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The Polo-like kinase Cdc5p and the WD-repeat protein Cdc20p/fizzy are regulators and substrates of the anaphase promoting complex in *Saccharomyces cerevisiae*

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Proteolysis mediated by the anaphase promoting complex (APC) has a crucial role in regulating the passage of cells through anaphase. Destruction of the anaphase inhibitor Pds1p is necessary for separation of sister chromatids, whereas destruction of the mitotic cyclin Clb2p is important for disassembly of the mitotic spindle, cytokinesis and re-replication of the genome. Pds1p proteolysis precedes that of Clb2p by at least 15 min, which helps to ensure that cells never re-replicate their genome before they have separated sister chromatids at the previous mitosis. What triggers Pds1p proteolysis and why does it not also trigger that of Clb2p? Apart from sharing a dependence on the APC, these two proteolytic events differ in their dependence on other cofactors. Pds1p proteolysis depends on a WD-repeat protein called Cdc20p, whereas Clb2p proteolysis depends on another, related WD protein called Hct1/Cdh1p. On the other hand, destruction of Clb2p, but not that of Pds1p, depends on the Polo-like kinase, Cdc5p. Cdc20p is essential for separation of sister chromatids, whereas Cdc5p is not. We show that both Cdc5p and Cdc20p are unstable proteins whose proteolysis is regulated by the APC. Both proteins accumulate during late G_2/M phase and disappear at a late stage of anaphase. Accumulation of Cdc20p contributes to activation of Pds1p proteolysis in metaphase, whereas accumulation of Cdc5p facilitates the activation of Clb2p proteolysis.

Keywords: APC/Polo/proteolysis/WD repeat

Introduction

The Anaphase Promoting Complex (APC) is a large, multi-subunit ubiquitination machine essential for the proteolysis of several proteins at the onset of and during anaphase (Irniger *et al.*, 1995; King *et al.*, 1995; Sudakin *et al.*, 1995; Zachariae *et al.*, 1996, 1997). These include mitotic cyclins, the anaphase inhibitors Pds1p (Cohen-Fix *et al.*, 1996) and Cut2p (Funabiki *et al.*, 1996), the cohesion Scc1p (Michaelis *et al.*, 1997) and Ase1p, a protein associated with the midzone of the mitotic spindle (Juang *et al.*, 1997). Proteolysis mediated by the APC is required for the separation of sister chromatids, for cytokinesis and for re-duplication of chromosomes in the subsequent cell cycle of budding yeast. The occurrence of these events presumably depends on the destruction of specific proteins by the APC. Destruction of Pds1p in budding yeast and Cut2p in fission yeast is required for sister chromatid separation, whereas destruction of mitotic cyclins is thought to have an important role in disassembly of the mitotic spindle and in the formation of the pre-replication complexes at DNA replication origins.

Ubiquitination of unstable proteins such as cyclins requires an ubiquitin activation enzyme (also called E1), an ubiquitin conjugating enzyme (E2) and an ubiquitin protein ligase activity (E3), which is thought to provide specificity to the reaction (Ciechanover, 1994). The multi-ubiquitin chain is recognized by the 26S proteasome that degrades the target proteins (Peters, 1994). The APC is thought to function as a cell-cycle-regulated ubiquitin protein ligase specific for substrates containing destruction boxes (Glotzer *et al.*, 1991; King *et al.*, 1995; Sudakin *et al.*, 1995) and it is generally believed that proteolysis mediated by the APC is largely, if not solely, regulated by the state of the APC itself, which is thought to switch from an inactive to an active state at the onset of anaphase. This simple picture of the APC switching from an off- to an on-state due to its phosphorylation state cannot, however, explain the fact that different APC substrates are degraded with very different kinetics. In budding yeast, Pds1p disappears shortly before the onset of anaphase, whereas Clb2p starts declining only during anaphase B, and yet both are clearly degraded via the APC. A similar 'programme' presumably occurs during animal cell cycles, in which destruction of cyclin A invariably precedes that of cyclin B (Hunt *et al.*, 1992). The ubiquitination reaction mediated by the APC is therefore more complicated than that suggested by previous biochemical experiments. It is not understood what distinguishes the destruction kinetics of different APC substrates. It could in principle be due to the state (e.g. phosphorylation) of its substrates, to changes in the activity of the APC itself or to the activity of as yet unknown cofactors in the ubiquitination process. Whatever its nature, the regulation is very likely to be important in ensuring an orderly progression through the cell cycle.

In this paper, we identify two proteins whose degradation towards the end of anaphase depends on the APC: the WD40 protein Cdc20p/fizzy and the protein kinase Cdc5p/Polo. Both are evolutionarily conserved cell cycle regulators. The *CDC20* gene encodes a WD-repeat protein related to *S.cerevisiae* Hct1/Cdh1p, *Drosophila* fizzy and fizzy-related, which were recently found to be involved in APC-dependent proteolysis of mitotic cyclins (Dawson *et al.*, 1995; Sigrist *et al.*, 1995; Schwab *et al.*, 1997; Sigrist and Lehner, 1997; Visintin *et al.*, 1997). The *CDC5* gene encodes a protein kinase of the Polo family (Kitada *et al.*, 1993), which is shown to have a function in control of the spindle function, chromosome segregation and

cytokinesis in various organisms (Sunkel and Glover, 1988; Llamazares *et al.*, 1991; Golsteyn *et al.*, 1995; Lee *et al.*, 1995; Ohkura *et al.*, 1995). Remarkably, Cdc5p and Cdc20p are not only substrates, but also regulators of APC-mediated proteolysis. Cdc20p is required for proteolysis of Pds1p and for the onset of anaphase A (Sethi *et al.*, 1991), whereas Cdc5p is required for the proteolysis of mitotic cyclins, for completion of anaphase B and for cytokinesis (Kitada *et al.*, 1993). We also show that accumulation of Cdc20p within the nucleus during metaphase plays an important role in triggering destruction of Pds1p by the APC and that a failure to degrade Cdc5p can interfere with terminating Clb2 proteolysis.

