 10% glycerol, 10mM EDTA, 0.0013% Bromophenol blue, 5% 2-mercaptoethanol). Standard methods were used for SDS-PAGE and protein transfer to nitrocellulose (Harlow and Lane 1988). Blots were stained with Ponceau S (Fisher, Santa Clara, CA) to confirm transfer and equal protein loading, then blocked for 30 minutes with blotto (4% dried milk, PBS, 0.2% Tween 20). Antibodies were used at 1:1200 for anti-

Clb2, 1:1000 for 12CA5 (BABCO, Berkeley, CA), 1:1000 for anti-βgal (Cappel, Durham, NC), and 1:1000 for anti-Cdc26 at either room temperature for 1 hour or 4°C overnight. Blots were washed 3 times 10 minutes in PBS with 0.2% Tween 20 (PBST) and then incubated with HRP-conjugated anti-rabbit or anti-mouse antibodies (Amersham Corp., Buckinghamshire, UK) at 1:5000 dilution in PBST. They were washed again and developed using Amersham ECL detection reagents following manufacturer's instructions. Cdc26 protein is not detectable by western, likely due to an inability to bind to nitrocellulose.

For immunoprecipitations, antibodies were used at 1:33 for 12CA5 and 1:50 for Cdc26. Lysates with primary antibody were rotated for 1 hour at 4°C, then transferred to a tube containing Protein A-Sepharose beads (Pharmacia, Pleasant Hill, CA) and rotated at 4°C for an additional hour. The beads were washed 2X with lysis buffer A, transferred to a new tube, and washed again with PBST, then resuspended in SDS sample buffer.

Sucrose Gradient Analysis

Solutions with lysis buffer A were prepared containing either 5% or 40% sucrose. Additional solutions with 13.75%, 22.5%, and 31.25% sucrose were made by combining various amounts of the 5% and 40% solutions. 950µl of each solution were layered into 1/2 X 2" Beckman Ultra-Clear Centrifuge tubes (Beckman, Palo Alto, CA). The gradient was incubated 12-16 hours at 4°C. Yeast extract (100µl), made as described above, was layered onto the gradient and spun at

50,000 rpm in a SW-55 rotor (Sorvall, Burbank, CA) for 4 hours at 4°C. Sixteen 300μl fractions were taken and 30μl of each fraction were loaded onto SDS-Page gels and analyzed by immunoblotting. Molecular weight markers were analyzed by Coomassie Blue staining (Fisher, Santa Clara, CA).

Table 2-1. Yeast strains

Strain	Relevant Genotype	Source
LH103	*MATa mec1-1 mad1Δ::HIS3 clb2::CLB2-LacZ bar1 YCp50/MAD1*	this work
AFS34 (W303-1a)	*MATa ade2-1 can1-100 ura3-1 leu2-3,112 his3-11,15 trp1-1*	Rothstein
YPH218	*MATa cdc16-1*	P. Hieter
YPH221	*MATa cdc23-1*	P. Hieter
LH127	*MATa cdc20-1 ura3-1 leu2-3,112 trp1-1*	this work
H160-3-3	*MATa cdc27-1*	L. Hartwell
H152-4-2	*MATa cdc26-1*	L. Hartwell
ELW65-93	*MATa cdc31-2YCp50/CDC31*	M. Winey
CMY763	*MATa cim3-1*	C. Mann
CMY765	*MATa cim5-1*	C. Mann
LH202	*MATa cim5-1 bar1*	this work
LH103-15	*MATa doc1-1 mec1-1 mad1Δ::HIS3 clb2::CLB2-LacZ bar1 YCp50/MAD1*	this work
LH103-66	*MATa cdc26-100 mec1-1 mad1Δ::HIS3 clb2::CLB2-LacZ bar1 YCp50/MAD1*	this work
LH225	*MATa cdc26-100 clb2::CLB2-LacZ bar1*	this work
LH226	*MATa doc1-1 clb2::CLB2-LacZ bar1*	this work
ADR58	*MATa pDK27 (GAL-CLB2)*	this work
ADR103	*MATa cdc16-1 pDK27 (GAL-CLB2)*	this work
LH227	*MATa cdc26-100 bar1 pDK27 (GAL-CLB2)*	this work
LH228	*MATa doc1-1 bar1 pDK27 (GAL-CLB2)*	this work
LH229	*MATa cdc26-100 bar1 ura3-1::CDC26myc-URA3 pDK27 (GAL-CLB2)*	this work
LH230	*MATa doc1-1 bar1 ura3-1::DOC1myc-URA3 pDK27 (GAL-CLB2)*	this work
LH209	*MATa bar1 pUb-R-βgal*	this work
LH210	*MATa bar1* pUb^{V76}*-V-*$e^{\Delta K}$*-βgal*	this work
LH211	*MATa doc1-1 bar1 pUb-R-βgal*	this work
LH212	*MATa doc1-1 bar1* pUb^{V76}*-V-*$e^{\Delta K}$*-βgal*	this work
LH213	*MATa cdc26-100 bar1 pUb-R-βgal*	this work
LH214	*MATa cdc26-100 bar1* pUb^{V76}*-V-*$e^{\Delta K}$*-βgal*	this work
LH215	*MATa cim3-1 bar1 pUb-R-βgal*	this work
LH216	*MATa cim3-1 bar1* pUb^{V76}*-V-*$e^{\Delta K}$*-βgal*	this work
LH231	*MATa bar1 pWAM10 (CDC16HA)*	this work
LH232	*MATa bar1 pRS239 (CDC23HA)*	this work
LH233	*MATa bar1 pJL25 (CDC27HA)*	this work
LH234	*MATa bar1 pLH32 (DOC1HA)*	this work
LH235	*MATa bar1 cdc26Δ::URA3 pWAM10 (CDC16HA)*	this work
LH236	*MATa bar1 cdc26Δ::URA3 pRS239 (CDC23HA)*	this work
LH237	*MATa bar1 cdc26Δ::URA3 pJL25 (CDC27HA)*	this work
LH238	*MATa bar1 cdc26Δ::URA3 pLH32 (DOC1HA)*	this work
LH241	*MATa bar1 pJL25 (CDC27HA) pLH32 (DOC1HA)*	this work

All strains from this work are isogenic to W303 (R. Rothstein) except where noted.

Results

Screening for New Mitotic cdc Mutants

We designed a strategy that enabled us to enrich for temperature sensitive budding yeast mutants that arrest in G1 or mitosis (Figure 2-1). We used *mec1-1*, a cell cycle checkpoint mutant, to devise conditions under which cycling cells would die while arrested ones would survive. *mec1-1* mutants lack the checkpoint that detects unreplicated DNA and DNA damage, and cannot arrest their cell cycle in the presence of unreplicated DNA (Weinert 1992). As a result, treating cycling *mec1-1* cells with DNA replication inhibitors, such as hydroxyurea, induces rapid cell death. In contrast, treating G1 or mitotically arrested *mec1-1* cells with hydroxyurea will not kill them. We exploited this difference to enrich for mutants that caused a temperature sensitive arrest in G1 or mitosis. This protocol does not enrich for most mutants arrested in S phase or G2, since their arrest requires the *MEC1*-dependent checkpoint. A *mec1-1* strain was mutagenized and the mutagenized cells were shifted to 37°C to allow potential cell division cycle mutants to prearrest, and then treated with hydroxyurea to kill the majority of cells which continued to progress through the cell cycle. Cells that arrest in either G1 or mitosis remain viable during the hydroxyurea treatment and form colonies on plating at the permissive temperature of 23°C.

We mutagenized 8 x 10^6 cells to 50% survival with ethyl methanesulfonate (EMS), and 40,000 survived the *mec1-1*

Figure 2-1. Mutant enrichment strategy

Schematic representation of the mutant enrichment strategy. Hydroxyurea, a drug that inhibits DNA synthesis, arrests wildtype cells in S-phase. Mutants that are defective in the checkpoint gene, *MEC1*, are unable to arrest in response to unreplicated DNA and die in the presence of hydroxyurea. If *mec1-1* cells are pre-arrested in G1 or mitosis, they remain viable after exposure to hydroxyurea.

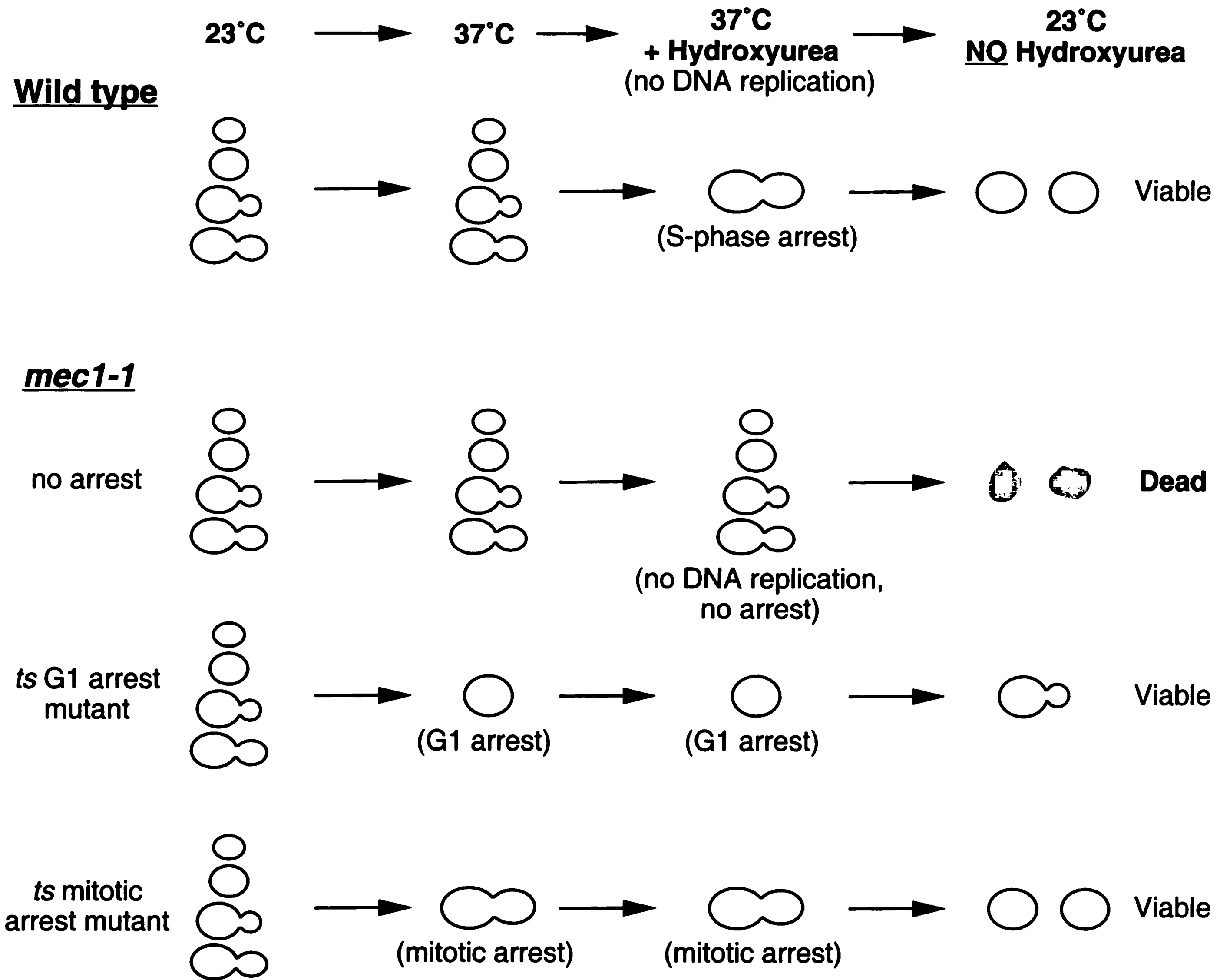


Figure 2-1

enrichment. Of those, 1968 (5%) were temperature sensitive. To distinguish mutants that arrested in mitosis from those that arrested in G1, we exploited the observation that mitotic cyclins are strongly expressed in mitosis but rapidly degraded as cells exit mitosis and enter G1 (Amon, et al. 1994). The temperature sensitive mutants were tested for expression of Clb2-lacZ, a protein fusion between the major mitotic cyclin (Clb2) and β-galactosidase (β-gal), whose activity can be easily monitored by exposing cells to a chromogenic substrate. Cells from mutants that expressed Clb2-lacZ strongly at 37°C (see Materials and Methods) were screened visually for their arrest phenotypes. We found 32 mutants that arrested with large budded cells at 37°C, a phenotype that is consistent with a mitotic arrest. These mutants fell into 11 complementation groups. Each group was tested against known mitotic mutants by complementation of temperature sensitivity and for rescue of temperature sensitivity by transformation with plasmids containing wild type copies of genes known to function in mitosis.

We isolated mitotic arrest mutations in nine genes (Table 2). One, *CDC31* (1 allele) is involved in spindle pole body duplication and *cdc31*^{ts} mutants arrest in mitosis with only one spindle pole body (Baum, et al. 1986, Byers 1981). Another, *PRP22* (2 alleles) codes for an RNA helicase involved in mRNA splicing (Company, et al. 1991). The alleles of *PRP22* isolated in this screen arrests in mitosis with no microtubules (data not shown). *TUB1* and *TUB3,* the genes that code for α-tubulin, each contain an intron. It is likely that the tubulin defect seen in these mutants is due to a failure to splice the mRNA's from these genes that leads to a deficiency in α-tubulin.

Table 2-2: Mitotic arrest mutants isolated by *mec1-1* enrichment

Gene	# alleles isolated	Biochemical Function	Reference
CDC16	16	APC component	(Irniger, et al. 1995, King, et al. 1995)
CDC23	3	APC component	(Irniger, et al. 1995)
CDC27	4	APC component	(King, et al. 1995)
CDC26	1	APC component	This work
DOC1	1	APC associated	This work
CIM3	1	proteasome component	(Ghislain, et al. 1993)
CDC20	1	unknown	(Sethi, et al. 1991)
CDC31	1	SPB component	(Baum, et al. 1986)
PRP22	2	splicing	(Company, et al. 1991)
uncloned	2	unknown	

Mutants were identified based on complementation of temperature sensitivity with previously identified mutants. They were also tested for rescue of temperature sensitivity by wildtype copies of known genes on plasmids.

Five of the genes we identified are involved in the proteolysis of cyclin that triggers the exit from mitosis. *CDC16* (16 alleles), *CDC23* (3 alleles), and *CDC27* (4 alleles) encode proteins that are involved in cyclin proteolysis and have been shown to form a multiprotein complex in yeast, frogs and clams (Irniger, et al. 1995, King, et al. 1995, Lamb, et al. 1994, Sudakin, et al. 1995). *CDC20* (1 allele) has also been implicated in cyclin proteolysis. Its homolog in *Drosophila*, fizzy, appears to be required for the proteolysis of Cyclin A and B (Dawson, et al. 1995, Sigrist, et al. 1995). In yeast, *cdc20* mutants arrest in metaphase and appear to have microtubule abnormalities (Byers and Goetsch 1974, Palmer, et al. 1989, Sethi, et al. 1991). *CIM3* (1 allele) is a subunit of the 26S proteasome, the multi-protein complex that degrades ubiquitinated proteins (Ghislain, et al. 1993).

Finally, we isolated one allele each of *CDC26* and a novel gene, which we named *DOC1* (Destruction of Cyclin B). *CDC26* is a mitotic arrest mutant, identified in the original Hartwell screen for cdc mutants (Hartwell, et al. 1970). The *CDC26* gene is essential only at 37°C; *cdc26Δ* cells grow well at 23°C (Araki, et al. 1992), a finding that we confirmed in the W303 strain background (data not shown).

CDC26 and *DOC1* are Required for Clb2 Proteolysis

Since the majority of mutants isolated in our screen identify genes involved in mitotic cyclin proteolysis, we tested the *cdc26* and *doc1* mutants for defects in this process. In order to determine if *CDC26* and *DOC1* were involved in cyclin proteolysis, we exploited the

observation that cyclin proteolysis begins in late mitosis and persists well into G1 (Amon, et al. 1994). Haploid yeast cells can be arrested in G1 by treatment with mating pheromone. Thus in *MATa* cells arrested in G1 by α-factor treatment, the B-type cyclin proteolysis machinery is active and ectopic expression of the *CLB2* gene does not lead to Clb2 protein accumulation. In a *cdc16-1* mutant, however, Clb2 protein does accumulate in G1-arrested cells as a result of a defect in the ubiquitination of Clb2 (Irniger, et al. 1995, Zachariae and Nasmyth 1996).

We integrated a copy of *CLB2* under the control of the inducible GAL promoter into wild type and mutant strains. *cdc16-1,* and the *doc1-1* and *cdc26-100* mutants isolated in our screen were arrested with α-factor and *CLB2* expression was induced with galactose. Accumulation of Clb2 was monitored by western blotting using anti-Clb2 antibodies. Figure 2-2A shows that although wildtype cells do not accumulate Clb2 at the α-factor arrest point, the *cdc16, doc1-1*, and *cdc26-100* mutants each accumulate Clb2 protein even at the permissive temperature, and that the amount of Clb2 increases still further in cells incubated at 37°C. This defect is rescued in *doc1-1* and *cdc26-100* by the integration of a wildtype copy of the corresponding gene at the *LEU2* locus (Figure 2-2A *doc1-1 DOC1* and *cdc26-100 CDC26*). Others have also identified *CDC26* as playing a role in cyclin proteolysis (Zachariae, et al. 1996).

The Clb2 destruction defect in *cdc26-100* and *doc1-1* appears to be specific to Clb2 and not a general defect in the proteolysis of ubiquitinated substrates. Figure 2-2B shows that *cdc26-100* and *doc1-1* cells have no defect in the degradation of two substrates that

require ubiquitination for destruction: Ub-R-βgal, which generates β-galactosidase with an N-terminal arginine, a substrate that is degraded by the N-end rule (Figure 2-2B, Ub-R-βgal) (Bachmair, et al. 1986), and Ub^{V76}-V-$e^{\Delta K}$-βgal, a fusion with a non-cleavable N-terminal ubiquitin (Figure 2-2B, UB^{V76}-βgal) (Johnson, et al. 1992). Cycloheximide was added to cells expressing Ub-R-βgal or Ub^{V76}-V-$e^{\Delta K}$-βgal to inhibit protein synthesis, and stability of the substrates was determined by western blotting with anti-β-galactosidase antibodies at different times after inhibiting protein synthesis. *cim3-1*, a mutant in a subunit of the 26S proteasome (Ghislain, et al. 1993) that degrades all ubiquitinated proteins, is defective in the degradation of both substrates (Figure 2-2B lanes 1-8).

To see if the mutant phenotype of *cdc26-100* and *doc1-1* cells reflected defects in mitotic proteolysis, we overexpressed a mitotic cyclin in these mutants. *cdc26-100* and *doc1-1* strains containing an integrated copy of *GAL-CLB2* are unable to grow on galactose at their permissive temperature (data not shown). We suggest that these strains are already crippled in their Clb2 destruction machinery at the permissive temperature, and that excess Clb2 overloads this machinery and results in a cell cycle arrest.

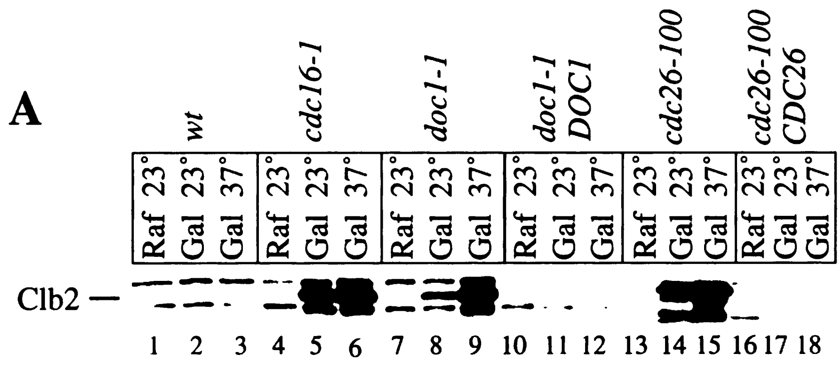
Cloning and Disruption of *DOC1*

Like previously identified mitotic cyclin proteolysis mutants, *doc1-1* mutants arrest as large budded cells at 37°C with a single nucleus and a short spindle (Figure 2-3A). A small number of *doc1-*

Figure 2-2. ***cdc26-100*** **and** ***doc1-1*** **mutants**

Phenotype of *cdc26* and *doc1* mutants. A) Stability of Clb2 in proteolysis mutants. The indicated strains contained an integrated copy of *pGAL-CLB2* and were grown in YEP + 2% raffinose media and arrested in G1 by exposure to α-factor for 3 hours at 23°C (Raf, 23°C). *CLB2* expression was induced by the addition of 2% galactose while maintaining the α-factor arrest at either 23°C (Gal, 23°C) or 37°C (Gal, 37°C) for 3 hours. The figure shows an exposure of a western blot of Clb2 using polyclonal anti-Clb2 antibodies. Wildtype, *cdc16-1, doc1-1*, *doc1-1* + *DOC1* (a *doc1-1* strain carrying a copy of *DOC1* integrated at *LEU2*), *cdc26-100*, *cdc26-100* + *CDC26* (a *cdc26-100* strain carrying a copy of *CDC26* integrated at *LEU2*).