Results

The location and abundance of Cdc5p and Cdc20p varies during the yeast cell cycle

Both *CDC5* and *CDC20* gene products are required for the destruction of mitotic cyclins during anaphase, but neither is a stable constituent of the APC. Their role during anaphase is presumably fundamental, as both proteins are conserved amongst all eukaryotes. *CDC20* encodes a protein composed of WD40 repeats, whereas *CDC5* encodes a protein kinase of the Polo family. These facts raise the possibility that *CDC20* and *CDC5* might be regulators of the APC which are not stably associated with it. As the first step towards investigating this notion, we replaced the endogenous *CDC5* and *CDC20* genes with versions tagged with multiple myc epitopes, which allowed us to analyse the cellular location and abundance of Cdc5p and Cdc20p as cells progress through the cell cycle.

Indirect immunofluorescence showed that both proteins accumulated within the nucleus during mitosis (Figure 1A and C). Cdc5p starts to appear after S phase and disappears shortly before cytokinesis, as chromosomes reach opposite spindle poles of the cell (Figure 1A). Cdc20p accumulates in the nucleus at around the same time as Pds1 protein disappears and remains there until cytokinesis has been completed (Figure 1C). Neither Cdc5p nor Cdc20p was detected in unbudded or small budded cells.

We also investigated these cell cycle fluctuations using immunoblotting to measure the abundance of Cdc5p and Cdc20p as unbudded G_1 cells isolated either by α-factor treatment (*CDC5–HA3*, Figure 2A) or by centrifugal elutriation (*CDC20–myc18*, Figure 2B) progress through the cell cycle. The concentration of both proteins was low from early G_1 to G_2, increased as cells formed bipolar mitotic spindles, remained high as cells separated sister chromatids and declined to low levels as cells completed cytokinesis. Unlike Pds1p, which disappears from cells slightly before sister separation (Cohen-Fix *et al.*, 1996; Figure 1C), Cdc5p and Cdc20p can still be detected in cells that have separated their sister chromatids to opposite spindle poles of the cell. This pattern of accumulation and proteolysis resembles that of the mitotic cyclin Clb2p (Ghiara *et al.*, 1991; Surana *et al.*, 1991). A similar pattern for Cdc5p has been observed by Hardy and Pautz (1996).

A significant fraction of Cdc5p co-localizes with spindle pole bodies in cells that have just formed bipolar spindles (Figure 1B). The co-localization of Cdc5p with spindle pole bodies was not detectable later in the cell cycle when cells undergo nuclear division.

Cdc5p and Cdc20p proteolysis depends on the APC

The similarity in the patterns of Cdc5p, Cdc20p and Clb2p accumulation during the cell cycle suggests that the rates of proteolysis of Cdc5p and Cdc20p, like that of Clb2p, are regulated during the cell cycle. We addressed this issue by expressing *CDC5* and *CDC20* transiently from the *GAL1-10* promoter and measuring protein levels at 30, 60 and 90 min after repression with glucose. Cdc5p and Cdc20p made in cells blocked in G_1 by α-factor are rapidly degraded, whereas Cdc5p made in cells blocked in a metaphase-like state by nocodazole is stable (Figure 3A–C). For reasons that will become apparent below, it is harder to interpret the results of such an experiment with Cdc20p.

The proteolysis of Cdc5p and Cdc20p that normally occurs during late anaphase fails to occur in *apc* mutants. Both proteins accumulate at high levels in ts *apc* mutant cells incubated at 37°C (data not shown). The APC probably has a direct role in their destruction. Proteolysis of both proteins in α-factor arrested cells was greatly reduced in arrested *cdc23-1* mutant cells (Figure 3A and C). Furthermore, mutation of sequences containing putative destruction boxes in both Cdc5p and Cdc20p (Figure 4A) slowed their rates of proteolysis. Deletion of an N-terminal extension in Cdc5p, which contains two putative destruction boxes but is neither necessary for activity nor conserved in other Polo-like kinases, greatly stabilized Cdc5p (Figure 4B). The N-terminus of Cdc20p contains also two putative destruction boxes, and deletion of both of them stabilized Cdc20p, whereas deletion of one of the destruction boxes of Cdc20p reduced its proteolysis (Figure 4C and data not shown). Finally, ubiquitination of Cdc5p in G_1 extracts was abolished by deletion of its N-terminal sequences and did not occur in extracts from *apc* mutants (Figure 5).

The phenotype of cdc5 and cdc20 mutants

To investigate the roles of Cdc5p and Cdc20p during anaphase, we carefully compared *cdc5* and *cdc20* mutant cells with wild-type cells as they progressed through the cell cycle. Sister chromatids have been shown to be paired in *cdc20* mutants by Guacci *et al.* (1994). They had done experiments using cycling culture shifted to 37°C for 3 h and had not shown kinetics of sister chromatid separation. We measured the kinetics of sister chromatid separation and the rates of disappearance of APC substrates. Unbudded G_1 cells were isolated by centrifugal elutriation (from asynchronous cultures growing at 25°C) and incubated at 37°C. *cdc20-3* mutant cells underwent DNA replication and formed bipolar spindles with identical kinetics (with regard to budding) as wild-type, but then failed to degrade Pds1p and Clb2p (data not shown) and to separate sister chromatids (Figure 6A). *cdc5(msd2-1)* mutants, in contrast to *cdc20-3*, degraded Pds1p (Figure 6B) and Clb5p (data not shown) and separated sister chromatids with wild-type kinetics (data not shown, but see below), but failed to degrade Clb2p (Figure 6B). They also failed to complete anaphase B. Unlike wild-type cells, in which sister chromatids move to opposite spindle poles of the cell,

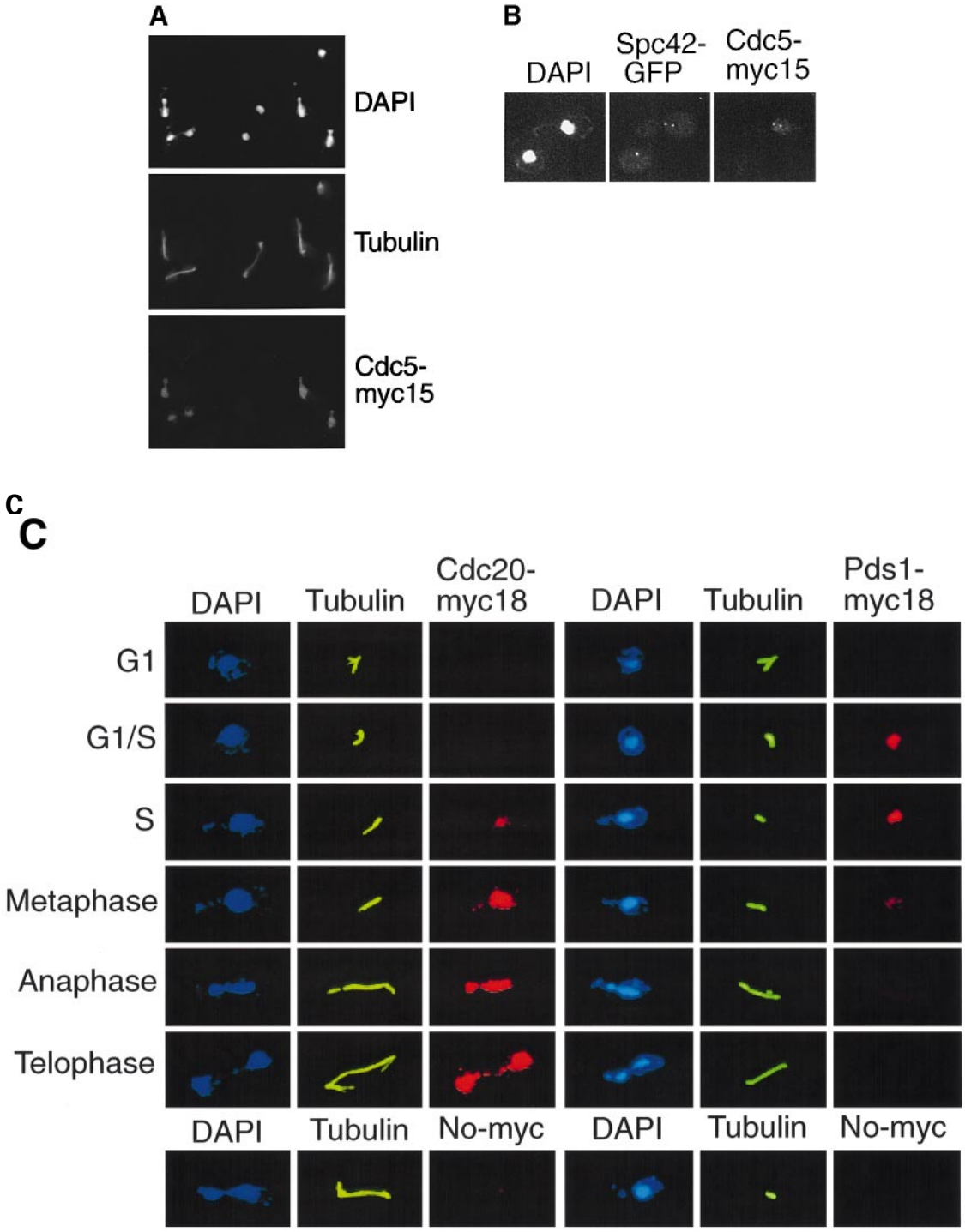


Fig. 1. Detection of Cdc5p and Cdc20p by immunofluorescence. Cells from asynchronous cultures were fixed with formaldehyde and analysed by indirect immunofluorescence. (**A**) Appearance of Cdc5p in mitotic cells. Cells of a *CDC5-myc15* strain (K6142) were stained with the anti-myc antibody to detect Cdc5-myc15p. DNA and spindles were stained with DAPI and an anti-tubulin antibody, respectively. (**B**) Association of Cdc5p with spindle pole bodies in early M-phase. Cells of a *CDC5-myc15 SPC42-GFP* strain (K6780) were double-stained with anti-myc and anti-GFP antibodies to visualize Cdc5p and the spindle pole body component Spc42p (Donaldson and Kilmartin, 1996) in the same cell. (**C**) Appearance of Cdc20p prior to the degradation of Pds1p. Cdc20p and Pds1p were detected with the anti-myc antibody in *CDC20-myc18* (K7143, left) and *PDS1-myc18* cells (K6445, right), respectively. DNA and spindles were detected by staining with DAPI and an anti-tubulin antibody. Cells were ordered according to cell cycle position from G_1 (top) to telophase (bottom).