B) Stability of β-galactosidase derivatives in proteolysis mutants. Wildtype, *doc1-1, cdc26-100* and *cim3-1* strains containing plasmids expressing either Ub-R-β-gal (lanes 1-4) or Ub^{V76}-V-$e^{\Delta K}$-βgal (Ub^{V76}-βgal) (lanes 5-8) under the control of the *GAL* promoter were grown in YEP + 2% raffinose and then arrested in G1 with α-factor for 3 hours. Expression from the plasmids was induced by the addition of 2% galactose for 2 hours. At time zero, cycloheximide was added to 10μg/ml and samples were taken at 15, 30, 45 minutes. The figure shows an exposure of a western blot using anti-β-galactosidase antibodies.



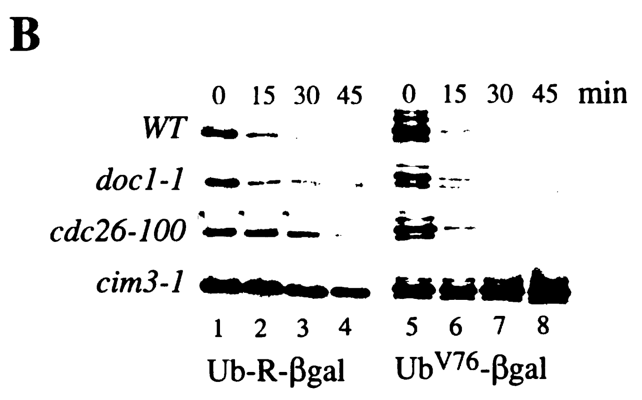


Figure 2-2

1 cells arrest in anaphase with separated DNA masses and a long spindle.

DOC1 was cloned by complementing the temperature sensitivity of *doc1-1* with a genomic yeast library on a centromeric vector (Figure 2-3B, T1). The minimal complementing region contains a single open reading frame coding for a protein of 283 amino acids. A construct containing the open reading frame of *DOC1* and 508 base pairs upstream, with a single myc tag on the C-terminus and no sequences 3' to the open reading frame, complements the temperature sensitivity and Clb2 proteolysis defect of the *doc1-1* mutant (Figure 2-3B, pLH24). In addition, a construct containing a frameshift at an Nde1 site 297 base pairs into the *DOC1* open reading frame, will not rescue the temperature sensitivity of *doc1-1* (Figure 2-3B, pLH26-1B). Disruption of *DOC1* by insertion of the *URA3* gene at the Nde1 site (Figure 2-3B, pLH26-1A) results in viable cells that grow poorly at 23°C, forming colonies in which most of the cells have large buds, and cannot grow at 37°C. Coincidentally, *DOC1* lies directly upstream of the *CSE1* gene, which is also implicated in cyclin proteolysis (Irniger, et al. 1995, Xiao, et al. 1993). These genes have distinct functions in proteolysis since plasmids that contain only the *CSE1* gene do not complement *doc1-1*. The Doc1 amino acid sequence is shown in Figure 2-3C. The Doc1 sequence lacks obvious motifs and shows no homology to proteins of known function, however, it does show homology to rat (Lee 1995) and mouse EST sequences (accession numbers: H33761 and W49295 respectively).

Figure 2-3. ***doc1-1*** **phenotype and sequence**

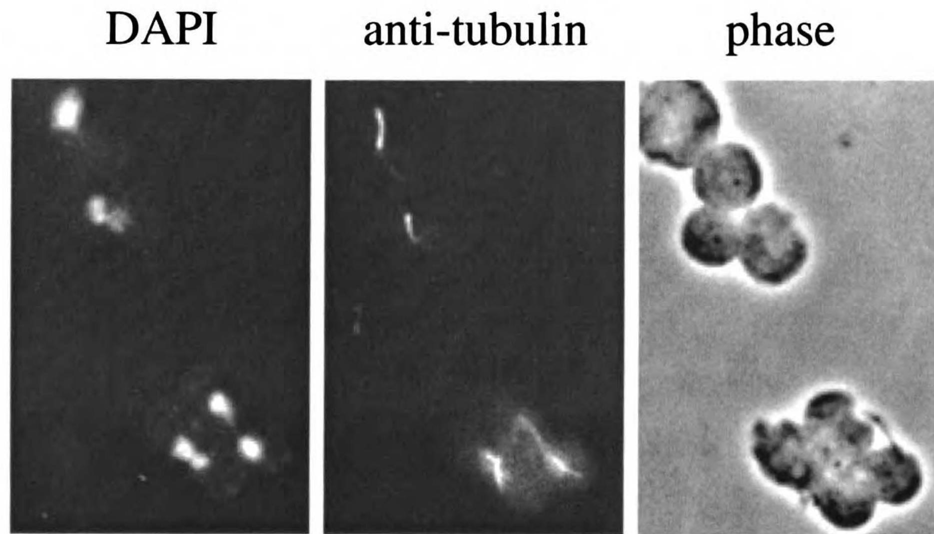
A) *doc1-1* arrest phenotype. The top two large-budded cells arrested with short spindles and a single DNA mass. The bottom two adjacent cells are large-budded. One has arrested with a short spindle and single DNA mass, the other appears to have undergone anaphase. *doc1-1* cells were grown to log phase at room temperature and then shifted to 37°C for 3 hours. The cells were fixed and stained with DAPI and anti-tubulin.

B) Structure of the *DOC1* locus and complementation of *doc1-1* temperature sensitivity with various plasmids. The shaded region represents the *DOC1* open reading frame and the hatched region represents the 5' end of the *CSE1* open reading frame. The arrows show the direction of transcription. The *DOC1* open reading frame with 508 base pairs upstream is sufficient to complement the temperature sensitivity of *doc1-1* at 37°C. TI, the rescuing plasmid from a YCp50 based library, contains approximately 640 base pairs upstream of the open reading frame of *DOC1* and approximately 1150 base pairs of the *CSE1* open reading frame. T1 was cut with Nde1 and blunted with the Klenow fragment of DNA polymerase I (New England Biolabs, Beverly, MA), producing a frameshift in the *DOC1* open reading frame (pLH26-1B). The frameshifted construct was unable to rescue *doc1-1*. pLH26-1A, in which *URA3* is inserted into the Nde1 blunted site, was also unable to rescue *doc1-1*.

C) Amino acid sequence of *DOC1*. This sequence corresponds to ORF YGL240W in the yeast genome database. The *DOC1* amino acid

sequence is aligned with rat and mouse EST sequences (accession numbers H33761 and W49295 respectively). Identities are boxed.

A



B

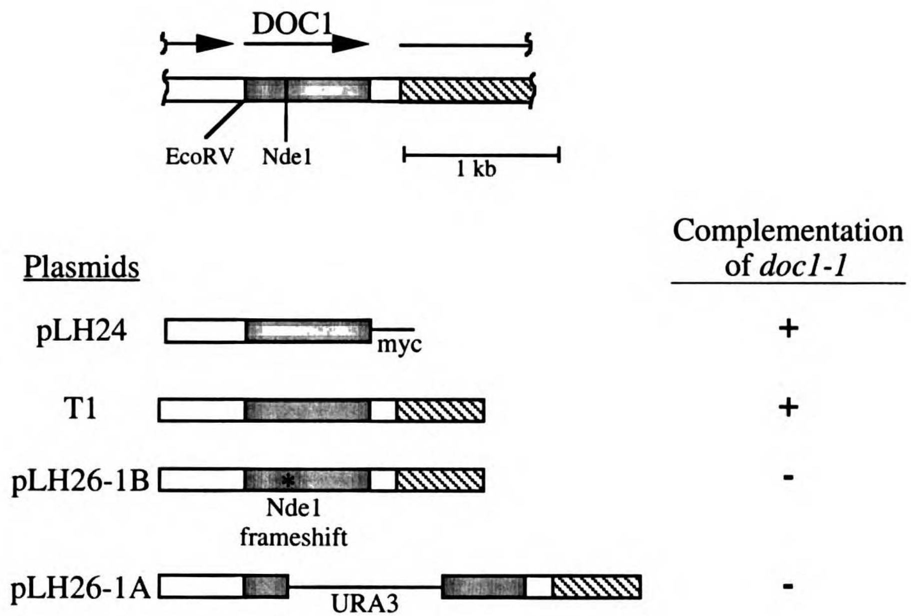


Figure 2-3

C

```
yeast doc1  MRQNKRRLYK RKKVLNKLTT EGGKNNTRFR SQIMDPIGIN KVLDHLAPSE
rat   EST   .......... .......... .......... .......... ...AVEVAGT
mouse EST   .......... .......... .......... .......... ..AAVEVAGT

            51                                                100
yeast doc1  LIKPVKSCHN KPSVLVLDDR IVDAATKDLY VNGFQEEIQY QNPTPENLQH
rat   EST   LRTGIVASQN NLKMTTPNKT PPGADPKQLE RTATVREIGS Q.........
mouse EST   LRTGIVASQN NLKMTTPNKT PPGADPKQLE RTATVREIGS Q.........

            101                                               150
yeast doc1  MFHQGIEILD SARMINVTHL ALWKPSSFKL GNPVDFALDD NYDTFWQSDG
rat   EST   .......... .......... AVWSLSSCKP GFGVDQLRDD NLETYWQSDG
mouse EST   .......... .......... AVWSLSSCKP GFGVDQLRDD NLETYWQSDG

            151                                               200
yeast doc1  GQPHQLDIMF SKRMDICVMA IFFSMIADES YAPSLVKVYA GHSPSDARFY
rat   EST   SQPHLVNIQF RRKTTVKTLC ILQTINLDES YTPSKISVRV GNI.....FH
mouse EST   SQPHLVNIQF SNSNWWSQVA GFTFLZ.... .......... ..........

            201                                               250
yeast doc1  KMLEVRNVNG WVALRFLDNR EDDQLLKCQF IRLLFPVNHE NGKDTHLRGI
rat   EST   NLQGSGNSIG

            251                                284
yeast doc1  RLYVPSNEPH QDTHEWAQTL PETNNVFQDA ILRZ
```

Figure 2-3

Cdc26 and Doc1 Associate with APC Components (Cdc16, Cdc23, and Cdc27)

Cdc16, Cdc23, and Cdc27 form a complex in yeast (Lamb, et al. 1994) and their homologs are components of a 20S complex in frog egg extracts (King, et al. 1995). This complex behaves as a ubiquitin ligase for B-type cyclins, and *CDC16, CDC23,* and *CDC27* are required for the ubiquitination of Clb2 in yeast (Zachariae and Nasmyth 1996). We therefore tested whether Cdc26 or Doc1 associated with Cdc16, Cdc23 or Cdc27. Extracts were made from exponentially growing wild type or *cdc26Δ* strains that had been transformed with centromeric plasmids containing hemagglutinin (HA)-tagged versions of *CDC16, CDC23, CDC27* and *DOC1*. Cdc26 was immunoprecipitated using polyclonal antibodies against Cdc26. The bound proteins were probed by western blotting with mouse monoclonal antibodies against HA (12CA5) to test whether the tagged proteins co-immunoprecipitate with Cdc26.

Figure 2-4 shows that immunoprecipitation of Cdc26 in a wild type strain results in the co-immunoprecipitation of HA-tagged Cdc16, Cdc23, Cdc27, and Doc1. No HA-tagged Cdc16, Cdc23, Cdc27, or Doc1 is detected when immunoprecipitations were performed on extracts made from a *cdc26Δ* strain. These experiments suggest that Cdc26 is a functional component of the APC, and this suggestion is strengthened by the observation that the Xenopus homolog of Cdc26 is a component of the purified APC (M. Kirschner, personal communication).

Figure 2-4. Cdc26 associates with Cdc16, Cdc23, Cdc27, and Doc1

Co-immunoprecipitation of Cdc26 with epitope tagged forms of Cdc16, Cdc23, Cdc27 and Doc1 ($Cdc16^{HA}$, $Cdc23^{HA}$, $Cdc27^{HA}$, and $Doc1^{HA}$). Wildtype (+) and *cdc26Δ* (Δ) strains containing centromeric plasmids expressing $Cdc16^{HA}$ (pWAM10) (lanes 1,2), $Cdc23^{HA}$ (pRS239) (lanes 3,4), $Cdc27^{HA}$ (pRS248) (lanes 5,6), or $Doc1^{HA}$ (pLH32) (lanes 7,8) were grown to log phase in selective media. Cdc26 was immunoprecipitated from those cell lysates using polyclonal anti-Cdc26 antibodies. Immunoprecipitates were analyzed by immunoblotting with 12CA5. The positions of molecular mass markers and $Cdc16^{HA}$, $Cdc23^{HA}$, $Cdc27^{HA}$, and $Doc1^{HA}$ are indicated.

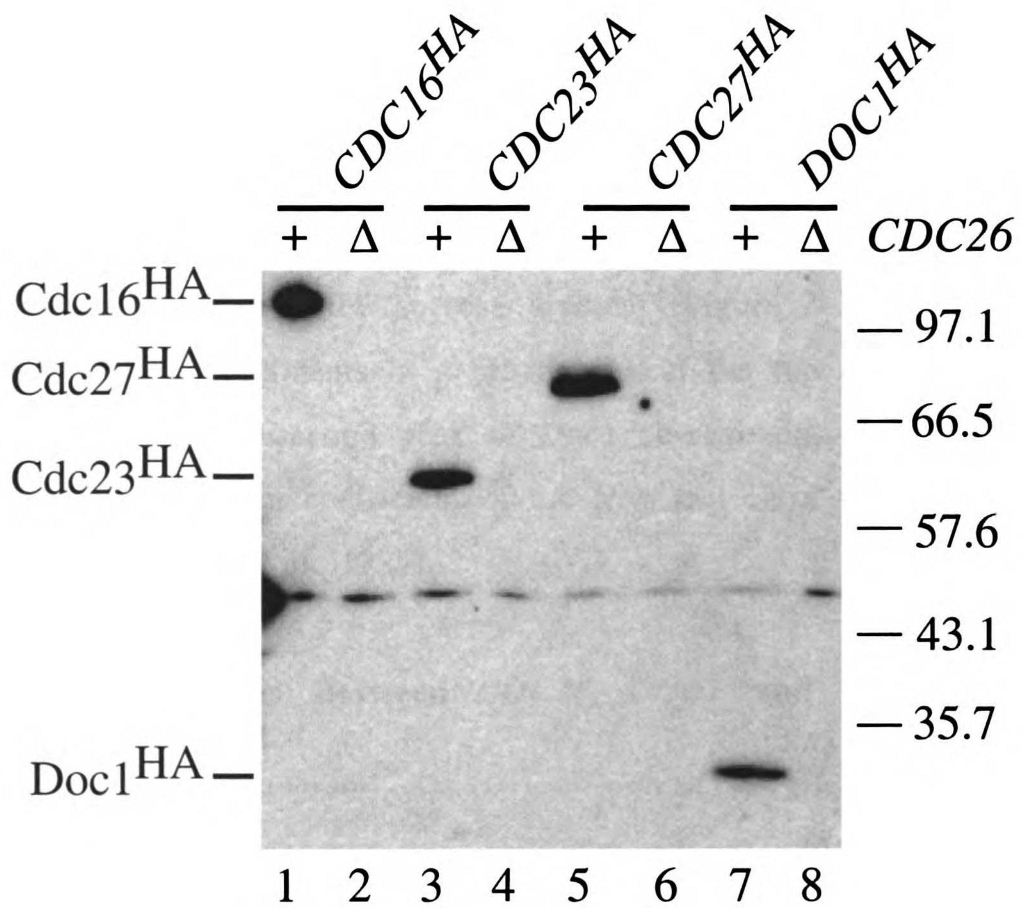


Figure 2-4

The association of Doc1 with Cdc26 could be interpreted in two ways. Either Doc1 is a component of the APC, or there are two pools of Cdc26, one associated with the APC, and one bound to Doc1. To distinguish these possibilities, we analyzed the sedimentation of Doc1 and Cdc27 in a sucrose gradient. Extracts from exponentially growing cells expressing both HA-tagged Doc1 and HA-tagged Cdc27 were separated on a 5-40% sucrose gradient (Figure 2-5). The majority of Doc1 sediments in a broad peak at the top of the gradient, however, a second peak of Doc1 co-sediments with Cdc27 at 20S, the sedimentation coefficient of the frog and clam APC (King, et al. 1995, Sudakin, et al. 1995).

Genetic Interactions Between *CDC26, DOC1* and Other Mitotic Mutants

Genetic interactions between mutants often indicate that they function in the same pathway. We found that double mutants of *cdc26-100* and *doc1-1* are synthetically lethal (Table 3), suggesting that the functions of Cdc26 and Doc1 overlap. In addition, *cdc26-100* is synthetically lethal with *cdc16-1* and *cdc23-1* and *cdc26-100 cdc27-1* double mutants have a lower permissive temperature than either single mutant. *doc1-1* double mutants with *cdc16-1* or *cdc23-1* have a lower permissive temperature than any of the single mutants. The interactions of *cdc26-100* and *doc1-1* with mutants involved in the ubiquitination of mitotic cyclins do not simply reflect synthetic interactions between any pair of mitotic mutants. Double mutants between *cdc26-100* or *doc1-1* and *cim5-1* (a subunit of the

Figure 2-5. Co-sedimentation of Docl with Cdc27

Co-sedimentation of Doc1 with Cdc27. A lysate from exponentially growing cells expressing both Doc1 and Cdc27 tagged with the HA epitope ($Doc1^{HA}$ and $Cdc27^{HA}$) was separated through a 5% to 40% sucrose gradient. Fractions were analyzed by immunoblotting with 12CA5. The slower migrating forms in fractions 5 to 7 of the Doc1HA panel are degradation products of a 12CA5 cross-reactive band.

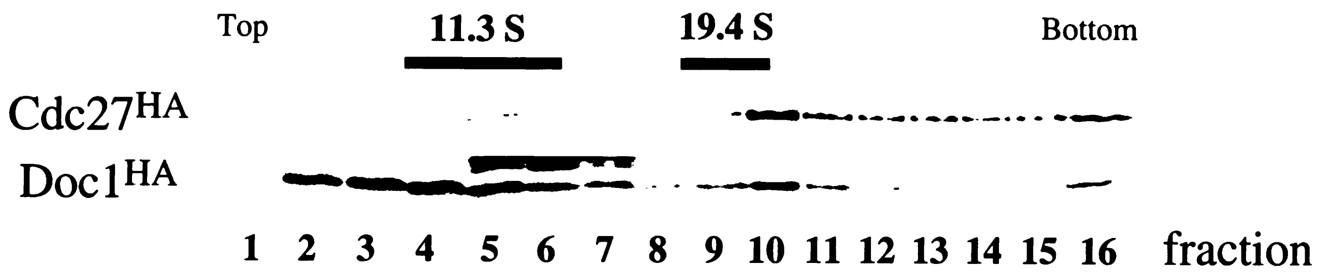


Figure 2-5

26S proteasome, required for the destruction of ubiquitinated proteins) (Ghislain, et al. 1993) have the same non-permissive temperature as the *cdc26-100* or *doc1-1* single mutants.

Table 3: Genetic interactions between *cdc26*, *doc1*, and other mitotic mutants

	cdc26-100	*doc1-1*	*cdc16-1*	*cdc23-1*	*cdc27-1*	*cim5-1*
Maximum permissive temperature, °C	**33**	**33**	30	30	33	37
Maximum permissive temperature of double mutant with *cdc26-100*, °C	**33**	dead	dead	dead	30	33
Maximum permissive temperature of double mutant with *doc1-1*, °C	dead	**33**	23	23	30	33

Synthetic interactions between *cdc26-100*, *doc1-1*, and various mitotic mutants. *cdc26-100* and *doc1-1* were mated to each other or to *cdc16-1*, *cdc23-1*, *cdc27-1*, or *cim5-1*. The resultant diploids were sporulated and tetrads were dissected and germinated at 23°C. They were then tested for growth at 23°C, 30°C, 33°C, 35°C, and 37°C. The genotype of viable spores was determined by complementation testing with appropriate mutant tester strains. The maximum permissive temperatures of the *cdc26-100* and *doc1-1* mutants are shown in bold face.

Discussion

We have identified two new components of the cyclin proteolysis machinery, Cdc26 and Doc1, and show that Cdc26 and Doc1 are physically associated with the APC/cyclosome, the multi-protein complex that acts as an E3 for cyclin ubiquitination (King, et al. 1995, Sudakin, et al. 1995, Zachariae and Nasmyth 1996). Similar results were recently obtained for *CDC26* by others (Zachariae, et al. 1996).

We isolated temperature sensitive mitotic mutants by using an enrichment that kills the majority of cells passing through S phase at the non-permissive temperature. Of the 32 temperature sensitive mitotic arrest mutations we isolated, 27 are in genes involved in the cyclin proteolysis machinery, one is in *CDC31*, a gene required for spindle pole body duplication, and two are in *PRP22*, which encodes a splicing function that is probably required to ensure adequate levels of α-tubulin synthesis. Why do proteolysis mutants dominate the metaphase arrest mutants isolated in both this screen and the original Hartwell screen for *cdc* mutants? We believe that three factors account for this observation. First, although defects in the mitotic spindle can arrest cells in mitosis by transiently activating the spindle assembly checkpoint, the checkpoint eventually adapts to persistent defects thus allowing cells to exit mitosis (K. Hardwick, A. Rudner, E. Wiess, M. Winey, and A.W. Murray, unpublished results). Since cells that adapt and leave mitosis with a defective spindle are likely to suffer lethal errors in chromosome segregation, mutants that cause spindle defects may have died during the prolonged

incubation at 37°C that was used during the mutant enrichment. Second, there appears to be considerable functional overlap among the components that assemble the mitotic spindle, so that mutations that inactivate single components do not cause a mitotic arrest. For example, strains lacking the Kar3 microtubule motor are viable, although mitosis in these cells is clearly abnormal (Meluh and Rose 1990). Finally, the integrity of some spindle functions, such as the protein kinase Mps1 (Hardwick, et al. 1996), are required for the spindle assembly checkpoint to detect defects in the spindle. As a result, lesions in these components would not arrest cells in mitosis.

cdc26 and *doc1* mutants arrest in metaphase and cannot destroy mitotic cyclins that are expressed in G1 arrested cells. Unlike mutations in the proteasome, *cdc26* and *doc1* mutants do not suffer general defects in ubiquitin mediated proteolysis. A substrate that is recognized by a destabilizing N-terminal amino acid or a non-cleavable N-terminal ubiquitin is degraded normally in *cdc26* and *doc1* mutants. Taken together, these observations suggest that the mitotic arrest of *cdc26* and *doc1* reflects their inability to degrade mitotic cyclins and proteins, such as Cut2 and Pds1, whose destruction is required for sister chromatid separation (Funabiki, et al. 1996, Yamamoto, et al. 1996). This conclusion is supported by both genetic and biochemical evidence for interactions of Cdc26 and Doc1 with the APC. Immunoprecipitates prepared with anti-Cdc26 antibodies contain Cdc16, Cdc23, and Cdc27 all of which are characterized components of the APC, as well as Doc1. In addition, a population of Doc1 co-sediments with Cdc27. This evidence suggests that both Cdc26 and Doc1 are associated with the APC. Since we

cannot easily measure the amounts of Cdc26 and Doc1 relative to other components of the proteasome, it is unclear whether Cdc26 and Doc1 are stoichiometric components of the APC. The observation that neither Cdc26 or Doc1 show any homology to cloned subunits of the biochemically purified Xenopus APC, suggests that Cdc26 and Doc1 cannot be tightly associated with all APC complexes (King, et al. 1995). The observation that *doc1-1* is synthetically lethal with *cdc26-100*, but not with mutants in other components of the APC, suggests that Doc1 and Cdc26 may play partially overlapping roles in cyclin proteolysis. Although *cdc26Δ* mutants grow well at 23°C, they are synthetically lethal with *cdc16-1* and *cdc23-1*, suggesting that Cdc26 may play a role in stabilizing the APC, and that this role is dispensable under optimal conditions but becomes essential under environmental stress or in the presence of defects in other APC components.

Defects in the mitotic spindle activate a spindle assembly checkpoint that arrests eukaryotic cells in mitosis (Hoyt, et al. 1991, Li and Murray 1991). Activation of the checkpoint prevents the destruction of mitotic cyclins and sister chromatid separation, suggesting that the principal action of the checkpoint is to inhibit the proteolysis of mitotic cyclins and proteins that play a role in maintaining the linkage between sisters. It is currently unclear whether this inhibition is due to inactivation of the APC itself or to reactions that protect specific substrates from a normally active APC in checkpoint-arrested cells. Further biochemical comparison between APC components in anaphase and checkpoint arrested extracts should help to resolve this issue.

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Chapter 3

Budding Yeast Cdc20: Target of the Spindle Checkpoint

Budding yeast Cdc20: a target of the spindle checkpoint

Lena H. Hwang, Lucius F. Lau, Dana L. Smith, Cathy A. Mistrot, Kevin G. Hardwick*, Ellen S. Hwang, Angelika Amon, Andrew W. Murray

L. H. Hwang, D. L. Smith, C. A. Mistrot, K.G. Hardwick and A. W. Murray: Departments of Physiology and Biochemistry, UCSF San Francisco, CA 94143-0444, U. S. A.

L. F. Lau, E. S. Hwang, A. Amon: Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA, 02142, U. S. A.

*Present address ICMB, University of Edinburgh, Edinburgh, EH9 3JR

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To Whom it My Concern:

The work in this chapter is primarily the work of Lena H. Hwang.

Lucius F. Lau, Ellen S. Hwang and Angelika Amon produced Figure 3-2. Dana L. Smith produced the two-hybrid data in Figure 3-1A. Kevin G. Hardwick provided us with antibodies to Mad1 and Mad3.

Lena Hwang produced Figures 3-1B and 3-1C, and Figure 3-3. She demonstrated the interaction of Cdc20 to Mad2 and Mad3 biochemically, isolated and identified the dominant mutants in *CDC20*, showed that these mutants no longer bind Mad2 and Mad3, and identified the mutations in the *CDC20* sequence.

Abstract

The spindle checkpoint regulates the cell division cycle by keeping cells with defective spindles from leaving mitosis. We used the two hybrid system to show that three proteins that are components of the checkpoint, Mad1, Mad2 and Mad3, interact with Cdc20, a protein required for exit from mitosis. Mad2 and Mad3 coprecipitate with Cdc20 at all stages of the cell cycle. The binding of Mad2 depends on Mad1 and that of Mad3 on Mad1 and Mad2. Overexpressing Cdc20 allows cells with a depolymerized spindle or damaged DNA to leave mitosis but does not overcome the arrest caused by unreplicated DNA. Mutants in Cdc20 that are resistant to the spindle checkpoint no longer bind Mad proteins, suggesting that Cdc20 is the target of the spindle checkpoint.

Materials and Methods

Yeast Strains, Media and Genetic Methods

All experiments in this paper were done in strains isogenic to W303. Yeast media and genetic manipulations were as described (Sherman et al.). Hydroxyurea (Sigma, St. Louis, MO) was used at 10mg/ml final concentration in media. α-factor (Bio-synthesis, Lewisville, TX) was used at 1μg/ml for *bar1-* strains and 10μg/ml for *BAR1* strains, from a stock solution at 10mg/ml in DMSO (Aldrich, Milwaukee, WI). Nocodazole (Sigma, St. Louis, MO) was used at 15μg/ml from a stock solution of 10mg/ml in DMSO.

Western Blot Analysis and Immunoprecipitations

Wild type or *cdc26Δ* strains contained the centromeric plasmid pLH68, which contains the *CDC20* gene with a triple hemagglutinin (HA) tag and a six histidine tag at its COOH terminus and the *URA3* gene. Cultures were grown in medium without uracil until mid log phase and then transferred to rich medium without further treatment, treated with 1 mg/ml of alpha factor to induce G1 arrest, with 15 mg/ml of nocodazole to activate the spindle checkpoint, or, in the case of *cdc26Δ* strains, shifted to 37°C to inactivate the APC.

Yeast extracts for immunoblotting and immunoprecipitations were made by bead beating cells 2 times 90 seconds in lysis buffer A

(50mM Hepes pH 7.6, 75mM KCl, 1mM $MgCl_2$, 1mM EGTA, 0.1% NP-40, 50mM NaF, 50mM β-glycerophosphate, 1mM PMSF and LPC (10mg/ml leupeptin, pepstatin, and chymostatin), Boehringer, Indianapolis, IN). The lysates were spun briefly to separate beads from the lysate and then cleared by centrifugation for 5 minutes in an Eppendorf micro-centrifuge at 4°C. This step was repeated 3 times. Protein concentrations of extracts were determined with the Bradford assay using bovine serum albumin as a standard. For immunoblotting, the extracts were adjusted to the same protein concentration and diluted with 2X SDS sample buffer (1X SDS sample buffer: 80mM Tris, pH 6.8, 2% SDS, 10% glycerol, 10mM EDTA, 0.0013% Bromophenol blue, 5% 2-mercaptoethanol). Standard methods were used for SDS-PAGE and protein transfer to nitrocellulose (Harlow and Lane, 1988). Blots were stained with Ponceau S (Fisher, Santa Clara, CA) to confirm transfer and equal protein loading, then blocked for 30 minutes with blotto (4% dried milk, PBS, 0.2% Tween 20). Antibodies were used at 1:1000 for 12CA5 (BABCO, Berkeley, CA), anti-Mad2 (R.H. Chen), and anti-Mad3 (K. Hardwick) at either room temperature for 1 hour or 4°C overnight. Blots were washed 3 times 10 minutes in PBS with 0.2% Tween 20 (PBST) and then incubated with HRP-conjugated anti-rabbit or anti-mouse antibodies (Amersham Corp., Buckinghamshire, UK) at 1:5000 dilution in PBST. They were washed again and developed using Amersham ECL detection reagents following manufacturer's instructions.

For immunoprecipitations, 12CA5 was used at 1:33. Lysates with primary antibody were rotated for 1 hour at 4°C, then transferred to a tube containing Protein A-Sepharose beads (Pharmacia, Pleasant Hill, CA) and rotated at 4°C for an additional hour. The beads were washed 2X with lysis buffer A, transferred to a new tube, and washed again with PBST, then resuspended in SDS sample buffer.

Bypass of Checkpoints by CDC20 Overexpression

The plasmid YIplac211GAL-CDC20 contains the wild-type *CDC20* open reading frame fused to the *GAL1* promoter and was directed to integrate into the *URA3* gene by digestion with NcoI. To examine the spindle and DNA replication checkpoints wild-type cells (A1015), cells carrying two copies of a *GAL-CDC20* fusion (A1016) or *GAL-CDC20 cdc23-1* (A1022) cells were grown to exponential phase and treated with nocodazole (15ug/ml) or hydroxyurea (10 mg/ml) in YEP raffinose at 23°C for 165 min. to induce cell cycle arrest before adding galactose to 2% to induce production of excess CDC20. Samples were taken at the indicated times after galactose addition to determine DNA content and the percentage of cells that had rebudded. To examine the DNA damage checkpoint the *cdc13-1* (A1017), *cdc13-1 GAL-CDC20* (A1018) and *cdc13-1 cdc23-1 GAL-CDC20* (A1023) strains were grown to exponential phase at 23°C and shifted to 33°C for 165 min in YEP raffinose medium before adding galactose. Samples were taken at the indicated times after galactose addition to determine DNA content and the fraction of cells that had rebudded. Viability was measured by adding 15 mg/ml of

nocodazole, 10 mg/ml of hydroxyurea or shifting the temperature to 33°C (for *cdc13-1*) for wild-type, *mad1::URA3* (A928), *mec1-1* (K2888) *cdc13-1,* or *cdc13-1 rad9::URA3* (K2554) strains. For corresponding strains that also contained *GAL-CDC20*, galactose was added to 2% at the time of the other addition or temperature shift. Samples were withdrawn at the indicated times and plated on YEP (yeast extract, peptone) glucose plates. Colonies were counted after 3 days. Induction of *GAL-CDC20* leads to spindle elongation in cells arrested by the *cdc13-1* mutation but not in cells arrested by hydroxyurea treatment (A. Amon, unpublished results, Lim and Surana 1996).

PCR Mutagenesis and Mutant Isolation

The plasmid pCM4 is derived from the *URA3* centromeric plasmid Ycplac33 and contains the wild type *CDC20* gene under the control of its own promoter. The open reading frame, 848 bases upstream, and 232 bases downstream were amplified by mutagenic polymerase chain reaction.

Polymerase chain reaction mixes contained 640pM pCM4 DNA, 10mM Tris-Cl pH8.8, 50mM KCl, 4.76mM MgCl2, 0.1% (v/v) Triton X-100, 56μM dATP, 90μM dCTP, 20μM dGTP, 140μM dTTP, 50pM of each primer (OCM20.5: 5' GACTAGTGGGTGATCTGGTCAGTCG 3' and OCM20.3: 5'CCCCCCGGGATGGTTTCTTCCAAGGCC 3'), 0.5mM MnCl2, and 8 units Taq polymerase in a total volume of 100μl. The reactions were placed through 30 cycles of 94°C for 1 minute, 55°C for 1

minute, and 72°C for 3 minutes, then a final cycle of 72°C for 10 minutes.

The product DNA gel purified and then mixed with a BstEII-SacI fragment from pCM4, which lacks all but 487 bases of the *CDC20* open reading frame, and transformed into the diploid strain DA2050A (*a/a bar1/bar1 mec1-1/mec1-1 CDC28-VF::LEU2/CDC28-VF::LEU2 GAL1-MPS1/GAL1-MPS1*). The *CDC28-VF* mutation blocks adaptation to the spindle assembly checkpoint (K. G. Hardwick, A. D. Rudner, A. W. Murray, unpublished data) whereas homozygozity at *MAT* and the *mec1-1* mutation have no effect on the behavior of checkpoint-resistant mutants. The transformation mix was plated on -URA plates containing 2% galactose. Plasmid DNA was recovered from colonies that grew on these plates, purified and amplified by transformation into *E. coli*, retransformed into DA2050A, and transformants were selected on -URA glucose plates. pCM4 derivatives that carried the checkpoint resistant *CDC20* were isolated.

Checkpoint bypass assays

Microcolony assay

CDC20 mutants were transformed into LH317 (*MATa ade2-1 his3-11,15 leu2-3,112 ura3 TRP1 GAL -MPS1).* The transformants were grown to saturation in -URA glucose medium, diluted 1000 fold, sonicated for 5 seconds to break up cell clumps, and 5 μl were spotted on a YEP 2% galactose plates. The plates were incubated at

30°C for 16 hours and the number of cell bodies plus buds were counted in 100 microcolonies.

Re-budding assay

CDC20 mutants were transformed into a *MATa bar1Δ* derivative of W303. The transformants were grown to saturation in -URA glucose medium, diluted 1000 fold, sonicated for 5 seconds to break up cell clumps, and 5 μl were spotted on a YPD plate containing 10 mg/ml benomyl. Plates were incubated at 16°C for 24 hours, and 100 large budded cells were examined for re-budding.

Rapid death assay

CDC20 mutants transformed into *MATa bar1Δ* were grown to saturation in -URA glucose medium. They were then diluted into YPD and allowed to recover at 23°C for 12 hours. Nocodazole was added to 15 mg/ml and cells were plated for viability on -URA glucose plates at the time of nocodazole addition and 2, 3.25, and 6 hours later.

Sequencing

The location of the mutation responsible for bypass of the spindle assembly checkpoint in *CDC20* was determined by ligating BstEII to BspEI fragments of each mutant into the wild type *CDC20* gene, replacing the wild type sequence. These constructs were tested for

their ability to allow growth of DA2050A (*a/a bar1/bar1 mec1-1/mec1-1 CDC28-VF::LEU2/CDC28-VF::LEU2 GAL1-MPS1/GAL1-MPS1*) on -URA galactose plates. All mutants contained mutations in the BstEII to BspEI region that were capable of conferring growth. This region was sequenced for all of the mutants.

Table 3-1. Yeast Strains

Strain	Relevant Genotype
A1015	*MATa ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1*
A1016	*MATa GAL-CDC20 (2X)*
A1022	*MATa cdc23-1 GAL-CDC20*
A1017	*MATa cdc13-1*
A1018	*MATa cdc13-1 GAL-CDC20*
A1023	*MATa cdc13-1 cdc23-1 GAL-CDC20*
A928	*MATa mad1Δ::URA3*
K2888	*MATa mec1-1*
K2554	*MATa cdc13-1 rad9::URA3*
DA2050	*MATa bar1Δ mec1-1 CDC28-VF::LEU2 GAL-MPS1*
	MATa bar1Δ mec1-1 CDC28-VF::LEU2 GAL-MPS1
LHH353	*MATa bar1Δ pLH68 (CDC20-6XHIS-3XHA)*
LHH359	*MATa bar1Δ mad1Δ::HIS3 pLH68 (CDC20-6XHIS-3XHA)*
LHH360	*MATa bar1Δ mad2Δ::LEU2 pLH68 (CDC20-6XHIS-3XHA)*
LHH361	*MATa bar1Δ mad3Δ::LEU2 pLH68 (CDC20-6XHIS-3XHA)*
LHH362	*MATa bar1Δ cdc26Δ::HIS3 pLH68 (CDC20-6XHIS-3XHA)*
LHH317	*MATa GAL-MPS1 TRP1*
LHH165	*MATa bar1Δ*

Results and Discussion

The spindle checkpoint improves the fidelity of chromosome segregation by delaying anaphase until all chromosomes are correctly aligned on the mitotic spindle (Rudner and Murray 1996, Wells 1996). Mutants in the *MAD* (mitosis arrest deficient) and *BUB* (budding uninhibited by benzimidazole) genes inactivate the checkpoint (Hoyt, et al. 1991, Li and Murray 1991) and overexpressing components of the checkpoint can arrest cells with normal spindles in mitosis (Hardwick, et al. 1996, Kim, et al. 1998, Li, et al. 1997). The checkpoint prevents ubiquitination and destruction of at least two types of protein: the B type cyclins, which activate the protein kinase activity of cyclin dependent kinase (Cdk1, known as Cdc28 in budding yeast and Cdc2 in fission yeast), and a protein required to maintain the linkage of sister chromatids (Pds1 in budding yeast and Cut2 in fission yeast) (Cohen-Fix, et al. 1996, Funabiki, et al. 1996, Hardwick and Murray 1995, Yamamoto, et al. 1996). Ubiquitination is catalyzed by a multiprotein complex called the cyclosome or anaphase promoting complex (APC) (Irniger, et al. 1995, King, et al. 1995, Sudakin, et al. 1995). The reactions that activate the APC are not understood, but cyclin B and Pds1/Cut2 destruction depends on Cdc20 and Hct1/Cdh1, two evolutionarily conserved members of the WD repeat family of proteins. Cdc20 preferentially promotes the destruction of Pds1/Cut2 and Hct1 promotes the destruction of B type cyclins (Schwab, et al. 1997, Visintin, et al. 1997). Unlike *HCT1*, *CDC20* is an essential gene and temperature sensitive *cdc20* mutants arrest in metaphase.

The interaction between Slp1 (the homolog of Cdc20) and Mad2 in fission yeast (Kim, et al. 1998), prompted us to investigate the interaction between Cdc20 and components of the spindle assembly checkpoint in budding yeast. In the two hybrid system, Mad1, Mad2 and Mad3 all show interactions with Cdc20 (Fig. 1A), suggesting that checkpoint proteins bind to Cdc20. We confirmed this suggestion by immunoprecipitating an epitope tagged version of Cdc20 and probing the immunoprecipitates with antibodies to Mad2 and Mad3. We examined four conditions: cells growing asynchronously, cells arrested in G1, cells arrested in mitosis by depolymerization of the spindle with nocodazole, and cells arrested in mitosis by *cdc26Δ*, a mutant that inactivates the APC (Hwang and Murray 1997, Zachariae, et al. 1996). Mad2 and Mad3 were both present in immunoprecipitates from strains carrying epitope tagged Cdc20 (Fig. 1B). We were unable to monitor the physical interaction between Mad1 and Cdc20 because free Mad1 binds to antibody coated beads in some control experiments. The amount of Mad2 and Mad3 precipitated with Cdc20 was highest in cells arrested in mitosis, lower in asynchronous cells, and still lower in cells arrested in G1. The increased association in mitotic cells does not depend on checkpoint activation, since cells arrested by inactivation of the APC showed the same interaction between Cdc20 and Mad proteins as cells arrested in mitosis by spindle depolymerization. We suspect that the different levels of Mad-Cdc20 association between mitotic and G1 cells reflect the level of Cdc20, which is high in mitosis and low in G1 (A. Amon, unpublished data).

Figure 3-1: Association of Mad1, Mad2 and Mad3 with Cdc20.