many sister chromatids arrested on either side of the bud neck in *cdc5* mutants. Thus, *cdc20-3* mutant cells fail to initiate anaphase, whereas *cdc5(msd2-1)* mutant cells fail only to complete its later stages. Importantly, further cell cycle progression does not take place in either mutant. Neither the *cdc5* nor the *cdc20* mutant undergoes cytokinesis or re-replicates its chromosomes (Pringle and Hartwell, 1981). The partial anaphase defect of *cdc5* mutant cells might be due to the leakiness of the *cdc5(msd2-1)* mutation. We analysed cells lacking Cdc5p, which were obtained by expressing *CDC5* solely from the *GAL1-10* promoter and repressing its expression prior to

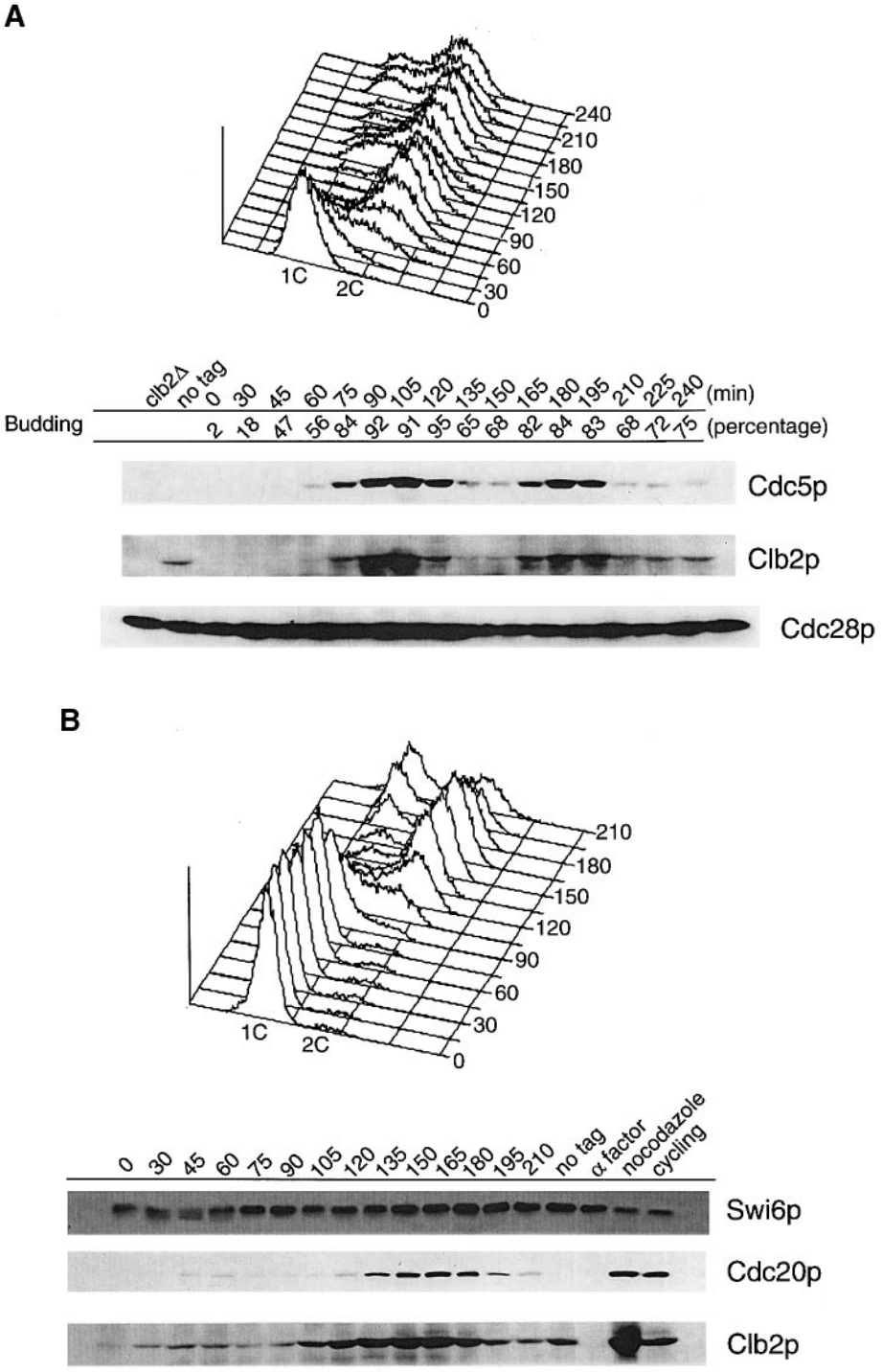


Fig. 2. Fluctuation of Cdc5p and Cdc20p protein levels during the cell cycle. (**A**) *CDC5–HA3* cells (K6019) were arrested in G_1 with α-factor and then released into fresh medium lacking α-factor at 25°C. Samples for flow-cytometric analysis of cellular DNA content (top panel) and immunoblot analysis of Cdc5–HA3p and Clb2p (bottom panel) were withdrawn at the indicated time points. Extracts from strains containing a deletion of *CLB2* (Δ*clb2*) or no epitope tag (wild-type) were used as controls. Cdc28p was detected as a loading control. (**B**) Small, unbudded G_1 cells of a *CDC20-myc18* strain (K7143) were isolated by centrifugal elutriation and inoculated into fresh medium at 25°C. Samples for flow-cytometric DNA analysis (top) and immunoblot analysis (bottom) of Cdc20-myc18p and Clb2p were taken at the indicated time points. Extracts from an asynchronous culture (cycling) or from cells arrested either in G_1 with α-factor, or in M-phase with nocodazole, were analysed in parallel. Swi6p was detected as a loading control.

elutriation. These cells separated sister chromatids with wild-type kinetics (Figure 6C), but failed to move chromatids to opposite spindle poles of the cells. Furthermore, extracts from cells arrested in mid-anaphase by depletion of Cdc5p were inactive in promoting the ubiquitination of Clb2p and Clb3p *in vitro* (Figure 6D). The arrest of *cdc5* mutants in mid-anaphase is not affected by deletion of *MAD2* (Li and Murray, 1991), which is necessary for arresting the cell cycle in response to spindle damage (data not shown).