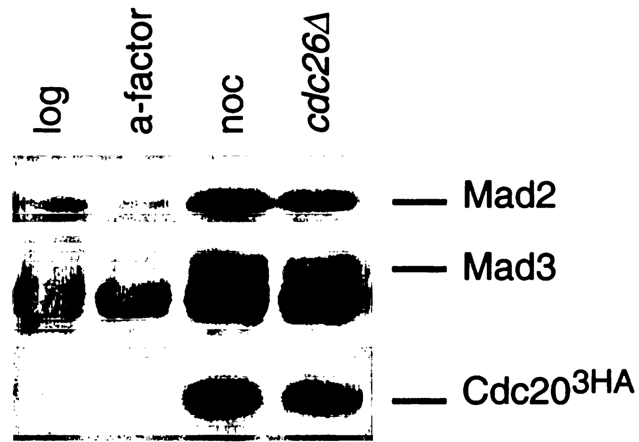
Two-Hybrid association: (A) Haploid strains containing fusions between the transcriptional activation domain and Cdc20 or Snf4 were crossed to strains containing fusions between the DNA binding domain and Mad proteins or Snf1 and the resulting diploids were assayed for β-galactosidase activity. Values are shown in Miller Units and are the average of three independent crosses. The fusions were constructed in the vectors pAS1-CYH2 (DNA binding domain) and pACTII (transcriptional activation domain) (Staudinger, et al. 1993) and all contained the full coding region, except for the Mad1 fusion which contains amino acids 313 to 750 of Mad1. The SNF1 and SNF4 fusions are control fusions to proteins involved in regulating sucrose metabolism. (B) Cell cycle regulation of Mad-Cdc20 interactions. Co-immunoprecipitation of Cdc 20 with Mad2 and Mad3. Strains containing a hemagglutinin (HA) epitope tagged version of Cdc20 were lysed, immunoprecipitated with antibodies to the HA epitope, and the immunoprecipitates were analyzed by protein immunoblotting with antibodies to Mad2, Mad3 or HA. Extracts were from exponentially growing cells, cells arrested in G1 by treatment with alpha factor, cells arrested in mitosis by treatment with nocodazole, or cells arrested in mitosis by the temperature sensitive *cdc26Δ* mutant (Hwang and Murray 1997, Zachariae, et al. 1996). The poly-clonal Mad3 antibodies recognize a background protein directly below the Mad3 protein. (C) Effect of *mad* mutants on Mad-Cdc20 interactions. Exponentially growing *mad1Δ*, *mad2Δ*,

and *mad3Δ* strains containing HA-tagged Cdc20 were lysed, immunoprecipitated with antibodies to the HA epitope, and the immunoprecipitates were analyzed by protein immunoblotting with antibodies to Mad2, Mad3 or HA.

A

BINDING DOMAIN FUSION	ACTIVATION DOMAIN FUSION			
	MAD1	MAD2	MAD3	SNF1
CDC20	13.2	24.5	128.0	0.3
SNF4	0.2	0.2	0.2	3.3

Figure 3-1A



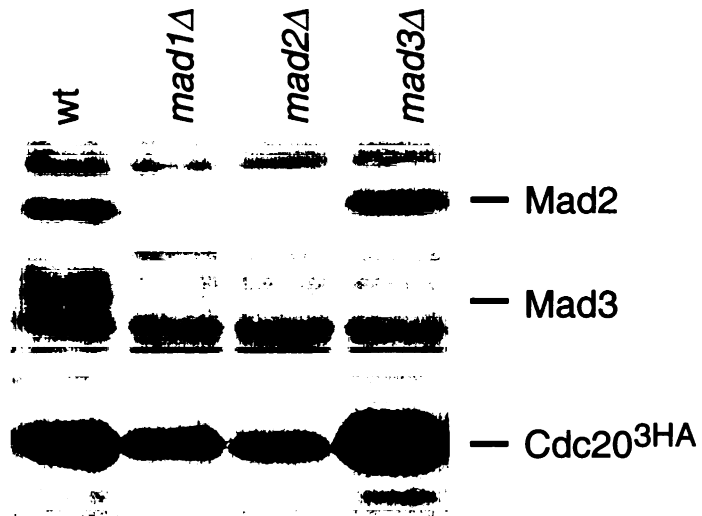


Figure 3-1B, C

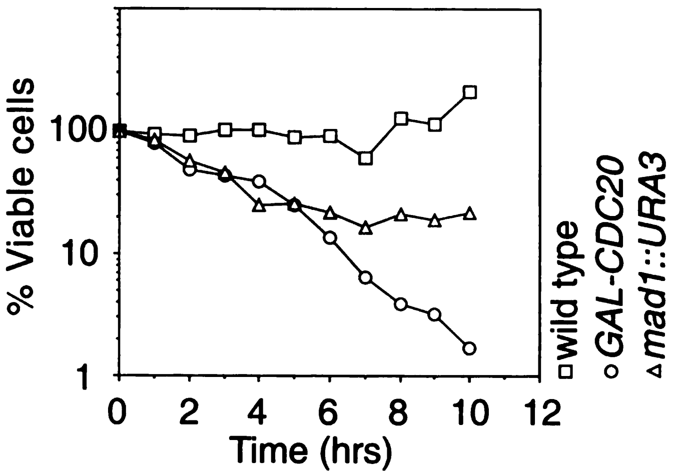
Because two Mad proteins associate with Cdc20, we asked whether they associate with Cdc20 independently or as part of a complex. In strains lacking Mad1, neither Mad2 nor Mad3 are associated with Cdc20; in strains lacking Mad2, Mad3 fails to bind Cdc20; whereas strains lacking Mad3 have unchanged levels of Mad2 associated with Cdc20 (Fig. 1C). Deletion of any one of the three *MAD* genes does not affect the amount of the remaining two Mad proteins(L. H. Hwang and A. W. Murray, unpublished data). These observations suggest that the association of Mad2 and Mad3 with Cdc20 depends on the presence of Mad1. For Mad2, the dependence on Mad1 probably reflects the existence of a Mad1-Mad2 complex, which has been detected in budding yeast (K. G. Hardwick, R.-H. Chen, A. W. Murray, unpublished data). The dependence of Mad3 binding on Mad1 suggests that Mad3 may also form part of this complex, but we cannot exclude the possibility that Mad3 binds to an interface created by the interaction of a Mad1-Mad2 complex with Cdc20. In some experiments, the level of Cdc20 in strains that lacked Mad1 or Mad3 was greater than it was in wild type strains, suggesting that association of Cdc20 with the Mad proteins may regulate the stability or synthesis of Cdc20.

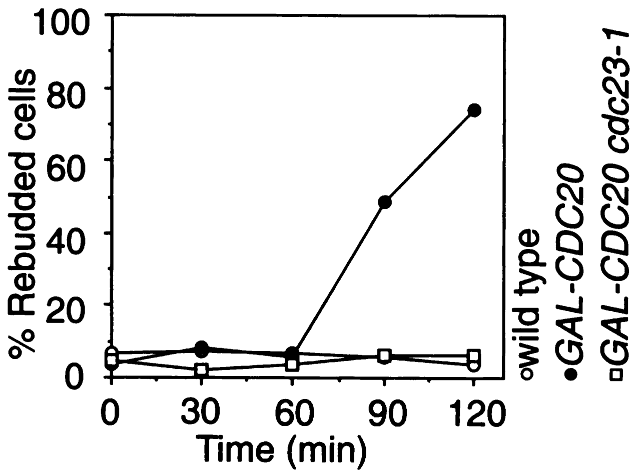
The association of Mad proteins with Cdc20 suggests that Cdc20 is the target for the spindle checkpoint. We therefore tested whether overexpressing Cdc20 overcame any cell cycle checkpoints. Cells overexpressing Cdc20 from the *GAL1* promoter are defective in two checkpoints: they no longer arrested in mitosis in response to spindle depolymerization (Fig. 2A) or to the DNA damage caused by the *cdc13-1* mutation (Fig. 2B and Lim and Surana 1996).

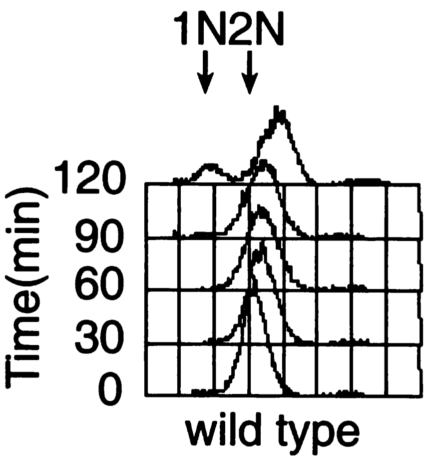
Figure 3-2: Cdc20 overexpression overcomes the spindle and DNA damage checkpoints.

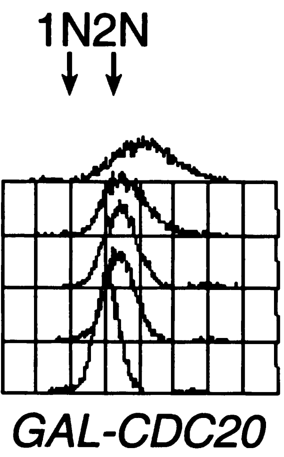
The indicated strains were subjected to (A) spindle depolymerization by nocodazole, (B) DNA damage caused by shifting the *cdc13-1* mutant to 33°C, or (C) inhibition of DNA replication by hydroxyurea. For measurement of rebudding and DNA content (morphological and biochemical criteria for exit from mitosis) cells were grown in raffinose-containing medium, arrested by drug or temperature treatment for 165 minutes, and then treated with 2% galactose to induce expression of Cdc20 from the *GAL1* promoter. Samples were taken at the indicated times after galactose addition. Arrows indicate the 1N DNA content of G1 cells and 2N content of G2 cells. The slow rightward shift of the DNA peak in nocodazole treated *cdc23-1* cells during the experiment is due to mitochondrial DNA replication. For measurements of cell viability, galactose was added to cells growing exponentially in raffinose containing medium at the same time that nocodazole or hydroxyurea was added or the cells were shifted to 33°C. At the indicated times, cells were removed and tested for their ability to give rise to colonies on glucose-containing medium.

A) Spindle checkpoint









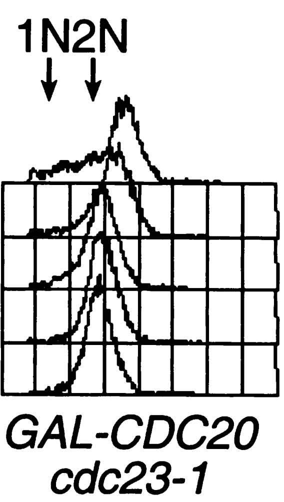


Figure 3-2A

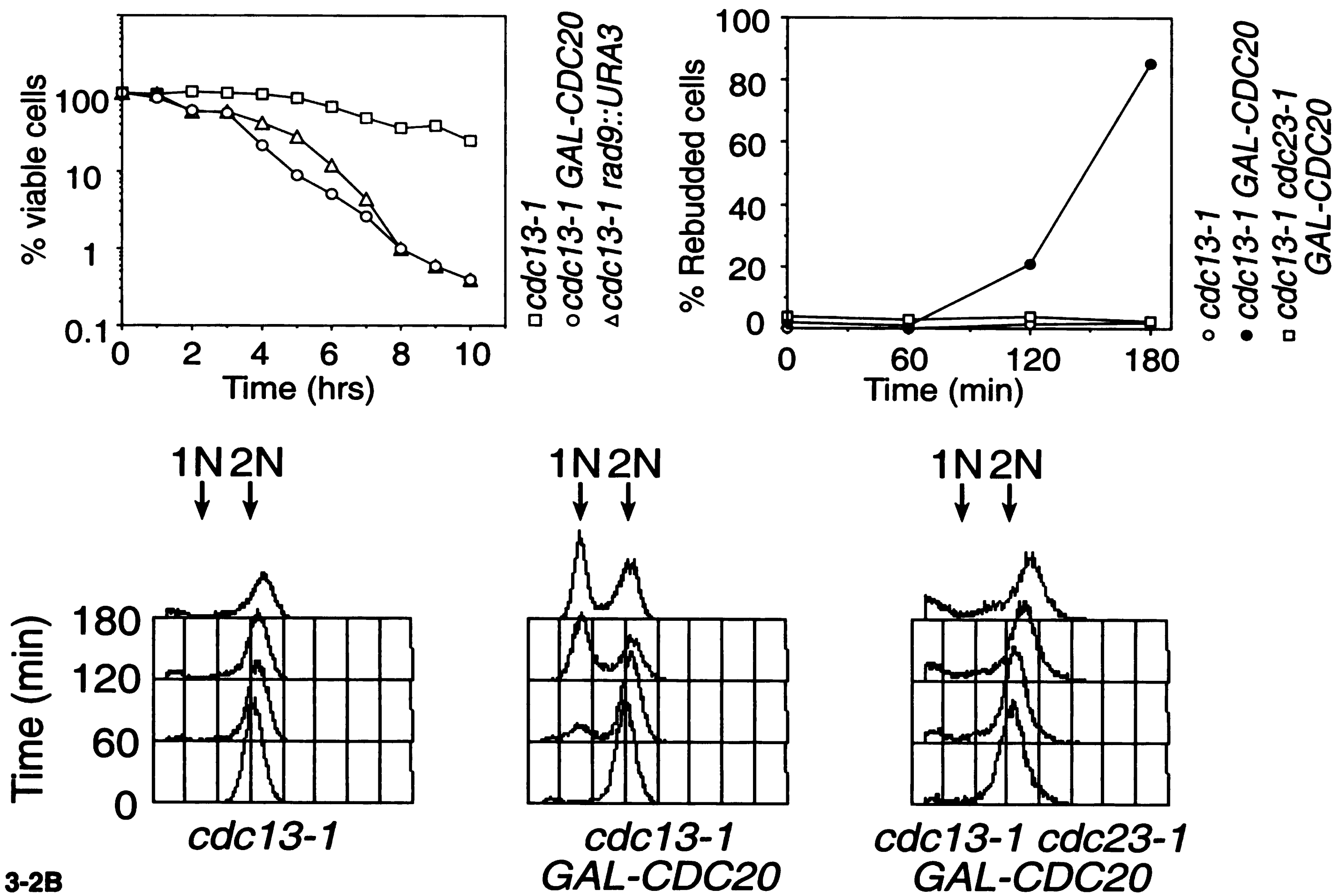


Figure 3-2B

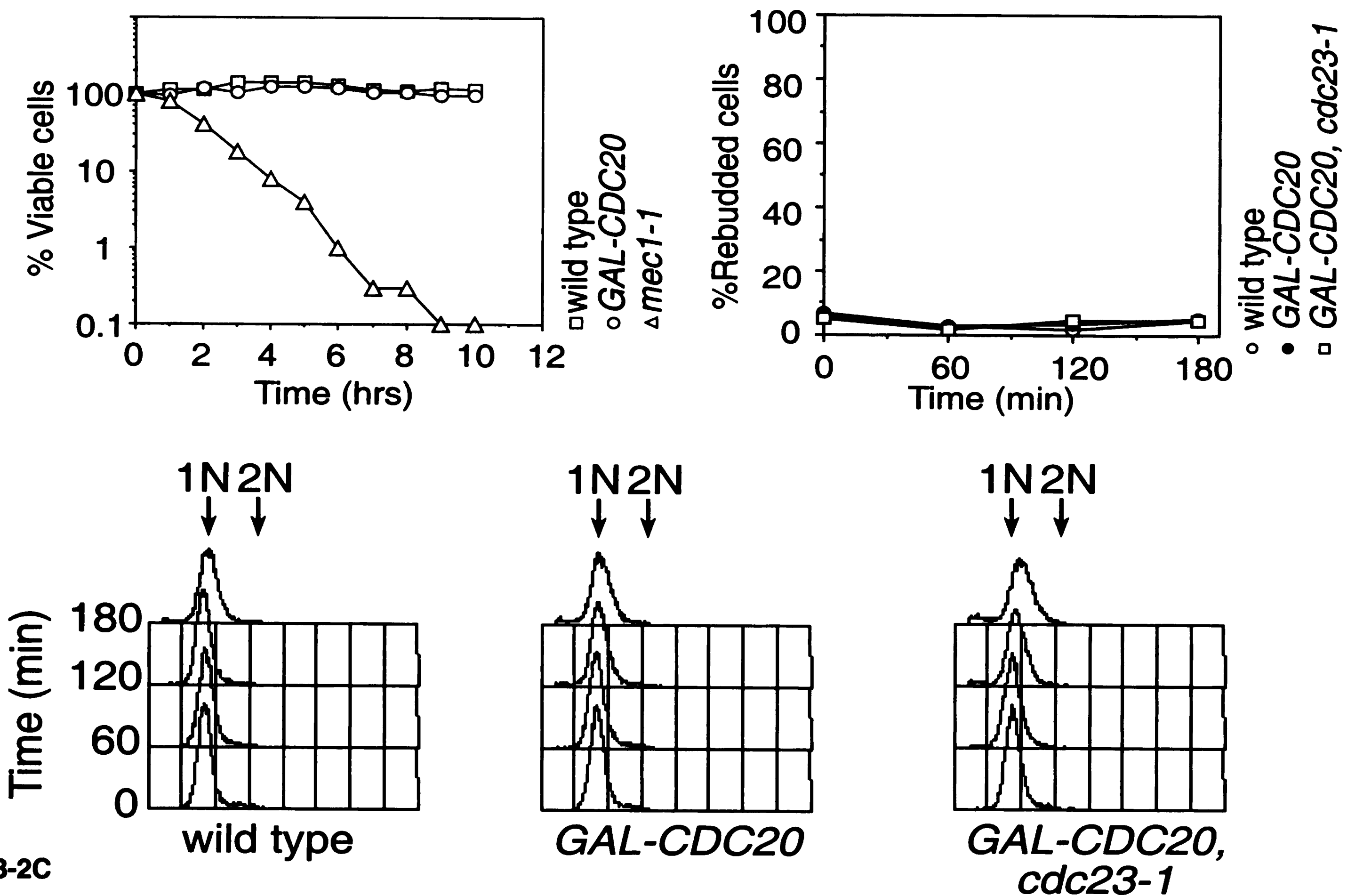


Figure 3-2C

Overexpression of Cdc20 bypasses checkpoints by activating the APC, since combining the *cdc23–1* mutation, which disrupts APC activity, with *GAL-CDC20* restores the mitotic arrest to cells with damaged spindles or DNA . Overexpressing Cdc20 does not overcome the cell cycle arrest caused by hydroxyurea, an inhibitor of DNA synthesis (Fig. 2C). These results are consistent with the idea that the spindle and DNA damage checkpoints arrest cells in mitosis by inhibiting Cdc20 and suggest that the DNA replication checkpoint uses another mechanism to arrest the cell cycle.

If Cdc20 is the target of the spindle checkpoint, it should be possible to isolate dominant mutations in Cdc20 that no longer respond to the checkpoint. Overexpression of *MPS1*, a component of the checkpoint, arrests cells in mitosis even though their spindle is still fully functional (Hardwick, et al. 1996). We mutagenized the *CDC20* gene, selected for mutants that overcame the mitotic arrest caused by Mps1 overexpression, and analyzed four of these mutants in detail. Mutant forms of Cdc20, like *mad* and *bub* mutants (Hardwick, et al. 1996), allow cells overexpressing Mps1 to proliferate more rapidly than control cells (Fig. 3A). The Cdc20 mutants also increase the rate at which cells exit mitosis and die after treatment with nocodazole (Fig. 3B). The checkpoint resistant Cdc20 mutants also weaken the DNA damage checkpoint although this effect is somewhat variable (L. H. Hwang and A.W. Murray, unpublished data). The checkpoint resistant mutants complement the temperature-sensitive growth defect of *cdc20-1* strains. In the cdc20-*1* strains that carry the mutant plasmids, the spindle checkpoint is inactive at 37°C, demonstrating that the checkpoint

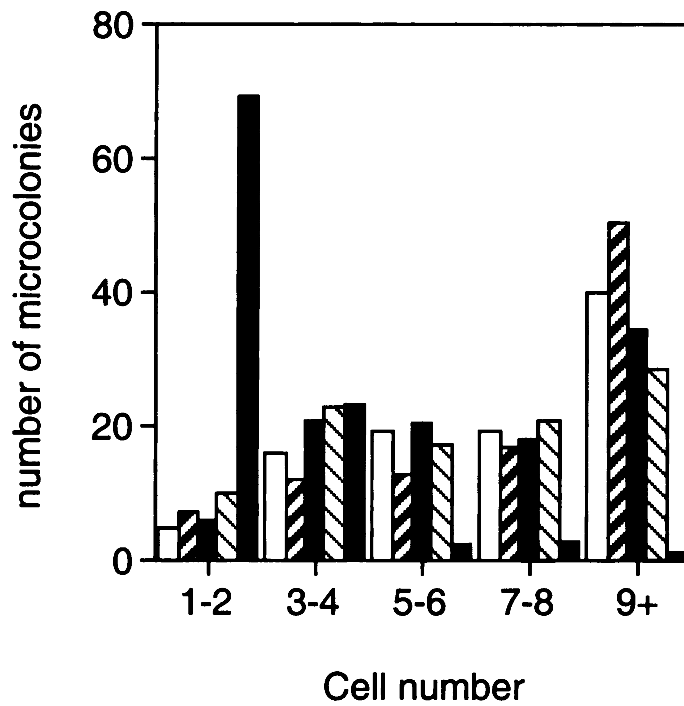
defect is not due to a dominant negative activity of the Cdc20 mutants (L. H. Hwang and A.W. Murray, unpublished data). The checkpoint resistant Cdc20 mutants greatly diminish the binding of Mad2 and Mad3 to Cdc20 (Fig. 3C) but have no effect on the overall level of Mad1, Mad2, or Mad3 in the cell (21). For all four mutants, the checkpoint-resistant phenotype is conferred by the mutations in the region of Cdc20 that Kim et al showed was required for interaction with Mad2 in fission yeast (Kim, et al. 1998). Sequencing this region revealed that all the mutants had changes in a short region of Cdc20 that corresponds to the region that contains the checkpoint-resistant mutation in Slp1 (Fig 3 D). Hoyt and his colleagues have also isolated a dominant allele of *CDC20* that bypasses the spindle assembly checkpoint but not the DNA damage checkpoint . It is not known if this mutant affects the interaction between Cdc20 and the Mad proteins.

The idea that the spindle checkpoint specifically inhibits Cdc20 is strengthened by comparing the sequences of Cdc20 and Hct1. These proteins have homologs in fission yeast, plants and animals and probably conserve the functional difference between Cdc20 and Hct1: Cdc20 is required for the proteolysis that drives the metaphase/anaphase transition and Hct1 is required for cyclin B proteolysis during G1 (Schwab, et al. 1997, Visintin, et al. 1997). Members of the Cdc20 family have conserved the region defined in fission yeast as the Mad2 interaction region (Kim, et al. 1998), but members of the Hct1 family lack this homology. It has been suggested that Mad2 inhibits exit from mitosis by binding directly to the APC (Li, et al. 1997) but we have been unable to detect binding

Figure 3-3: Dominant Cdc20 mutants that overcome the spindle checkpoint

(A) Cdc20 mutants proliferate despite overexpression of Mps1. Strain LH317 (*MATa ade2-1 his3-11,15 leu2-3,112 ura3 TRP1 GAL-MPS1)* containing centromeric plasmids with wild-type or mutant *CDC20* were grown to saturation and diluted onto galactose-containing plates. After 16 hours, the number of cell bodies plus buds in 100 microcolonies was counted. (B) Cdc20 mutants rebud and die rapidly when treated with nocodazole. Exponentially growing cells of a *MATa bar1Δ* derivative of W303 containing centromeric plasmids with wild type or mutant *CDC20* were treated with 15 mg/ml of nocodazole for 0, 2, 3.25 and 6 hours and then plated for viability. The values are expressed as a percentage of the viability before nocodazole addition. (C) Checkpoint resistant *CDC20* mutants have diminished binding of Mad2 and Mad3. Exponentially growing cultures containing HA-tagged wild type or mutant *CDC20* were lysed, lysates were immunoprecipitated with antibodies to the HA epitope, and the immunoprecipitates were analyzed by Western blotting with antibodies to Mad2, Mad3, or HA. (D) Sequence changes in dominant, checkpoint resistant *CDC20* alleles. The figure shows the changes from the wild type sequence in four mutants and the comparison with the sequence of Slp1 (the fission yeast homolog of Cdc20) and the checkpoint resistant Slp1 mutant described by Kim et al (Kim, et al. 1998). The *CDC20-120* allele also includes two changes outside this region, T226S and T247I and we do not know if these changes are required to confer the mutant phenotype.

A) MPS1 overexpression



■ Wild Type *CDC20*

□ *CDC20-106*

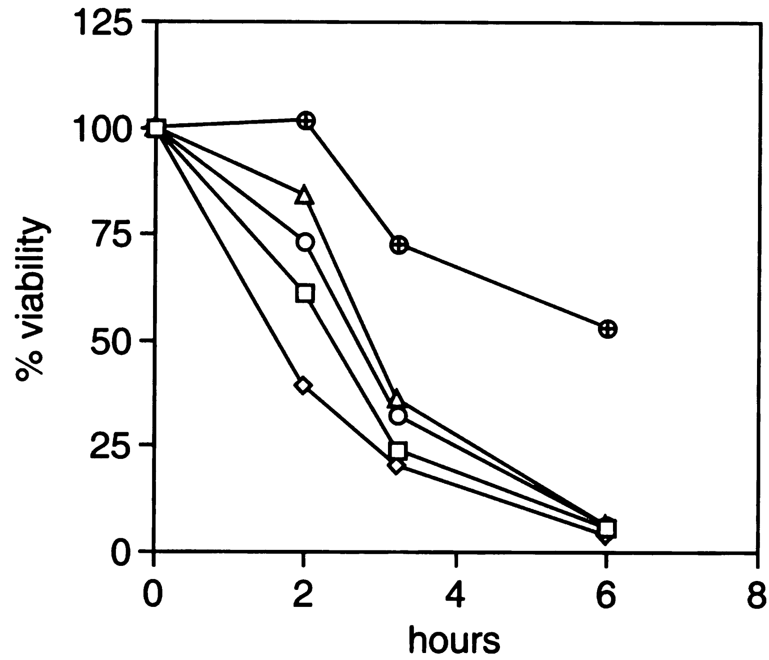
▨ *CDC20-107*

■ *CDC20-120*

▧ *CDC20-127*

Figure 3-3A

B) Spindle depolymerization



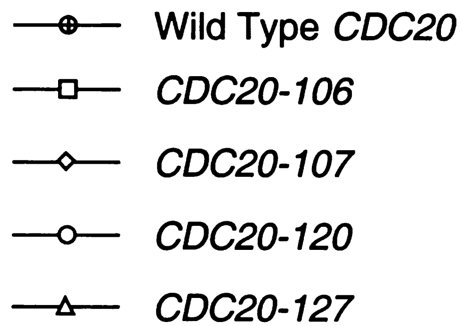


Figure 3-3B

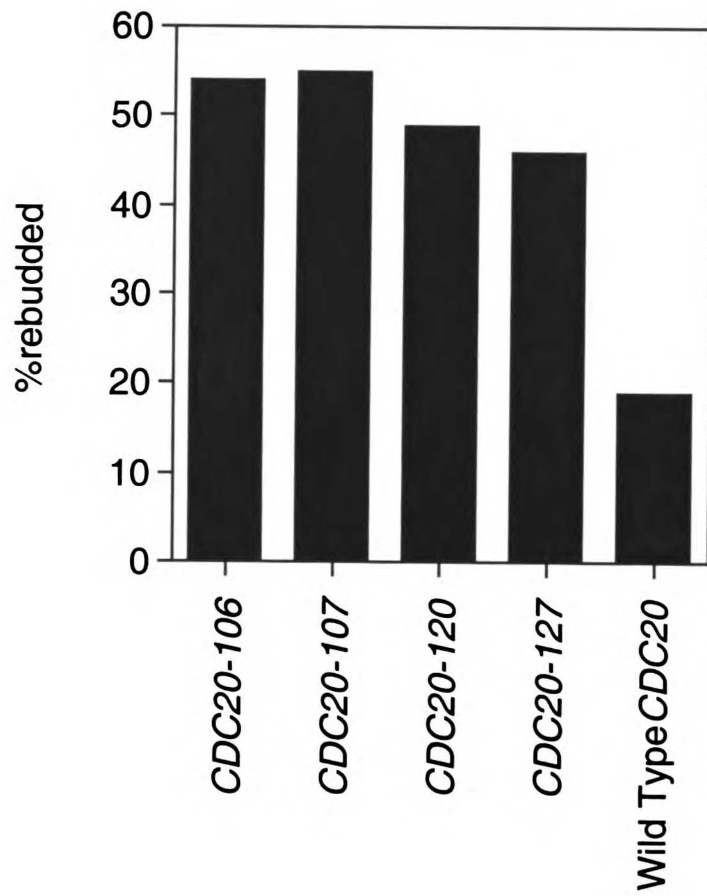


Figure 3-3B

Figure 3-3C

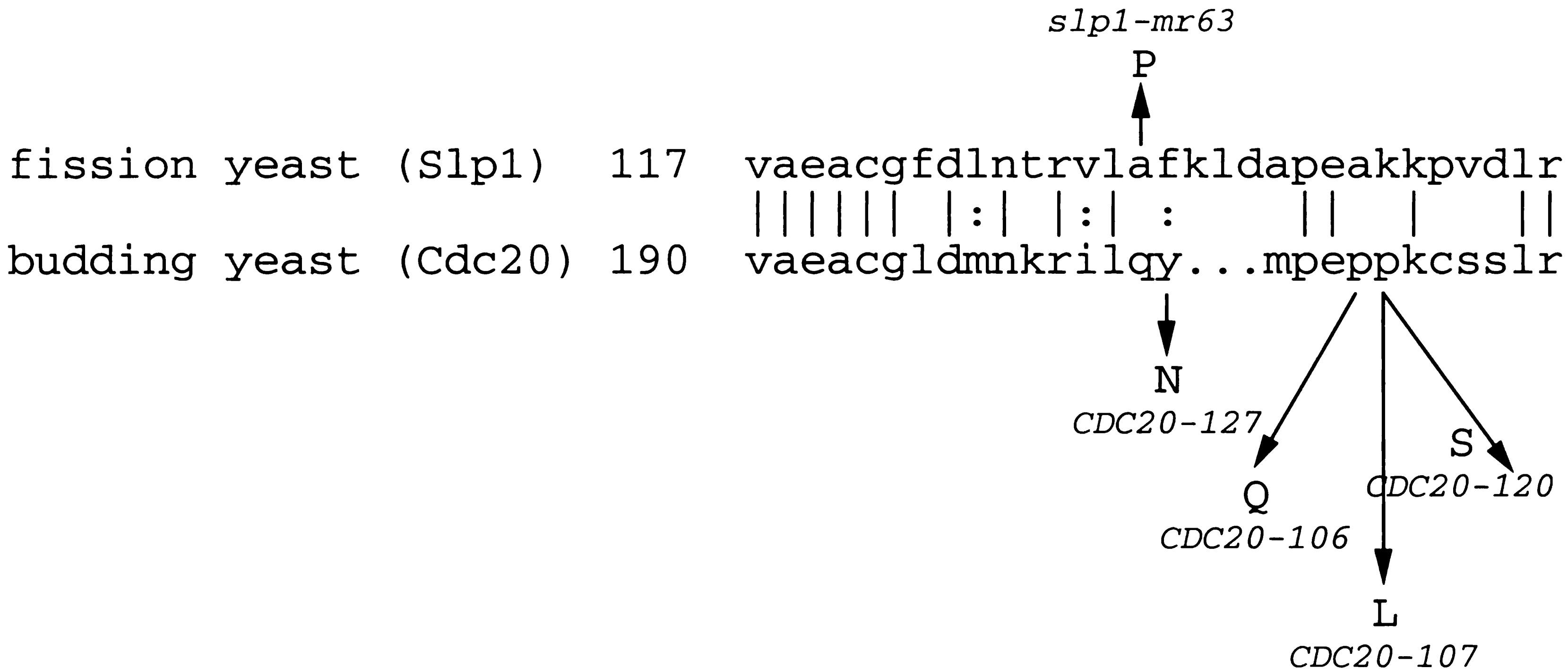


Figure 3-3D

of Mad2 to the APC in yeast (L. H. Hwang and A.W. Murray, unpublished data).

We do not understand how the checkpoint regulates Cdc20 activity. Although Mad2 and Mad3 are bound to Cdc20, this association does not change on activation of the checkpoint. Perhaps modification of one of the Mad proteins converts it into a form that can inhibit as well as bind to Cdc20. Although we have not detected modification of Mad2 and Mad3, Mad1 becomes hyperphosphorylated on activation of the checkpoint (Hardwick and Murray 1995). An alternative possibility is that other, undiscovered proteins, show checkpoint-dependent binding to Mad2 or Mad3 and inhibit Cdc20 activity.

Overexpression of Cdc20 and dominant Cdc20 mutants interfere with both the DNA damage and spindle checkpoints. The role of Cdc20 in the DNA damage checkpoint is independent of the Mad proteins since *mad* mutants have an intact DNA damage checkpoint (K. G. Hardwick, C. A. Mistrot, A. W. Murray, unpublished data). We speculate that in all eukaryotes the spindle checkpoint prevents the onset of anaphase by inhibiting Cdc20. In contrast, inhibition of mitotic exit by the DNA damage checkpoint is likely to be confined to organisms like budding yeast that lack a clearly defined transition between G2 and mitosis. In animals and fission yeast, DNA damage can arrest cells in G2 thus keeping them from entering mitosis and condensing their chromosomes. In budding yeast, the mitotic spindle assembles normally in cells with damaged or unreplicated DNA. Thus the checkpoints that detect these defects must be able to prevent the exit from rather than the entry into

mitosis. The budding yeast Pds1 protein may be involved in this evolutionary shift in the target of the DNA damage checkpoint. Destruction of Pds1 and its fission yeast homolog (Cut2) are required to separate sister chromatids (Cohen-Fix, et al. 1996, Funabiki, et al. 1996), and in budding yeast Pds1 is required for the DNA damage checkpoint in G2 (Cohen-Fix and Koshalnd 1997, Yamamoto, et al. 1996). One explanation for this dual requirement is that Pds1 acts as both a substrate and an inhibitor of the APC. In undamaged cells, Pds1 binding to the activated APC would partially inhibit APC activity, but because the APC can target Pds1 for destruction, cells could rapidly escape this inhibition. DNA damage would induce modifications of Pds1 that increased its ability to inhibit the APC. As a consequence, Pds1 and mitotic cyclins would be stable, sister chromatids would not separate, and cells would remain in metaphase. This model explains why an indestructible form of Pds1 arrests budding yeast in metaphase. A similar mutation in Cut2, the fission yeast homolog of Pds1, prevents sister chromatid separation but does not prevent cyclin B destruction and the exit from mitosis. This difference correlates with the different organization of the cell cycle in the two yeasts.

Acknowledgments

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for valuable discussions. We are especially grateful to T. Matsumoto for encouragement, helpful discussions and for communicating unpublished results. Supported by a National Science Foundation Fellowship (L. H. H.) a grant from N.I.H (A. A.) and grants from N.I.H., the March of Dimes, and the David and Lucile Packard Foundation (A. W. M).

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Chapter 4

The Development of a Reconstituted Assay for APC Activity in *S. cerevisiae*

The regulation of APC activity in *S. cerevisiae*: Development of an APC assay

Lena H. Hwang and Andrew W. Murray

Abstract

The exit from mitosis is induced by the activation of the anaphase promoting complex (APC). It has recently been shown that the spindle assembly checkpoint inhibits exit from mitosis by inhibiting Cdc20, an activator of the APC. In order to study the regulation of the APC by the spindle assembly checkpoint, we developed a purified, reconstituted assay to measure APC activity in *Saccharomyces cerevisiae*. The APC assay appears to reflect Hct1/Cdh1-dependent APC activity and not Cdc20-dependent activity. Modifications to the assay are required to study Cdc20-associated APC activity and regulation of that activity by the spindle assembly checkpoint.

Introduction

Exit from mitosis is driven by the targeted degradation of key regulatory proteins. These include the mitotic cyclins and proteins that inhibit sister chromatid separation, Cut2 in fission yeast and Pds1 in budding yeast (reviewed in King, et al. 1996).

A short amino-terminal sequence on each of these proteins, called the destruction box (King, et al. 1996), targets them for ubiquitin-mediated proteolysis (reviewed in Ciechanover 1994). This process utilizes four factors, a ubiquitin-activating enzyme (E1), a ubiquitin-carrier protein (E2), a ubiquitin-protein ligase (E3), and the 26S proteosome. The E1 activates ubiquitin through the hydrolysis of ATP, forming a thiol ester with ubiquitin. The ubiquitin is then transferred to an E2, and the E2 either directly ubiquitinates the substrate or utilizes an E3 to help transfer the ubiquitin onto the target protein. The ubiquitin of the ubiquitin-substrate complex itself then becomes a substrate for this process and a multi-ubiquitin chain is formed onto the target protein. This multi-ubiquitin chain targets the protein to the 26S proteosome for destruction.

In budding yeast there is a single major E1, encoded by *UBA1* (Dohmen, et al. 1995). The E2 for mitotic cyclins and Pds1 has not been definitively determined, although homologues of *UBC4* and *UBCX* are capable of acting *in vitro* as the E2 for cyclin in other systems (King, et al. 1995, Sudakin, et al. 1995). The E3 was identified in clam and frog egg extracts as a large 20S complex called

the cyclosome or anaphase promoting complex (APC) (King, et al. 1995, Sudakin, et al. 1995). In frogs, the APC contains eight subunits: *CDC16, CDC23, CDC27, BIME, APC2, APC4, APC5*, and *APC7* (King, et al. 1995, Peters, et al. 1996, Yu, et al. 1998). This complex has also been found in yeast with four additional subunits: Apc9, Doc1, Apc11, and Cdc26 (Hwang and Murray 1997, Irniger, et al. 1995, Zachariae and Nasmyth 1996, Zachariae, et al. 1998, Zachariae, et al. 1996).

Recently, accessory factors for the APC have been identified that appear to be possible substrate and cell cycle specific activators of the APC. These factors form a well conserved family of WD-40 repeat proteins, Cdc20 and Hct1/Cdh1 in budding yeast (Schwab, et al. 1997, Visintin, et al. 1997), Fizzy and Fizzy-related in *Drosophila* (Sigrist, et al. 1995, Sigrist and Lehner 1997), Slp1 and Srw1 in fission yeast (Kim, et al. 1998), and X-FZY and X-FZR in *Xenopus* (Lorca, et al. 1998).

Cdc20 and Hct1/Cdh1 have been shown to be limiting, substrate specific activators of the APC. Over-expression of *CDC20* causes the destabilization of Pds1 in logarithmically growing cells or cells arrested in S-phase (Visintin, et al. 1997). Over-expression of *CDH1* in these conditions destabilizes Clb2 (the major mitotic cyclin) (Schwab, et al. 1997, Visintin, et al. 1997). However, the substrate specificity of both accessory factors appears to be lost in mitosis, or the expression of one of the factors activates the other. Over-expression of either protein in mitosis destabilizes both Clb2 and Pds1.

Human CDC20 and CDH1 have been shown to bind directly to the APC and are capable of activating both interphase and mitotic

APCs when present in excess (Fang, et al. 1998). Interestingly, human CDC20 confers a strict requirement for a destruction box, whereas hCDH1 has a much more relaxed specificity for the destruction box.

Recent work has identified Cdc20 as the target of the spindle assembly checkpoint, a checkpoint that monitors proper assembly of the mitotic spindle (Hwang, et al. 1998, Kim, et al. 1998). In response to defects in the spindle or mis-attached chromosomes, the checkpoint arrests or delays the cell cycle to allow time for repair and proper attachment (reviewed in Rudner and Murray 1996). It had been hypothesized that the checkpoint delayed progression through mitosis by inhibiting the activity of the APC. Identification of Cdc20 as the target of the checkpoint supported that hypothesis. However, to directly examine the regulation of the checkpoint on the activity of the APC, a reconstituted assay for APC activity was required.

The ubiquitination of cyclins has been reconstituted *in vitro* in clam and frog egg extracts. In clam egg extracts, partially purified fractions provided the non-specific E1, a specific E2 activity (E2-C), and a large mitosis specific complex that acts as an E3, the cyclosome (Sudakin, et al. 1995). In frog egg extracts, recombinant human E1 and E2 (hUBC4) are capable of reconstituting cyclin ubiquitination activity with either a purified fraction from mitotic extracts or with immunoprecipitates of the E3 using antibodies against components of the anaphase promoting complex (King, et al. 1995).

Ubiquitination of cyclins in yeast had been examined using cell free extracts. Using this system, the APC is inactive in extracts from

cells arrested due to activation of the spindle assembly checkpoint (Zachariae and Nasmyth 1996). It is unclear, however, if the APC is itself inactive, or if a soluble inhibiting activity is present in the extract. In order to study the inhibition of the APC by the spindle checkpoint, we developed a purified, reconstituted assay for APC activity.

Materials and Methods

Yeast Strains, Plasmids and Media

All yeast strains are derivatives of W303 unless otherwise noted. Standard yeast media was used. JD490, a yeast strain expressing *CUP1-UBA1-6XHIS* was provided by Jurgen Dohmen (University of Dusseldorf). A recombinant bacterial expression plasmid for *UBC4* was provided by Vincent Chao.

Alpha-factor (Bio-synthesis, Lewisville, TX) was used at 1μg/ml from a stock solution at 10 mg/ml in DMSO (Aldrich, Milwaukee, WI). Nocodazole (Sigma, St. Louis, MO) was used at 15μg/ml from a stock solution of 10 mg/ml in DMSO.

Purification of Uba1

JD490 was grown overnight in media lacking leucine at 30°C. The overnight culture was diluted and grown to logarithmic phase in 1 liter of media lacking leucine and then resuspended in 2 liters of YPD and grown for 2 hours at 30°C. The CUP1 promoter was induced with 250μM $CuSO_4$ (final concentration) for 4 hours. Cells were harvested and washed twice in PBS then resuspended in a small volume of PBS and frozen in liquid nitrogen. Extracts were made by grinding frozen cells with liquid nitrogen in a mortar and pestle for 30 minutes and resuspending in lysis buffer A (50mM Hepes pH 7.6,

75mM KCl, 1mM $MgCl_2$, 1mM EGTA, 0.1% NP-40, 50mM NaF, 50mM β-glycerophosphate, 1mM PMSF and LPC (10 mg/ml leupeptin, pepstatin, and chymostatin), Boehringer, Indianapolis, IN). The extract was cleared by spinning in a TL100.3 at 45K for 1 hour.