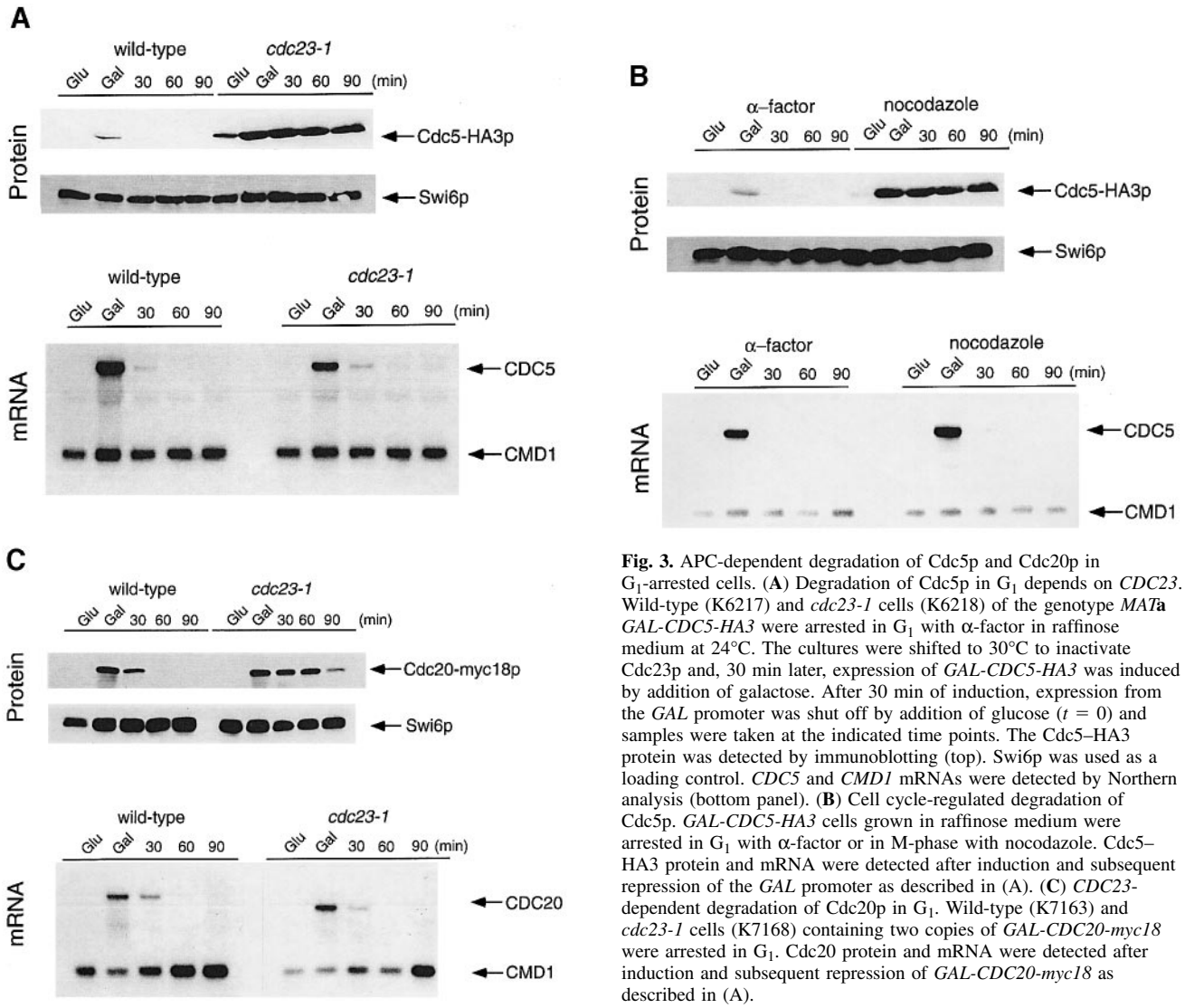


Fig. 3. APC-dependent degradation of Cdc5p and Cdc20p in G_1-arrested cells. (**A**) Degradation of Cdc5p in G_1 depends on *CDC23*. Wild-type (K6217) and *cdc23-1* cells (K6218) of the genotype *MAT***a** *GAL-CDC5-HA3* were arrested in G_1 with α-factor in raffinose medium at 24°C. The cultures were shifted to 30°C to inactivate Cdc23p and, 30 min later, expression of *GAL-CDC5-HA3* was induced by addition of galactose. After 30 min of induction, expression from the *GAL* promoter was shut off by addition of glucose ($t = 0$) and samples were taken at the indicated time points. The Cdc5–HA3 protein was detected by immunoblotting (top). Swi6p was used as a loading control. *CDC5* and *CMD1* mRNAs were detected by Northern analysis (bottom panel). (**B**) Cell cycle-regulated degradation of Cdc5p. *GAL-CDC5-HA3* cells grown in raffinose medium were arrested in G_1 with α-factor or in M-phase with nocodazole. Cdc5–HA3 protein and mRNA were detected after induction and subsequent repression of the *GAL* promoter as described in (A). (**C**) *CDC23*-dependent degradation of Cdc20p in G_1. Wild-type (K7163) and *cdc23-1* cells (K7168) containing two copies of *GAL-CDC20-myc18* were arrested in G_1. Cdc20 protein and mRNA were detected after induction and subsequent repression of *GAL-CDC20-myc18* as described in (A).

Effects of ectopic expression of CDC20 and CDC5

To investigate the significance of oscillations in the abundance of Cdc5p and Cdc20p, we expressed each protein at inappropriate stages of the cell cycle. In the case of *CDC20*, we used a strain containing three additional copies of *CDC20* under control of the *GAL1-10* promoter. We isolated unbudded G_1 cells by centrifugal elutriation from an asynchronous culture growing in raffinose medium and incubated them either in the presence of glucose, which represses *GAL1-10*, or galactose, which induces it. In glucose, Pds1p accumulated in most if not all cells during late G_1 and disappeared at around the same time as the separation of sister chromatids, whereas in galactose, Pds1p accumulated in only a small minority of cells during mitosis and disappeared from these cells earlier than it did in glucose (Figure 7A). The lack of Pds1p accumulation due to ectopic *CDC20* expression did not occur in cells which contain a disruption of *CDC26* (Figure 7B) and are defective in APC function (Zachariae *et al*., 1996). This suggests that premature accumulation of Cdc20p causes precocious Pds1p proteolysis, which is mediated by the APC.

Ectopic expression of *CDC20* had, in contrast, little or no effect on the timing of sister chromatid separation, which occurred with similar kinetics in glucose and galactose medium in cells carrying *GAL-CDC20*. This result may seem to be surprising, because it is currently thought that Pds1p prevents precocious separation of sister chromatids at least in nocodazole arrested cells. We have recently found, however, that deletion of *PDS1* does not in fact cause precocious separation of sister chromatids under normal growth conditions (R.Ciosk, C.Michaelis, W.Zachariae, A.Shevchenko, M.Mann and K.Nasmyth, submitted). Ectopic expression of *CDC20* is nevertheless deleterious to cell proliferation. Strains carrying three copies of *GAL-CDC20* can divide only once in galactose medium at 25°C.

We also investigated the effects of expressing *CDC20* from the *GAL1-10* in cells arrested with nocodazole in an M phase-like state (Figure 7C). The disruption of microtubules by this drug is normally detected by a surveillance mechanism (checkpoint) that prevents destruction of Pds1p and Clb2p and the loss of cohesion between sister chromatids. Induction of *CDC20* in a culture arrested with nocodazole caused the disappearance of Pds1p within 1 h in 75% of the cells. It also caused sister chromatid separation in 25% of the cells. Thus, accumulation of Cdc20p to higher than normal levels interferes with the mechanism by which mitotic surveillance mechanisms arrest cell cycle progress.