Uba1-6XHis was purified by passing the extract over a Co^{2+} column that had been equilibrated in lysis buffer B (lysis buffer A lacking $MgCl_2$ and EGTA). The column was then washed with 5 column volumes of wash buffer (lysis buffer B containing 300mM NaCl and 10% glycerol), followed by 5 column volumes of wash buffer including 10mM imidazole, and then 3 column volumes of wash buffer with 20mM imidazole. The protein was eluted with 5 column volumes of elution buffer (wash buffer with 100mM imidazole), taking 1ml aliquots. Peak fractions were combined and placed over a PD-10 column that had been equilibrated with QA (20mM Tris-HCl pH7.6, 100mM KCl, 1mM $MgCl_2$, 1mM DTT). Protein was eluted using QA.

In Vitro Ubiquitination Assay

Yeast extracts were prepared by bead beating cells in lysis buffer A in a BioSpec Beadbeater 8. The APC was immunoprecipitated from 125 to 625 μg of yeast lysate with monoclonal 12CA5 antibodies (which recognize the hemagglutinin tagged Cdc16 or Cdc27) or polyclonal antibodies to Cdc26. 12CA5 and anti-Cdc26 antibodies were used at a 1:400 dilution with 5 μl of Protein A-Sepharose beads (Pharmacia) for each reaction.

Immunoprecipitates were washed twice with lysis buffer A, twice with high salt QA (QA with 250mM KCl), and twice with QA. After the final wash, 3.5 pmol Uba1, 47 pmol Ubc4, 0.25 to 0.5 μl ^{125}I labeled sea urchin cyclin B^{12-91}, 1 nmol bovine ubiquitin (Sigma), and 1 mM ATP were added to the beads in a reaction volume of 15 μl. The reaction was incubated at 25°C for 15 minutes and the ubiquitin conjugates were resolved on a 7.5-15% gradient gel. The conjugates were visualized by autoradiography using BioMaxMS (Kodak).

Extract mixing experiments were performed by preparing extracts as described above. Extracts from cells arrested with either α-factor or nocodazole were mixed in varying ratios starting with 625μg of extract containing hemagglutinin tagged protein to 625μg, 1250μg, or 3125μg of extract lacking the tagged protein. The APC from mixed extracts was immunoprecipitated as described above for 2 hours at 4°C, and assayed for APC activity.

Inhibition of partially purified APC was determined by immunoprecipitating the APC as above, washing three times with lysis buffer A, and then incubating the immunoprecipitate with additional extract for 5 minutes at 25°C. The immunoprecipitate was washed twice with lysis buffer A, twice with high salt QA, and twice with QA. The APC assay was performed as described above and the activity of active APC incubated with lysis buffer, α-factor extract or nocodazole extract was compared.

Table 4-1. Yeast Strains

Strain	Genotype	Source
LHH105	MATa bar1Δ ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100	This work
LHH311	MATa bar1Δ pWAM10 (CDC16HA)	This work
LHH233	MATa bar1Δ pJL25 (CDC27HA)	This work
LHH207	MATa cdc26Δ::URA3	This work
LHH205	MATa bar1Δ pLH36 (GAL-HACDC20)	This work
JD490	MATa CUP1-UBA1-6HIS	J. Dohmen

All strains from this work are isogenic to W303 (R. Rothstein).

Results

An assay for APC activity in yeast

We developed a reconstituted assay for ubiquitination of mitotic cyclins to study the regulation of this process in budding yeast. Uba1, the major E1 in *S. cerevisiae* was purified from strains over-expressing a 6-His tagged version of the protein. A recombinant yeast E2, Ubc4 was purified from bacteria, and the E3 was purified by immunoprecipitating the APC from yeast extracts.

It has been shown in other systems that the E1 and E2 for mitotic cyclins are not regulated through the cell cycle. Only the E3 appears to regulated, exhibiting activity in mitosis, but not in interphase (King, et al. 1995, Sudakin, et al. 1995).

In yeast, Amon *et al.* elegantly demonstrated that mitotic cyclin destruction is turned on in late mitosis and remains on into G1 until the G1 cyclin Cdk has been activated (Amon, et al. 1994). Mitotic cyclins are therefore unstable in α-factor arrested cells. Extracts from strains expressing hemagglutinin (HA) tagged components of the APC, either *CDC16* or *CDC27*, were made from cells that had been arrested in early G1 using α-factor. The APC was immunoprecipitated onto Protein A beads and washed extensively, and the assay was performed on these beads. Purified Uba1, Ubc4, ubiquitin, and ATP were added to the beads. In addition, the substrate used was an iodinated amino terminal fragment of sea urchin cyclin B (^{125}I-CycB$^{12\text{-}91}$) that contains the cyclin destruction box (Holloway, et al. 1993).

Figure 4-1 shows that all of the components of the assay are required for ubiquitination of the cyclin B substrate. If all components are present, three higher molecular weight conjugates of the cyclin B substrate are evident. In this figure, the first conjugate is masked by the high signal of un-conjugated substrate. The conjugates form an approximately 7kD ladder, indicative of ubiquitination. There is a low level of ubiquitination in the absence of ATP (Figure 4-1, lane 5). This is likely due to the purification of activated Uba1-ubiquitin during the isolation of Uba1 from yeast. The activation of ubiquitin is the only ATP requiring step in the ubiquitination of proteins.

APC activity during the spindle assembly checkpoint

It has long been hypothesized that the spindle assembly checkpoint arrests cells in mitosis by inhibiting the destruction of mitotic cyclins and Pds1. To determine if the APC is active against mitotic cyclins when cells are arrested due to activation of the spindle assembly checkpoint, we immunoprecipitated the APC from cells that had been treated with nocodazole to activate the checkpoint. While the APC immunoprecipitated from α-factor arrested cells is highly active, the APC immunoprecipitated from spindle checkpoint arrested cells is inactive (Figure 4-2A).

We then examined whether the inhibiting activity of the spindle assembly checkpoint was dominant to an active APC by mixing extracts from α-factor arrested cells that expressed hemagglutinin tagged Cdc27 with extracts from nocodazole treated

Figure 4-1. Purified components reconstitute ubiquitination of cyclin B substrate.

Purified yeast Uba1, recombinant yeast Ubc4, bovine ubiquitin, ATP, and immunopurified APC reconstitute the ubiquitination of sea urchin ^{125}I-cyclin B (amino acids 12-91). The APC was immunoprecipitated from a strain containing a hemagglutinin tagged *CDC27* (LHH233) that had been arrested in G1 with α-factor (lanes 2-5, 7) or mock immunoprecipitated from a strain lacking the tagged construct (LHH105, lanes 1 and 6). The immunoprecipitates were washed and incubated with 3.5 pmol Uba1, 47 pmol Ubc4, 1 nmol bovine ubiquitin (Sigma),1 mM ATP, and 0.5μl ^{125}I labeled sea urchin cyclin B^{12-91} as indicated above in a final reaction volume of 15 μl. The reactions were incubated at 25°C for 15 minutes and the ubiquitin conjugates were resolved on a 7.5-15% gradient gel. The positions of conjugates are marked. Molecular weight markers are indicated in kilodaltons.

	1	2	3	4	5	6	7
Uba1	-	-	+	+	+	+	+
Ubc4	-	+	-	+	+	+	+
Ubiquitin	-	+	+	-	+	+	+
ATP	-	+	+	+	-	+	+
APC	-	+	+	+	+	-	+
^{125}I-Cyc B (12-91)	+	+	+	+	+	+	+

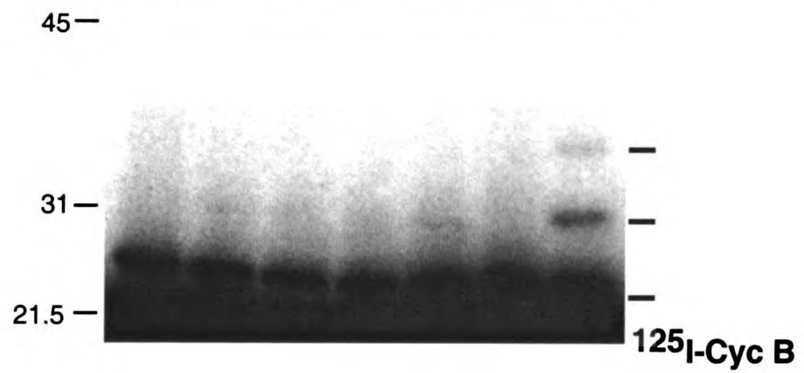


Figure 4-1

cells that lacked a tagged version of Cdc27. The α-factor extract was mixed with increasing ratios of the nocodazole extract (by protein concentration) and incubated at 4°C for 2 hours. The APC was then immunoprecipitated using antibodies to the hemagglutinin tag. An equal amount of nocodazole extract could strongly inhibit an active G1 APC, and five fold excess of a nocodazole extract could completely inhibit the APC (Figure 4-2B, lanes 1-5). Mixing α-factor extracts with lysis buffer or additional α-factor extract had no effect on activity (data not shown). To determine if an APC in a spindle checkpoint arrested extract could be activated by an α-factor extract, we did the reciprocal experiment by mixing extracts from nocodazole treated cells that expressed hemagglutinin tagged Cdc27 with α-factor extracts that did not. Even a five fold excess of α-factor extract was incapable of activating the APC in a nocodazole treated extract (Figure 4-2B, lanes 6-10).

To determine if the inhibitory activity of the spindle checkpoint could inhibit an purified, active APC, we first isolated an active APC from G1 cells by immunoprecipitating the APC and washing extensively. This partially purified APC was then incubated with extracts from cells that had either been treated with nocodazole or α-factor. After incubation, the APC was washed again and assayed for ubiquitination activity. While incubation of an active APC with an α-factor extract had no effect on its activity, a nocodazole treated extract was able to inhibit the APC in the presence and absence of ATP (Figure 4-2C).

Figure 4-2. Inhibition of the APC by the spindle assembly checkpoint

(A) The APC from a spindle checkpoint arrested extract is inactive. A strain containing a hemagglutinin tagged *CDC16* (LHH311) was grown to logarithmic phase at 25°C, then split and arrested with either α-factor or nocodazole for 3 hours. Extracts were prepared from these cells and the APC was immunoprecipitated using 12CA5. APC activity was measured as described in Materials and Methods. An asterisk indicates a background band present in the non-ubiquitinated substrate.

(B) Mixing spindle checkpoint arrested extract with G1 arrested extract inhibits APC activity. Strains expressing the hemagglutinin tagged *CDC16* were arrested with α-factor and cell lysates were prepared. These lysates were mixed with either lysis buffer (lane 1) or increasing amounts of lysate from a strain lacking the tagged construct that had been arrested with nocodazole (lanes 3-5). In a reciprocal experiment, cell lysates from strains expressing the hemagglutinin tagged *CDC16* that had been arrested with nocodazole were mixed with increasing amounts of cell lysate from an untagged strain that had been arrested with α-factor (lanes 6-10). Each immunoprecipitation contained 625 μg of the tagged lysate.

(C) An active APC is partially inhibited by a spindle checkpoint arrested extract. The APC was immunoprecipitated from G1 arrested extracts and washed extensively. It was then incubated for 5

minutes at 25°C with either lysis buffer (lane 1) or extracts from cells that had been arrested with nocodazole (lanes 2 and 3) or α-factor (lanes 4 and 5). The incubations were done either in the presence or absence of an ATP regenerating system. The incubation period was restricted to 5 minutes to prevent degradation of proteins at 25°C.

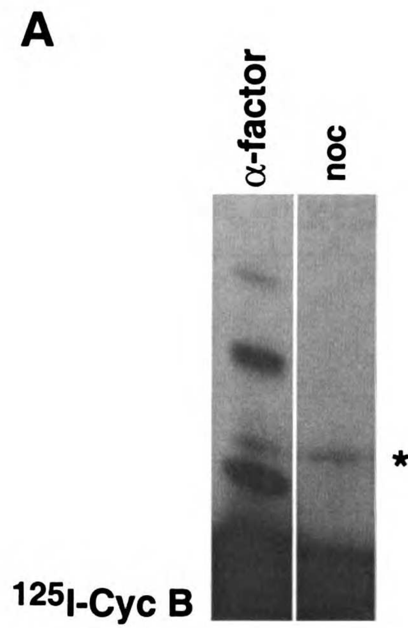


Figure 4-2A

B

tagged extract	α–f	-	α–f	α–f	α–f		noc	-	noc	noc	noc
untagged extract	-	noc	noc	noc	noc		-	α–f	α–f	α–f	α–f
fold excess (untagged : tagged)	-	-	1X	2X	5X		-	-	1X	2X	5X

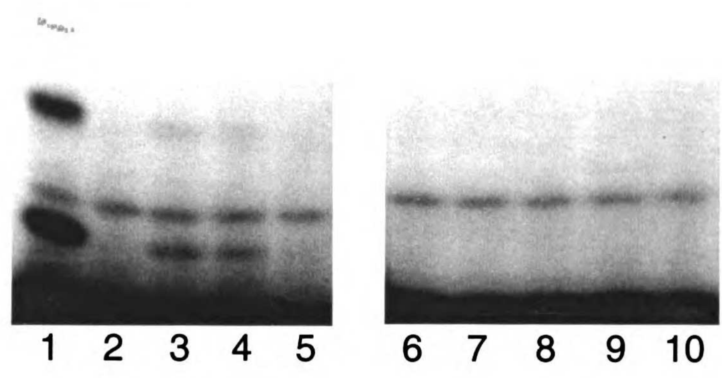


Figure 4-2B

C

incubating extract: none, noc, noc, α-f, α-f

ATP: +, -, +, -, +

Figure 4-2C

The specificity of the spindle checkpoint's ability to inhibit the APC was determined by comparing its ability to inhibit to the APC to that of an extract arrested in mitosis by other means. Extracts were made from cells arrested in metaphase using a *cdc26Δ* mutant that lacks APC activity at the non-permissive temperature (Hwang and Murray 1997, Zachariae, et al. 1996). Anaphase extracts were made by over-expressing a non-destructible version of *CLB2, Δ176CLB2*. Both of these extracts inhibit the activity of the APC as well as a spindle checkpoint arrested extract. The inhibition of the APC by the checkpoint extract is not specific to the checkpoint, but appears to be an activity present in all mitotically arrested cells.

Each of these extracts arrest with high levels of mitotic Cdc28 activity. To determine if Clb2-Cdc28 kinase activity was capable of inhibiting the activity of the APC, we isolated the APC from α-factor arrested cells that had been induced to express *Δ176CLB2*, under the control of the galactose promoter. Thirty minutes after induction APC activity was reduced, and after 90 minutes, no APC activity was detectable (Figure 4-3). This decrease in APC activity may be due to direct inhibition by the Δ176Clb2-Cdc28 kinase. Alternatively, Δ176Clb2-Cdc28 kinase activity drives cells to exit G1, the APC may be inhibited by other factors induced by progression through the cell cycle.

Figure 4-3. The APC is inhibited by Clb2-Cdc28 kinase

The APC in G1 arrested cells is inhibited by the expression of a non-degradable *CLB2*. A strain containing *GAL-Δ176CLB2* and a hemagglutinin tagged Cdc16 (LHH196) was arrested in G1 with α-factor. Expression of *Δ176CLB2* was induced with the addition of 2% galactose, and time points were taken at 0, 30, 60, and 90 minutes after galactose addition. The APC was immunoprecipitated with 12CA5 at each time point and assayed for activity.

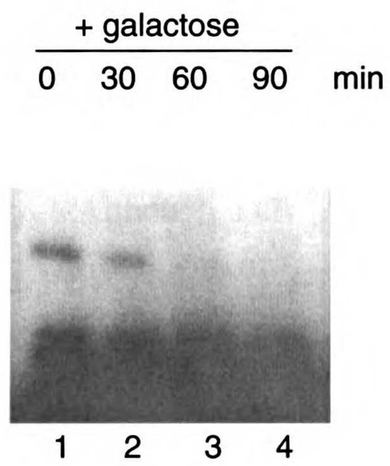


Figure 4-3

Cdc20-associated APC activity

To determine if the APC activity we see is Cdc20 associated activity, we expressed a hemagglutinin tagged version of Cdc20 under the control of the galactose promoter. Strains containing the tagged Cdc20 were arrested with α-factor or grown exponentially and expression was induced with the addition of galactose. The APC was immunoprecipitated using either anti-Cdc26 antibodies, to determine the bulk level of APC activity, or 12CA5, to measure Cdc20 associated APC activity. Although bulk APC activity is robust in both logarithmically growing cells and cells arrested in G1, there is no APC activity immunoprecipitated with the hemagglutinin tagged Cdc20 (Figure 4-4). This is not surprising for α-factor arrested cells, as Cdc20 is unstable in G1, however, the tagged Cdc20 accumulated to high levels in exponentially growing cells (data not shown). In addition, incubating an active APC from a G1 arrested extract with extracts from cells over-expressing Cdc20 has no effect on the activity of the APC (data not shown).

Figure 4-4. The assay does not reflect Cdc20-associated APC activity

Immunoprecipitation of Cdc20 does not co-immunoprecipitate APC activity. A strain containing a hemagglutinin tagged *CDC20* under the control of the galactose promoter (LHH205) was either grown to logarithmic phase or arrested with α-factor, and *HACDC20* expression was induced by the addition of 2% galactose. Cell lysates were prepared and immunoprecipitations were performed with either 12CA5 or α-Cdc26 antibody. Immunoprecipitates were assayed for APC activity as described in Materials and Methods. The positions of conjugates are marked.

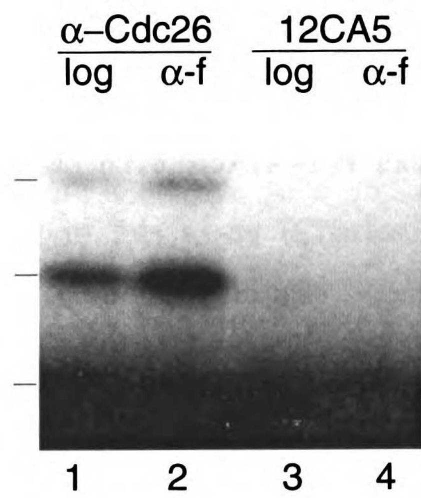


Figure 4-4

Discussion

We have developed a purified, reconstituted assay for APC activity towards the amino terminus of cyclin B using purified Uba1, Ubc4, and an immunopurified APC. The APC is the only regulated component in the assay and it appears to be active in G1 and inactive in a spindle checkpoint arrest, reproducing the regulation seen *in vivo*. However, the assay does not appear to reflect Cdc20 associated activity, and more likely reflects Hct1/Cdh1 associated APC activity. Recently, Charles *et al.* have shown that extracts from *hct1Δ* cells have no APC activity in this assay (Charles, et al. 1998). Furthermore, Cdc20 immunoprecipitates contain no APC activity in these assays, which may be due to limitations of the assay or an inability to ubiquitinate the cyclin B substrate.

Hct1/Cdh1 Associated APC activity

Hct1/Cdh1 promotes the destruction of Clb2 and other substrates late in mitosis and through G1 (Schwab, et al. 1997, Visintin, et al. 1997). Recent evidence has shown that Cdc28 kinase activity is capable of inhibiting Hct1/Cdh1, possibly by directly phosphorylating Hct1/Cdh1 and inhibiting its association with the APC (Zachariae, et al. 1998). In our experiments, active APC was inhibited by extracts from cells arrested by the spindle assembly checkpoint, an APC mutant, or high level expression of a non-degradable form of Clb2. All of these treatments arrest cells with high levels of mitotic Cdc28 activity. In addition, α-factor arrested

extracts that express a non-degradable form of Clb2, with high Cdc28 associated kinase activity, have decreased APC activity. The inhibition of APC activity that we see after exposure to mitotically arrested extracts or non-degradable Clb2 is likely due to inhibition of Hct1/Cdh1 in our assay. Jaspersen *et al.* have shown that this APC assay is responsive to the addition of recombinant Hct1, and that activation of the APC by recombinant Hct1 is inhibited if the Hct1 has previously been phosphorylated by Cdc28.

Spindle Assembly Checkpoint

Work in yeast, *Xenopus,* and humans has identified Cdc20 as the likely target of the spindle assembly checkpoint. In all of these systems, Cdc20 has been found to bind to checkpoint components, Mad1, Mad2 and Mad3 in budding yeast (Hwang, et al. 1998), and Mad2 in fission yeast, *Xenopus,* and human (Fang, et al. 1998, Kallio, et al. 1998, Kim, et al. 1998). In budding and fission yeast, dominant mutants in either *CDC20* or *slp1* that no longer bind Mad2, are capable of bypassing activation of the spindle checkpoint (Hwang, et al. 1998, Kim, et al. 1998). In *Xenopus* egg extracts, human MAD2 associates with the APC through CDC20, forming an inactive complex (Fang, et al. 1998). The spindle assembly checkpoint likely operates by directly inhibiting the ability of Cdc20 to activate the APC.

It is not known if the spindle assembly checkpoint also inhibits Hct1/Cdh1 associated APC activity. Temperature sensitive yeast mutants in *CDC20* arrest in mitosis with sister chromatids held together and mitotic cyclin levels high (Shirayama, et al. 1998).