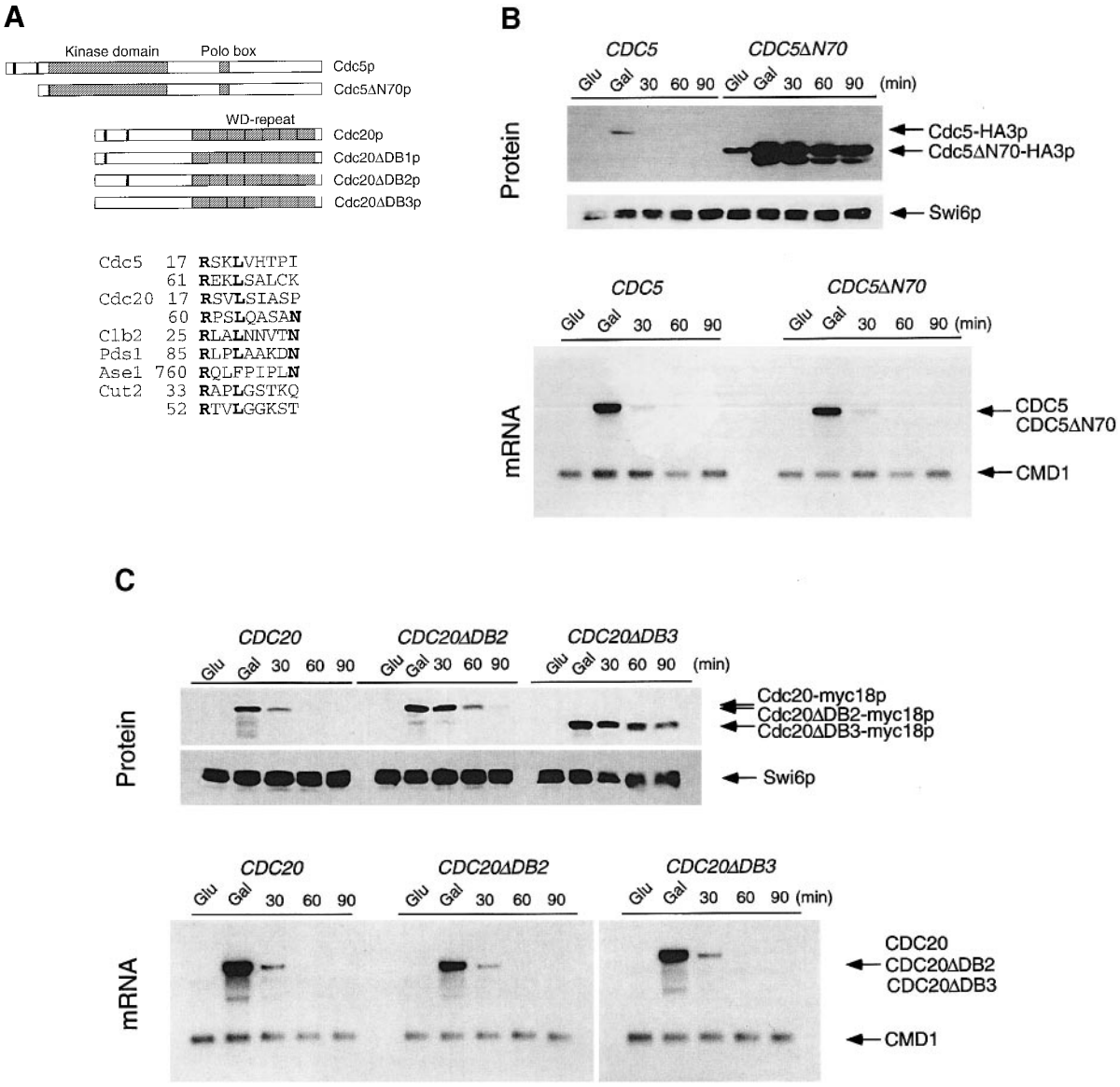


Fig. 4. Degradation of Cdc5p and Cdc20p depends on sequences containing destruction boxes. (**A**) Localization of putative destruction boxes in Cdc5p and Cdc20p (black bars) and alignment with destruction boxes of other APC substrates. (**B**) Degradation of Cdc5p in G_1 depends on the N-terminal 70 amino acids. Cells expressing either HA3-tagged Cdc5p (*GAL-CDC5-HA3*, K6217) or a version lacking the NH_2-terminal 70 amino acids (*GAL-CDC5ΔN70-HA3*, K6614) under the control of the *GAL* promoter were arrested in G_1 with α-factor. The Cdc5 proteins and their mRNAs were detected after induction with galactose and subsequent repression with glucose, as described in Figure 3A. (**C**) Efficient degradation of Cdc20p in G_1 depends on a sequence related to destruction boxes. Cells expressing from the *GAL* promoter a myc18-tagged Cdc20p (*GAL-CDC20-myc18*, K7162) or versions lacking the putative destruction box(es) (*GAL-CDC20ΔDB2-myc18*, K7302 and *GAL-CDC20ΔDB3-myc18*, K7304) were arrested in G_1 with α-factor. The Cdc20 proteins and their mRNAs were detected after induction and subsequent repression of the *GAL* promoter as described above.

To investigate the effects of ectopic Cdc5p accumulation, we analysed the consequences of expressing from the *GAL1-10* promoter a version (*CDC5ΔN70*) lacking the N-terminal sequences that confer its ubiquitination by the APC (Figure 5). Galactose-induced expression of *CDC5ΔN70* was deleterious but not lethal to otherwise wild-type cells. Because Cdc5p is essential for the onset of Clb2p proteolysis but not for that of Clb5p (data not shown), and because B-type cyclins can inhibit the cyclin proteolysis (Amon, 1997), we investigated the effects of expressing *CDC5ΔN70* in cells lacking *CLB5*. We found that this caused cells to arrest within 4 h, in a G_2-like state, lacking both mitotic spindles and Clb2p (Figure 7D), a phenotype which resembles that of mutants lacking all four mitotic B-type cyclins (Clbs1, 2, 3 and 4). Although induction of *CDC5ΔN70* did not cause an immediate cessation of cell division, it did cause the number of cells possessing Clb2p to decline within 60 min. Remarkably, the lethality due to expression of *CDC5ΔN70* was reversed by mutations that reduced APC activity; induction of *CDC5ΔN70* caused wild-type but not Δ*cdc26* or *cdc23-1* mutants to arrest division when grown at 25°C. In contrast, the phenotype was not reversed by deletion of *SIC1*, which encodes a Cdk inhibitor (Schwob *et al*., 1994). These data suggest that hyper-activation of the Cdc5p kinase