Therefore, it appears that it would be sufficient for the checkpoint to inhibit Cdc20 activity to maintain cells in a metaphase arrest. However, there is some evidence that Mad3 in budding yeast associates with both Cdc20 and Hct1/Cdh1 (D. Smith personal communication), indicating a role for Hct1/Cdh1 in the spindle assembly checkpoint.

The present assay could be used to examine if certain components of the checkpoint, such as Mad3, have any effect on Hct1/Cdh1 dependent APC activity. However, in order to study the regulation of the spindle assembly checkpoint on the APC, we need a Cdc20-dependent APC assay. The limitations of the present assay could be detection of ubiquitination by Cdc20 associated APC. If the robustness of ubiquitination by Cdc20-dependent APC is lower than that of Hct1/Cdh1-dependent APC, the assay may be unable to detect ubiquitination by a Cdc20-dependent APC. If this is the case, the reaction could be enhanced by the addition of exogenous Cdc20. Preliminary experiments with reticulocyte translated Cdc20, indicates that addition of Cdc20 is not sufficient to activate the APC in our assay. In addition, as discussed previously, immunoprecipitation of Cdc20 does not yield APC activity.

Substrate specificity may also be a issue in our assay. Although over-expression of *CDC20* in mitosis leads to the destabilization of both Pds1 and Clb2, Cdc20 may be a poor activator of the APC towards a cyclin substrate. Pds1 has been shown to be an *in vivo* substrate of Cdc20 dependent ubiquitination and would be a good candidate substrate for Cdc20 associated APC activity *in vitro*.

Conclusion

We have developed a reconstituted assay for APC activity that likely reflects Hctl/Cdhl dependent APC ubiquitination. This assay can be used to study the regulation of the APC in G1, and possibly against substrates that lack a destruction box, including Cdc20. Modification of this assay to detect Cdc20-dependent APC activity will enable us to study the regulation of the APC by the spindle assembly checkpoint. A number of components of the checkpoint have been identified in yeast, and two-hybrid analysis indicate that they may form a large complex (D. Smith personal communication). Formation of this complex at the kinetochores of unattached chromosomes may be the signal that leads to cell cycle arrest. Further studies using the APC assay and purified components of the checkpoint will help to elucidate the mechanisms used by the cell to regulate exit from mitosis.

Acknowledgments

The development of the APC assay was done in collaboration with Sue Jaspersen, Julia Charles, and Rachel Tinker-Kulberg. We thank Alex Szidon for providing the cyclin B substrate, J. Dohmen for the *UBA1* expressing plasmid, and V. Chao for the bacterial *UBC4* expressing plasmid.

Chapter 5

Conclusion

This thesis explores the regulation of the exit from mitosis in *Saccharomyces cerevisiae.* In a screen developed to isolate novel mitotic arrest mutants, I identified two new components of the anaphase promoting complex (APC), Cdc26 and Doc1. Both of these proteins are involved in the destruction of cyclin B and other proteins during the exit from mitosis. I also identified Cdc20, a regulator of the APC, as the target of the spindle assembly checkpoint. Components of the checkpoint, Mad2 and Mad3, associate with Cdc20, and dominant mutants in *CDC20* that are capable of bypassing the spindle assembly checkpoint no longer associate with those proteins. Finally, in order to further study the regulation of the spindle assembly checkpoint on the activity of Cdc20, I developed an *in vitro* reconstituted assay for APC activity. Unfortunately, this assay appears to reflect Hct1/Cdh1 dependent APC activity instead of Cdc20 dependent activity.

How does the spindle assembly checkpoint inhibit the activity of Cdc20 to produce an arrest in mitosis? In order to address this question, a Cdc20 dependent APC assay must be developed. In *Xenopus*, it appears that excess recombinant XMAD2 alone is sufficient to inhibit XFZY dependent APC activity. However, only tetrameric XMAD2 is capable of this inhibition. Preliminary experiments in yeast suggest that recombinant Mad2 is not capable of inhibiting the APC. Is tetramerization of Mad2 required for inhibition and is the role of the other components of the checkpoint to effect the tetramerization of Mad2? *S. cerevisiae* is an ideal system to analyze the spindle assembly checkpoint, as a large

number of the components of the checkpoint have been identified and cloned.

In *S. cerevisiae,* it appears that Cdc20 associates with the checkpoint components at all stages in the cell cycle, and not just during activation of the checkpoint. This differs from *Xenopus,* where the association of XMAD2 and XFZY is seen only during checkpoint activation. Analyzing the Cdc20/Mad complex in yeast during activation of the checkpoint to determine any changes in modifications or subunit composition would help to elucidate the mechanism of inhibition as well as the differences between yeast and frogs.

Finally, how does localization of Cdc20 and the checkpoint components affect their activities? Cdc20 localizes to kinetochores from late prophase to telophase in HeLa and PtK1 cells. Mad2, Bub1 and Bub3 have been shown to localize to unattached kinetochores, and a single unattached kinetochore is able to inhibit progression into anaphase. How does a single unattached kinetochore inhibit the entire pool of Cdc20 in a cell? One possibility is that the pool of Cdc20 is sampled at each kinetochore continuously. If a kinetochore is unattached, the spindle assembly checkpoint will inactivate each Cdc20 protein that the kinetochore comes into contact with. The inactivation would have a limited half life, and eventually, the Cdc20 protein would be reactivated unless it again came into contact with an unattached kinetochore. This model could be tested by tethering Cdc20 to the kinetochore by creating fusion proteins that bind the centromere, to prevent Cdc20 from sampling all kinetochores. If these fusion proteins were active and unable to sample for

unattached kinetochores, a small number of unattached kinetochores would be unable to prevent the progression into anaphase.

The identification of Cdc20 as an activator of the APC and as the target of the spindle assembly checkpoint allows us to address at a molecular level how activation of the spindle assembly checkpoint induces a cell cycle arrest.

Appendix A

The Spindle Assembly Checkpoint in Anaphase

Appendix A

The spindle assembly checkpoint is inactive in anaphase

Introduction

Progression through the cell cycle is monitored by checkpoints that ensure proper replication and segregation of chromosomes. In mitosis, disruption of the spindle or a single unattached kinetochore will result in an arrest or delay prior to the initiation of anaphase. This arrest is mediated by the spindle assembly checkpoint to allow time for the cells to correct the defect before proceeding through anaphase, preventing chromosome mis-segregation (reviewed in Rudner and Murray 1996).

Components of the spindle assembly checkpoint were first identified in *Saccharomyces cerevisiae*. The *MAD1-3* (mitotic arrest deficient) and *BUB1-3* (budding uninhibited by benzimidazole) genes were identified in genetic screens for genes that were essential for mitotic arrest in response to depolymerization of the mitotic spindle (Hoyt, et al. 1991, Li and Murray 1991). In a screen for spindle pole body duplication mutants, a protein kinase, *MPS1* was identified that is required for both spindle pole body duplication and the spindle assembly checkpoint (Weiss and Winey 1996). Homologues to a number of these genes have been found in fission yeast, *Xenopus*, mouse, and human, indicating that this checkpoint is well conserved

through evolution (Chen, et al. 1996, Jin, et al. 1998, Kim, et al. 1998, Li and Benezra 1996, Taylor, et al. 1998, Taylor and McKeon 1997).

It has recently been shown that a number of the spindle checkpoint proteins are localized to the kinetochores of unattached chromosomes. The *Xenopus* homologue of *MAD2, XMAD2* localizes to unattached and mis-aligned kinetochores in prometaphase and to all kinetochores in cells treated with nocodazole (Chen, et al. 1996). Once the kinetochores become attached, XMAD2 binding disappears. This kinetochore specific localization has also been seen for human BUB1 and BUB3 (Taylor, et al. 1998, Taylor and McKeon 1997), indicating that the signal for the spindle assembly checkpoint originates at the kinetochores of unattached chromosomes.

Once the signal is generated at the kinetochore, it must be transduced to the cell cycle machinery in order to affect an arrest. This transduction pathway is still unclear, however, Mad1, a protein that associates with Mad2, becomes hyper-phosphorylated when the spindle checkpoint is activated (Hardwick and Murray 1995). This phosphorylation was used to formulate a rough ordering of the pathway, placing *MAD2, BUB1, BUB3,* and *MPS1* upstream of *MAD1*, and *MAD3* and *BUB2* downstream. *MPS1,* a protein kinase, constitutively activates the spindle checkpoint when over-expressed, leading to extensive hyper-phosphorylation of Mad1. It is unclear, however, if Mad1 hyper-phosphorylation is required for checkpoint activation. Over-expression of a dominant mutant of the Bub1 kinase, *BUB1-5* is able to activate the spindle checkpoint in the absence of Mad1 hyper-phosphorylation (Farr and Hoyt 1998).

Ultimately, activation of the spindle assembly checkpoint results in a cell cycle arrest at metaphase. Chapter three described the identification of Cdc20 as the target of the checkpoint. Activation of the checkpoint inhibits the activity of Cdc20, a substrate specific activator of the anaphase promoting complex (APC), preventing the initiation of anaphase (Hwang, et al. 1998, Kim, et al. 1998).

The spindle assembly checkpoint ensures that cells do not progress through anaphase in the presence of damage to the spindle or mis-attached chromosomes. It is important that cells maintain a metaphase arrest during checkpoint activation. If cells progress into anaphase, sister chromosomes lose their cohesion, and mis-segregated chromosomes cannot be re-segregated properly. It seems likely, therefore, that the spindle checkpoint would be unnecessary in anaphase. However, detection of mis-segregated chromosomes or damage to the spindle in anaphase may allow cells to inhibit cell division when these defects occur, preventing the production of aneuploid cells. We attempted to determine if the spindle assembly checkpoint can be activated in anaphase.

Materials and Methods

Yeast strains and Media

All yeast strains are derivatives of W303. Standard yeast media was used (Sherman, et al. 1974). Nocodazole (Sigma, St. Louis, MO) was used at 15μg/ml from a stock solution of 10 mg/ml in DMSO.

Cycloheximide (Sigma) was used at 10μg/ml from a stock solution of 10mg/ml.

Assay for checkpoint maintenance in anaphase

Nocodazole addition to Δ176CLB2 arrested cells

LHH198 (*MATa GAL-Δ176CLB2::URA3 bar1Δ ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100)* was grown to logarithmic phase in 30 ml of YEP + 2% raffinose. The culture was split into three 10ml cultures. The first culture was arrested with 15μg/ml nocodazole at 22.5°C for 4.5 hours; the second culture with 2% galactose at 22.5°C for 4.5 hours; the third culture with 2% galactose for 3.5 hours, then nocodazole was added for an additional hour.

To determine the half life of Clb2, cycloheximide was added after the 4.5 hour incubation, and 0.75ml aliquots were taken at 0, 5, 10, 30, and 60 minutes. An equal volume of ice cold 20mM NaN_3 was added to each aliquot and incubated on ice for 5 minutes. The cells were pelleted and frozen in liquid nitrogen. Cell pellets were bead beaten in 60μl 2X SDS sample buffer (1X SDS sample buffer: 80mM Tris, pH 6.8, 2% SDS, 10% glycerol, 10mM EDTA, 0.0013% Bromophenol blue, 5% 2-mercaptoethanol). Standard methods were used for SDS-PAGE and protein transfer to nitrocellulose (Harlow and Lane 1988). Cell lysates from the zero time point were also probed for Mad1 hyperphosphorylation. Clb2 and Mad1 were detected as described previously (Hardwick and Murray 1995, Hwang and Murray 1997).

MPS1 over-expression in cdc15-1 arrested cells

MATα cdc15-1 GAL-MPS1::URA3 CDC28-VF was grown overnight in YPD then diluted into YEP + 2% raffinose and grown to logarithmic phase. The culture was then shifted to 37°C for 3.75 hours, until greater than 90% of the cells appeared to be arrested as large budded cells (time=0). At that time, the culture was split into two, galactose (final concentration 2%) was added to half and raffinose (final concentration 2%) was added to the other half. Both cultures were incubated at 37°C for 30 minutes (time=30 minutes), and then each culture was again split in half. One half of each culture was maintained at 37°C and the other was shifted down to 22.5°C. Aliquots of 1.5 ml were collected at 60, 90, 120, and 150 minutes. The cells were pelleted and frozen in liquid nitrogen. Cell lysates were made by bead beating cells in 50μl lysis buffer A (50mM Hepes pH 7.6, 75mM KCl, 1mM $MgCl_2$, 1mM EGTA, 0.1% NP-40, 50mM NaF, 50mM β-glycerophosphate, 1mM PMSF and LPC (10 mg/ml leupeptin, pepstatin, and chymostatin), Boehringer, Indianapolis, IN). Protein concentrations of extracts were determined with the Bradford assay using bovine serum albumin as a standard (Bradford 1976). For immunoblotting, the extracts were adjusted to the same protein concentration and diluted with 2X SDS sample buffer. Clb2 and Mad1 were detected as described above.

Results

We examined the ability of the spindle assembly checkpoint to be activated in anaphase using two different anaphase arrests. Cells were arrested using either over-expression of a non-degradable form of *CLB2, Δ176CLB2* or by the temperature sensitive *cdc15-1* mutant. Activation of the checkpoint was determined by monitoring stability of Clb2, the major mitotic cyclin, and hyper-phosphorylation of Mad1. Clb2 protein is rapidly degraded during the exit from mitosis. When the spindle assembly checkpoint is activated, Clb2 protein is stabilized.

Nocodazole does not induce the checkpoint in *Δ176CLB2* arrested cells

The degradation of Clb2 is required for the progression through anaphase (Surana, et al. 1993). LHH198, a strain containing *Δ176CLB2* under the control of the *GAL1-10* promoter was arrested in anaphase with the addition of galactose. Nocodazole, a drug that depolymerizes microtubules, was added to disrupt the mitotic spindle, and potentially activate the spindle checkpoint. After an hour, no anaphase spindles could be detected (data not shown). Cycloheximide was then added to inhibit protein synthesis, and samples were taken to determine the half life of Clb2 (Figure A-1A, lanes 6-10).

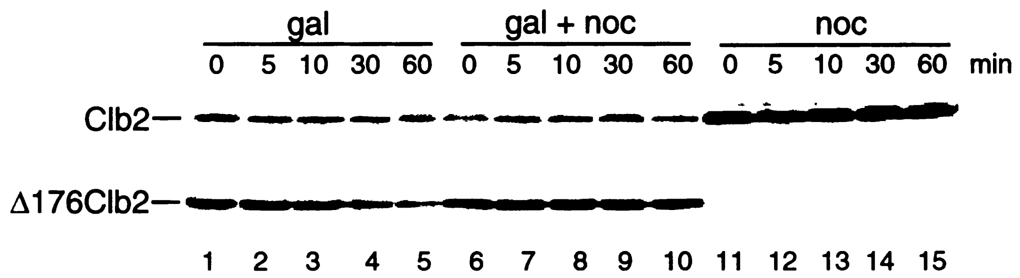
The half life of Clb2 could not be determined with the time points taken in this experiment. Longer time points would be

Figure A-1: The spindle checkpoint cannot be activated in a *Δ176CLB2* arrest

(A) LHH198 (*MATa GAL-Δ176CLB2::URA3 bar1Δ ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100)* was grown to logarithmic phase in YEP + 2% raffinose. The culture was split into three: the first was arrested with 2% galactose for 4.5 hours (lanes 1-5); the second was arrested with 2% galactose for 3.5 hours, then nocodazole was added to 15μg/ml for one hour (lanes 6-10); the third was arrested with 15μg/ml nocodazole for 4.5 hours (lanes 11-15). Cycloheximide was then added to 10μg/ml, and samples were taken at the indicated times after cycloheximide addition. The samples were analyzed by Western blot for Clb2 and Δ176Clb2 protein using a polyclonal anti-Clb2 antibody.

(B) Hyperphosphorylation of Mad1. Cell lysates from the zero time point above (Figure A-1A, lanes 1, 6, and 11) were probed for Mad1 hyperphosphorylation using polyclonal anti-Mad1 antibodies.

A



B

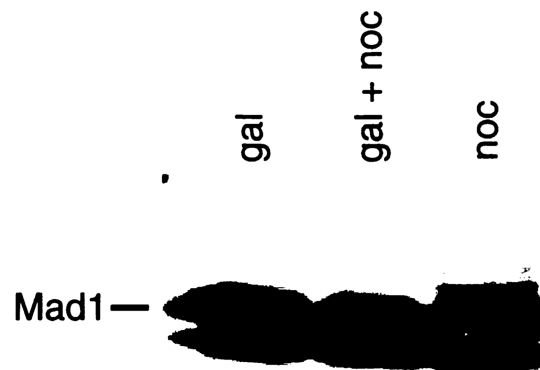


Figure A-1

required to determine the half life. However, it is clear that the addition of nocodazole to the *Δ176CLB2* arrested cells did not stabilize Clb2 to the same level as nocodazole addition to logarithmically growing cells (Figure A-1A, compare lanes 6 and 11). In fact, the addition of nocodazole for one hour has no effect on the level of Clb2 protein in *Δ176CLB2* arrested cells (Figure A-1A, compare lanes 1 and 6). Furthermore, the addition of nocodazole to *Δ176CLB2* arrested cells does not result in Mad1 hyperphosphorylation (Figure A-1B, lane 2)

GAL-MPS1 does not induce the checkpoint in *cdc15-1* arrested cells

Once sister chromosomes are segregated, the checkpoint may be unable to generate a signal at the kinetochore. Therefore, depolymerization of the spindle, and loss of kinetochore attachment in anaphase may not signal activation of the checkpoint at that time. Over-expression of *MPS1,* a spindle checkpoint component, constitutively activates the checkpoint in the absence of any spindle defect (Hardwick, et al. 1996). To determine if the checkpoint can be constitutively activated by over-expression of *MPS1* during anaphase, *MPS1* under the control of the *GAL1-10* promoter was expressed in a *cdc15-1* strain.

CDC15 encodes a protein kinase whose activity is required for progression through anaphase (Schweitzer and Philippsen 1991). *cdc15-1* temperature sensitive mutants were arrested in anaphase at the non-permissive temperature, and *MPS1* expression was induced

with galactose. Cells were either maintained at the *cdc15-1* arrest point at 37°C (Figure A-2, lanes 4-7), or were released from the arrest at 22.5°C (Figure A-2, lanes 8-11). Spindle checkpoint activation was ascertained by monitoring Clb2 protein levels and Mad1 hyperphosphorylation. If over-expression of *MPS1* can activate the checkpoint in anaphase, cells should remain arrested in anaphase after release from the *cdc15-1* arrest.

Cells arrested in anaphase by *cdc15-1* are unable to activate the spindle checkpoint with the over-expression of *MPS1*. Clb2 protein levels fall when cell are released from the *cdc15-1* arrest after *GAL-MPS1* induction (Figure A-2, lanes 8-11), indicating exit from mitosis. In fact, over-expression of *MPS1* appears to have no effect on the rate of exit after release from the *cdc15-1* arrest (Figure A-2, compare lanes 8-11 to lanes 17-20). Clb2 protein levels rise at 150 minutes, indicating entry into the next mitosis (Figure A-2, lanes 11 and 20). Timing of Clb2 protein accumulation is also not affected by *MPS1* expression.

Furthermore, Mad1 is not hyper-phosphorylated in *cdc15-1* arrested cells expressing high levels of *MPS1* (Figure A-2, lanes 4-7). Only after cells are released from the *cdc15-1* arrest and enter the next mitosis, are they able to hyper-phosphorylate Mad1 (Figure A-2, lane 11).

Conclusion

Preliminary experiments indicate that the spindle assembly checkpoint cannot be activated in anaphase. Depolymerization of the

Figure A-2: The spindle checkpoint cannot be activated in a *cdc15-1* arrest.

MATα cdc15-1 GAL-MPS1::URA3 CDC28-VF was grown to logarithmic phase in YEP + 2% raffinose at 22.5°C and then shifted to 37°C for 3.75 hours to arrest cells in anaphase. The culture was split into two, 2% galactose was added to half and 2% raffinose was added to the other half. Both cultures were incubated at 37°C for 30 minutes to allow induction of *GAL-MPS1* and then each culture was again split in half. One half of each culture was maintained at 37°C and the other was shifted down to 22.5°C. Samples were taken at 0, 30, 60, 90, 120, and 150 minutes after galactose or raffinose addition. In lane 1, the strain was grown to logarithmic phase at 22.5°C and *GAL-MPS1* was induced with the addition of 2% galactose for 3 hours at 22.5°C.

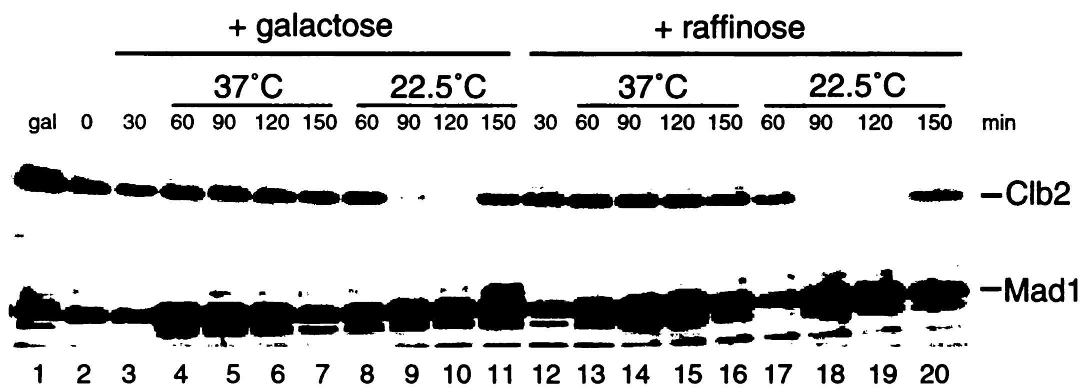


Figure A-2

spindle is unable to activate cells arrested in anaphase by the expression of a non-degradable Clb2. In addition, constitutive activation of the checkpoint by the over-expression of *MPS1* is unable to activate the checkpoint in *cdc15-1* arrested cells.

The activation of the checkpoint was detected by monitoring Clb2 stability and Mad1 hyper-phosphorylation. However, the phosphorylation state of Mad1 is only correlated with activation of the checkpoint, and has not been shown to be required (Hardwick and Murray 1995). In fact, Mad1 hyper-phosphorylation is not seen in a *BUB1-5* mutant that constitutively activates the checkpoint (Farr and Hoyt 1998). Clb2 protein levels indicate that cells released from a *cdc15-1* arrest, but expressing *GAL-MPS1* are exiting mitosis, however, additional hallmarks of cell cycle progression, such as rebudding and accumulation of cells with a G1 DNA content, should be examined. In addition, there are some indications that the *GAL1-10* promoter is inefficient at 37°C. The level of *GAL-MPS1* induction during a *cdc15-1* arrest at 37°C should be determined.

How could the spindle checkpoint be inactivated in anaphase? A number of mitotic regulatory proteins are degraded during the progression through mitosis. Pds1 (budding yeast) and Cut2 (fission yeast) must be degraded in order for cells to initiate anaphase (Cohen-Fix, et al. 1996, Funabiki, et al. 1996). Perhaps an essential component of the spindle checkpoint is also targeted for degradation at metaphase, inactivating the checkpoint in anaphase. Alternatively, inhibition of Cdc20, the target of the checkpoint, may be ineffective in anaphase, and Hct1/Cdh1, the homologue of Cdc20 that targets Clb2 for degradation late in mitosis may be unresponsive

to activation of the checkpoint. Elucidation of the mechanism by which cells turn off the checkpoint may give clues to the regulation of other signal transduction cascades.

References

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

Araki, H., Awane, K., Ogawa, N. and Oshima, Y. 1992. The *CDC26* gene of *Saccharomyces cerevisiae* is required for cell growth only at high temperature. *Mol. Gen. Genet.* 231: 329-331

Bachmair, A., Finley, D. and Varshavsky, A. 1986. In vivo half-life of a protein is a function of its amino-terminal residue. *Science* 234: 179-185

Baum, P., Furlong, C. and Byers, B. 1986. Yeast gene required for spindle pole body duplication: homology of its product with Ca2+-binding proteins. *Proc Natl Acad Sci U S A* 83: 5512-6

Bradford, M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254

Byers, B. 1981. Multiple roles of the spindle pole bodies in the life cycle of *Saccharomyces cerevisiae*. *Mol. Genet. Yeast* 16: 119-133

Byers, B. and Goetsch, L. 1974. Duplication of spindle plaques and integration of the yeast cell cycle. *Cold Spring Harbor Symp. Quant. Biol.* 38: 123-131

Charles, J., Jaspersen, S. L., Tinker-Kulberg, R. L., Hwang, L., Szidon, A. and Morgan, D. O. 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., Waters, J. C., Salmon, E. D. and Murray, A. W. 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

Cohen-Fix, O. and Koshalnd, D. 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., Peters, J. M., Kirschner, M. W. and Koshland, D. 1996. Anaphase initiation in Saccharomyces cerevisiae is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. *Genes Dev* 10: 3081-93

Company, N., Arenas, J. and Abelson, J. 1991. Requirement of the RNA helicase-like protein PRP22 for realease of messenger RNA from spliceosomes. *Nature* 349: 487-493

Dawson, I. A., Roth, S. and Artavanis-Tsakonas, S. 1995. The Drosophila cell cycle gene fizzy is required for normal degradation of cyclins A and B during mitosis and has homology to the CDC20 gene of Saccharomyces cerevisiae. *J Cell Biol* 129: 725-37

Dohmen, R. J., Stappen, R., McGrath, J. P., Forrova, H., Kolarov, J., Goffeau, A. and Varshavsky, A. 1995. An essential yeast gene encoding a homolog of ubiquitin-activiating enzyme. *J. Biol. Chem.* 270: 18099-109

Fang, G., Yu, H. and Kirschner, M. W. 1998. 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., Yu, H. and Kirschner, M. W. 1998. Direct Binding of CDC20 Protein Family Members Activates the Anaphase-Promoting Complex in Mitosis and G1. *Molecular Cell* 2: 163-171

Farr, K. A. and Hoyt, M. A. 1998. Bub1p kinase activates the Saccharomyces cerevisiae spindle assembly checkpoint. *Mol Cell Biol* 18: 2738-47

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

Ghislain, M., Udvardy, A. and Mann, C. 1993. S. cerevisiae 26S protease mutants arrest cell division in G2/metaphase. *Nature* 366: 358-62

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

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

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

Hartwell, L. H., Culotti, J. and Reid, B. 1970. Genetic control of cell division in yeast, I. Detection of mutants. *Proc. Natl. Acad. Sci. USA* 66: 352-359

Hartwell, L. H. and Smith, D. 1985. Altered fidelity of mitotic chromosome transmission in cell cycle mutants of S. cerevisiae. *Genetics* 110: 381-95

Hershko, A., Ganoth, D., Pehrson, J., Palazzo, R. E. and Cohen, L. H. 1991. Methylated ubiquitin inhibits cyclin degradation in clam embryo extracts. *J. Biol. Chem.* 266: 16376-16379

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

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

Hwang, L. H., Lau, L. F., Smith, D. L., Mistrot, C. A., Hardwick, K. G., Hwang, E. S., Amon, A. and Murray, A. W. 1998. Budding yeast Cdc20: a target of the spindle checkpoint. *Science* 279: 1041-4

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

Jin, D. Y., Spencer, F. and Jeang, K. T. 1998. Human T cell leukemia virus type 1 oncoprotein Tax targets the human mitotic checkpoint protein MAD1. *Cell* 93: 81-91

Johnson, E. S., Bartel, B., Seufert, W. and Varshavsky, A. 1992. Ubiquitin as a degradation signal. *EMBO J.* 11: 497-505

Kallio, M., Weinstein, J., Daum, J. R., Burke, D. J. and Gorbsky, G. J. 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

Kim, S. H., Lin, D. P., Matsumoto, S., Kitazano, A. and Matsumoto, T. 1998. Fission yeast slp1+ encodes the effector of the Mad2-dependent spindle checkpoint. *Science* 279: 1045-7

King, R. W., Deshaies, R. J., Peters, J. M. and Kirschner, M. W. 1996. How Proteolysis Drives the Cell Cycle. *Science* 274: 1652-1659

King, R. W., Peters, J. M., Tugendreich, S., Rolfe, M., Hieter, P. and Kirschner, M. W. 1995. A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. *Cell* 81: 279-88

Lamb, J. R., Michaud, W. A., Sikorski, R. S. and Hieter, P. A. 1994. Cdc16p, Cdc23p and Cdc27p form a complex essential for mitosis. *Embo J* 13: 4321-8

Lee, N. H. e. a. 1995. Comparative expressed sequence tag analysis of differential gene expression profiles in PC-12 cells before and after nerve growth factor treatment. *Proc. Natl. Acad. Sci. USA* 92: 8303-8307

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

Li, Y. and Benezra, R. 1996. Identification of a human mitotic checkpoint gene: hsMAD2. *Science* 274: 246-8

Li, Y., Gorbea, C., Mahaffey, D., Rechsteiner, M. and Benezra, R. 1997. MAD2 associates with the cyclosome/anaphase-promoting complex and inhibits its activity. *Proc. Natl. Acd. Sci.* 94: 12431-12436

Lim, H. H. and Surana, U. 1996. Cdc20, a beta-transducin homologue, links RAD9-mediated G2/M checkpoint control to mitosis in Saccharomyces cerevisiae. *Mol Gen Genet* 253: 138-48

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

Meluh, P. B. and Rose, M. D. 1990. *KAR3*, a kinesin-related gene required for yeast nuclear fusion. *Cell* 60: 1029-1041

Morgan, D. O. 1995. Principles of CDK regulation. *Nature* 374: 131-134

Murray, A. W. and Kirschner, M. W. 1989. Dominoes and Clocks: the union of two views of cell cycle regulation. *Science* 246: 614-621

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

Nurse, P. 1990. Universal control mechanism regulating cell cycle timing of M-phase. *Nature* 344: 503-508

Palmer, R. E., Koval, M. and Koshland, D. 1989. The dynamics of chromosome movement in the budding yeast Saccharomyces cervisiae. *J. Cell Biol.* 109: 3355-3366

Peters, J. M., King, R. W., Hoog, C. and Kirschner, M. W. 1996. Identification of BIME as a Subunit of the Anaphase-Promoting Complex. *Science* 274: 1199-1201

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

Schwab, M., Lutum, A. S. and Seufert, W. 1997. Yeast Hct1 is a regulator of Clb2 cyclin proteolysis. *Cell* 90: 683-93

Schweitzer, B. and Philippsen, P. 1991. *CDC15*, an essential cell cycle gene in *Saccharomyces cerevisiae*, encodes a protein kinase domain. *Yeast* 7: 265-273

Sethi, N., Monteagudo, M. C., Koshland, D., Hogan, E. and Burke, D. J. 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

Shirayama, M., Zachariae, W., Ciosk, R. and Nasmyth, K. 1998. The Polo-like kinase Cdc5p and the WD-repeat protein Cdc20/fizzy are regulators and substrates of the anaphase promoting complex in *Saccharomyces cerevisiae*. *EMBO* 17:

Sigrist, S., Jacobs, H., Stratmann, R. and Lehner, C. F. 1995. Exit from mitosis is regulated by Drosophila fizzy and the sequential destruction of cyclins A, B and B3. *Embo J* 14: 4827-38

Sigrist, S. J. and Lehner, C. F. 1997. Drosophila fizzy-related down-regulates mitotic cyclins and is required for cell proliferation arrest and entry into endocycles. *Cell* 90: 671-81

Sikorski, R. S. and Hieter, P. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122: 19-27

Smith, D. B. and Johnson, K. S. 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* 67: 31-40

Staudinger, J., Perry, M., Elledge, S. J. and Olson, E. N. 1993. Interactions among vertebrate helix-loop-helix proteins in yeast using the two-hybrid system. *J Biol Chem* 268: 4608-11

Sudakin, V., Ganoth, D., Dahan, A., Heller, H., Hersko, J., Luca, F., Ruderman, J. V. and Hershko, A. 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

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

Taylor, S. S., Ha, E. and McKeon, F. 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 McKeon, F. 1997. Kinetochore localization of murine Bub1 is required for normal mitotic timing and checkpoint response to spindle damage. *Cell* 89: 727-35

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

Wan, J., Xu, H. and Grunstein, M. 1992. CDC14 of *Saccharomyces cerevisiae*. Cloning, sequence analysis, and transcription during the cell cycle. *J. Biol. Chem.* 267: 11274-11280

Weinert, T. A. 1992. Dual cell cycle checkpoints sensitive to chromosome replication and DNA damage in the budding yeast Saccharomyces cerevisiae. *Radiat Res* 132: 141-3

Weiss, E. and Winey, M. 1996. The S. cerevisiae SPB duplication gene *MPS1* is part of a mitotic checkpoint. *J. Cell Biol.* 132: 111-123

Wells, W. A. E. 1996. The spindle-assembly checkpoint: aiming for a perfect mitosis, every time. *TICB* 6: 228-234

Xiao, Z., McGrew, J. T., Schroeder, A. J. and Fitzgerald-Hayes, M. 1993. *CSE1* and *CSE2*, two new genes required for accurate mitotic chromosome segregation in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 13: 4691-4702

Yamamoto, A., Guacci, V. and Koshland, D. 1996. Pds1p is required for faithful execution of anaphase in the yeast, Saccharomyces cerevisiae. *J Cell Biol* 133: 85-97

Yamamoto, Y., A., Guacci, V. and Koshland, D. 1996. 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

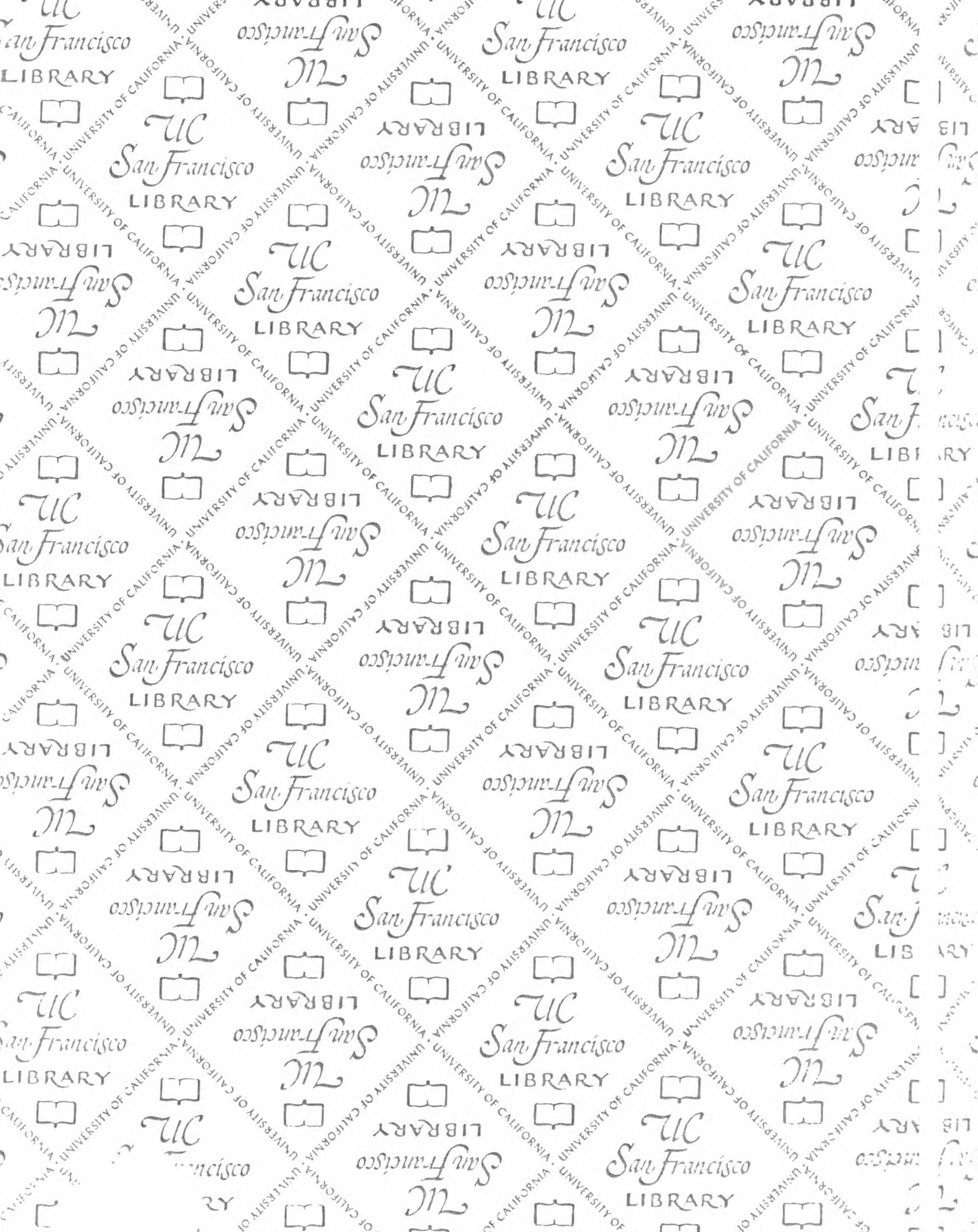
Yu, H., Peters, J. M., King, R. W., Page, A. M., Hieter, P. and Kirschner, M. W. 1998. Identification of a Cullin Homology Region in a Subunit of the Anaphase-Promoting Complex. *Science* 279: 1219-1222

Zachariae, W. and Nasmyth, K. 1996. TPR proteins required for anaphase progression mediate ubiquitination of mitotic B type cyclins in yeast. *Mol. Biol. Cell* 7: 791-801

Zachariae, W., Schwab, M., Nasmyth, K. and Seufert, W. 1998. Control of Cyclin Ubiquitination by CDK-Regulated Binding of Hct1 to the Anaphase Promoting Complex. *Science* 282: 1721-1724

Zachariae, W., Shevchenko, A., Andrews, P. D., Ciosk, R., Galova, M., Stark, M. J. R., Mann, M. and Nasmyth, K. 1998. Mass Spectrometric Analysis of the Anaphase-Promoting Complex from Yeast: Identification of a Subunit Related to Cullins. *Science* 279: 1216-1219

Zachariae, W., Shin, T. H., Galova, M., Obermaier, B. and Nasmyth, K. 1996. Identification of subunits of the anaphase-promoting complex of Saccharomyces cerevisiae. *Science* 274: 1201-4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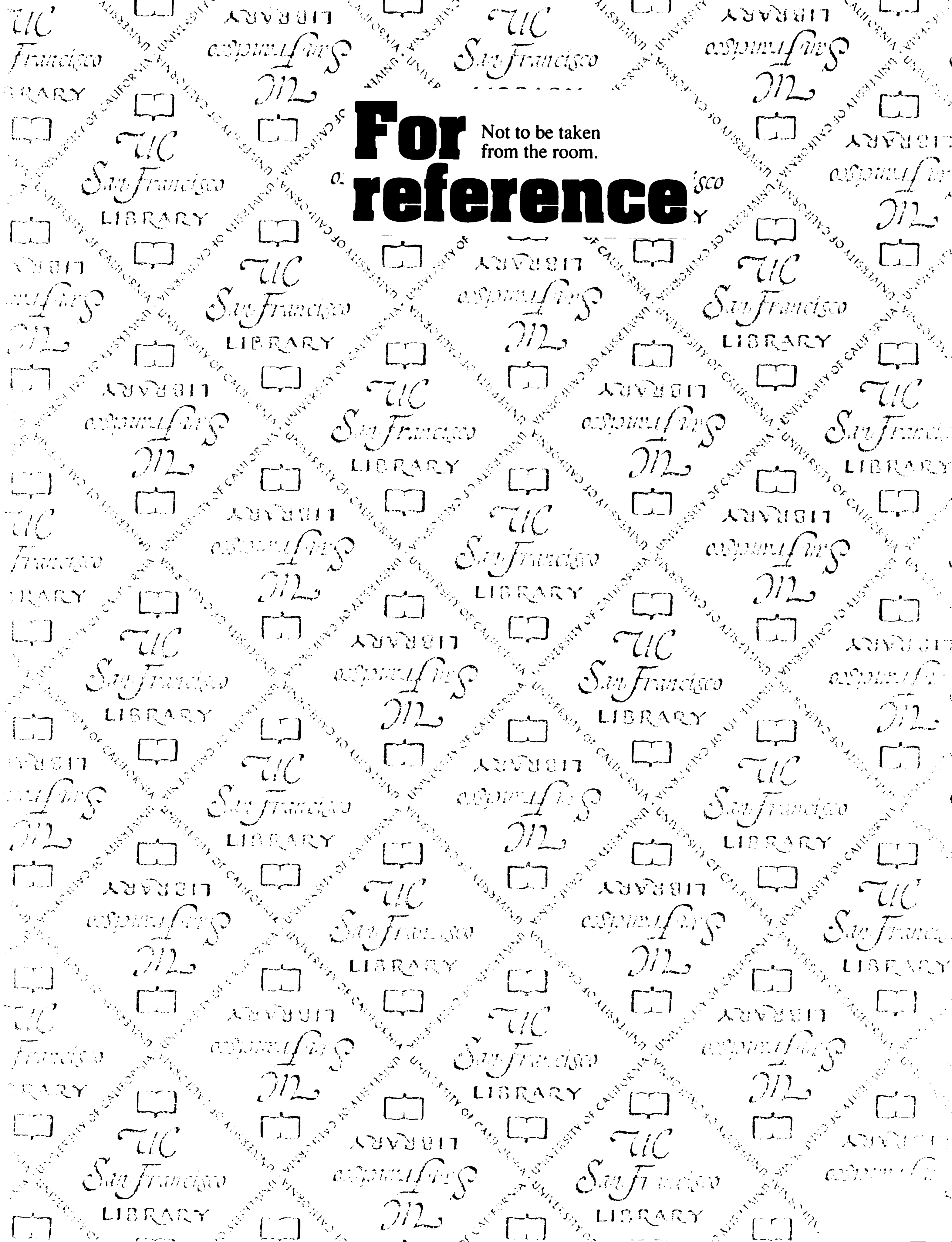
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