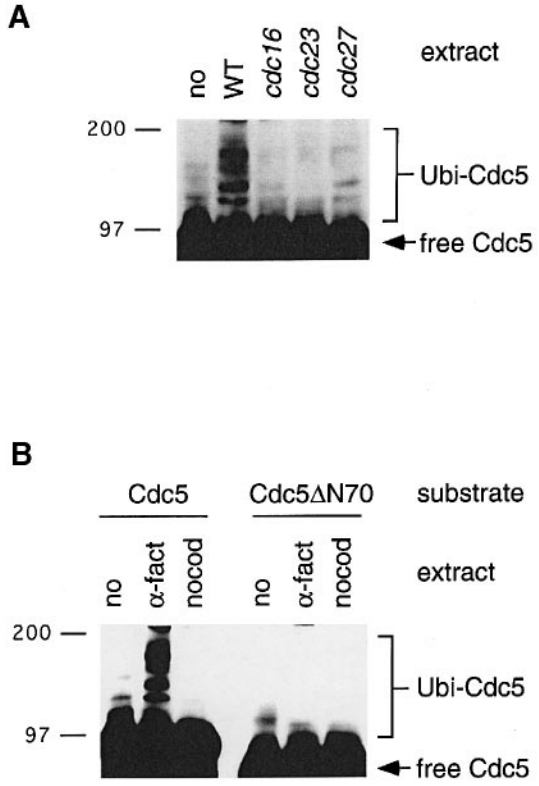


Fig. 5. Ubiquitination of Cdc5p *in vitro*. (**A**) Ubiquitination of Cdc5p *in vitro* depends on the APC. Wild-type and mutant strains (*cdc16-123, cdc23-1, cdc27-1*) of the genotype *MAT***a** Δ*pep4* Δ*bar1* were arrested in G_1 with α-factor at 24°C and then shifted to 37°C for 30 min. Uniform G_1 arrest was confirmed by flow-cytometric analysis (data not shown). Extracts were prepared and incubated with ATP and a small amount of an extract from a strain overexpressing Cdc5–HA3p as described in Materials and methods. Cdc5p–ubiquitin conjugates were detected by immunoblotting using the anti-HA antibody. (**B**) Ubiquitination of Cdc5p *in vitro* requires the N-terminal 70 amino acids and is cell-cycle regulated. Extracts were prepared from wild-type cells arrested either in G_1 with α-factor or in M-phase with nocodazole, and incubated with ATP and either Cdc5–HA3p or Cdc5ΔN70–HA3p-containing extracts. Reactions were analysed by immunoblotting.

is sufficient, at least in the absence of Clb5p, to induce APC-mediated proteolysis of Clb2p.

Causes of cell cycle arrest in cdc20 mutants

It was reported that Pds1p plays an important role in blocking anaphase in *cdc20*-arrested cells, as evidenced by elongated spindles and segregated chromosomes in *cdc20 pds1* double mutants (Yamamoto *et al.*, 1996b). To test whether the failure of *cdc20* mutants to separate sister chromatids is solely due to their failure to degrade Pds1p, we compared the timing of sister chromatid separation of *cdc20-3* Δ*pds1* double mutants with wild-type and *pds1* disruption cells. Unbudded G_1 cells were isolated by centrifugal elutriation and incubated at 37°C. Unlike *cdc20* single mutants, which fail totally to separate sister chromatids (see Figure 6A), a large fraction of the *cdc20-3* Δ*pds1* double mutant cells did so (Figure 8A). Despite this bypass of the sister chromatid separation defect, the double mutant cells failed to undergo cytokinesis and to re-replicate their genomes. They also failed to disassemble their mitotic spindles and to degrade Clb2p. Sister separation in *cdc20-3* Δ*pds1* mutants appeared to be less efficient than in wild-type cells. In wild-type cells the curves for budding and sister separation are parallel, but separated by 30 min (Michaelis *et al.*, 1997). In *cdc20-3* Δ*pds1* double mutants, sister separation commences later. Furthermore, the curves for budding and sister separation are not parallel, suggesting that sister separation does not occur as efficiently as budding. The slow and inefficient separation of sister chromatids in the double mutants could be due to the *pds1* mutation. Pds1p is thought to be needed for anaphase (Yamamoto, 1996a). To investigate this more rigorously, we compared the timing of sister separation of *cdc20-3* Δ*pds1* double mutants with that of Δ*pds1* single mutants and *cdc15-2* Δ*pds1* double mutants (R.Ciosk, C.Michaelis, W.Zachariae, A.Shevchenko, M.Mann and K.Nasmyth, submitted); loss of *CDC15* function does not affect sister separation but does prevent cytokinesis. The timing of sister separation was similar if not identical in these three mutants. It therefore appears that, whereas Cdc20p is normally essential for sister chromatid separation, it has little or no role in cells lacking Pds1p. Destruction of Pds1p is therefore Cdc20p's sole task as far as sister separation is concerned.

There are two explanations for the bypass of a *cdc20* defect by deletion of *PDS1*. The simplest is that Cdc20p is directly involved, along with the APC, in mediating proteolysis of Pds1p, which must be destroyed if cells are to initiate anaphase. An alternative explanation is suggested by the partial failure of *pds1* mutants to arrest their cell cycle in response to spindle damage. Approximately 50% of *pds1* mutant cells fail to arrest the separation of sister chromatids in the presence of nocodazole after 5 h (Yamamoto *et al.*, 1996b), but none proceeds to degrade mitotic cyclins. It is possible, therefore, that the failure of *cdc20-3* mutants to enter anaphase is an indirect consequence of damage to the mitotic apparatus, which is detected by a surveillance mechanism that blocks proteolysis of Pds1p and other APC substrates. This hypothesis would be consistent with the report that *cdc20-1* mutants arrest with abnormally thick mitotic spindles (Sethi *et al.*, 1991). However, it is unclear whether the spindle phenotype is due to a primary defect or merely due to the extended arrest in a metaphase-like state. In *cdc20-3* mutant cells, the spindle looks normal shortly after their failure to degrade Pds1p. To investigate the 'spindle damage' hypothesis, we analysed the effect of deleting *MAD2* and *BUB2* on the arrest of *cdc20-3* mutants. *MAD2* and *BUB2* are known to be required for cell cycle arrest in response to spindle damage (Hoyt *et al.*, 1991; Li and Murray, 1991). We constructed *cdc20-3* Δ*bub2* and *cdc20-3* Δ*mad2* strains and incubated unbudded G_1 cells isolated by centrifugal elutriation at 37°C. Neither double mutant separated sister chromatids (Figure 8B and C). However, we did notice that deletion of *BUB2* caused the level of Pds1p to drop gradually. Thus, whereas deletion of *PDS1* allows *cdc20-3* mutants to separate sister chromatids, neither deletion of *BUB2* nor of *MAD2* has the same effect.

Discussion

New substrates of the APC

Many of the key events from metaphase to the end of anaphase are mediated by proteolysis catalysed by the

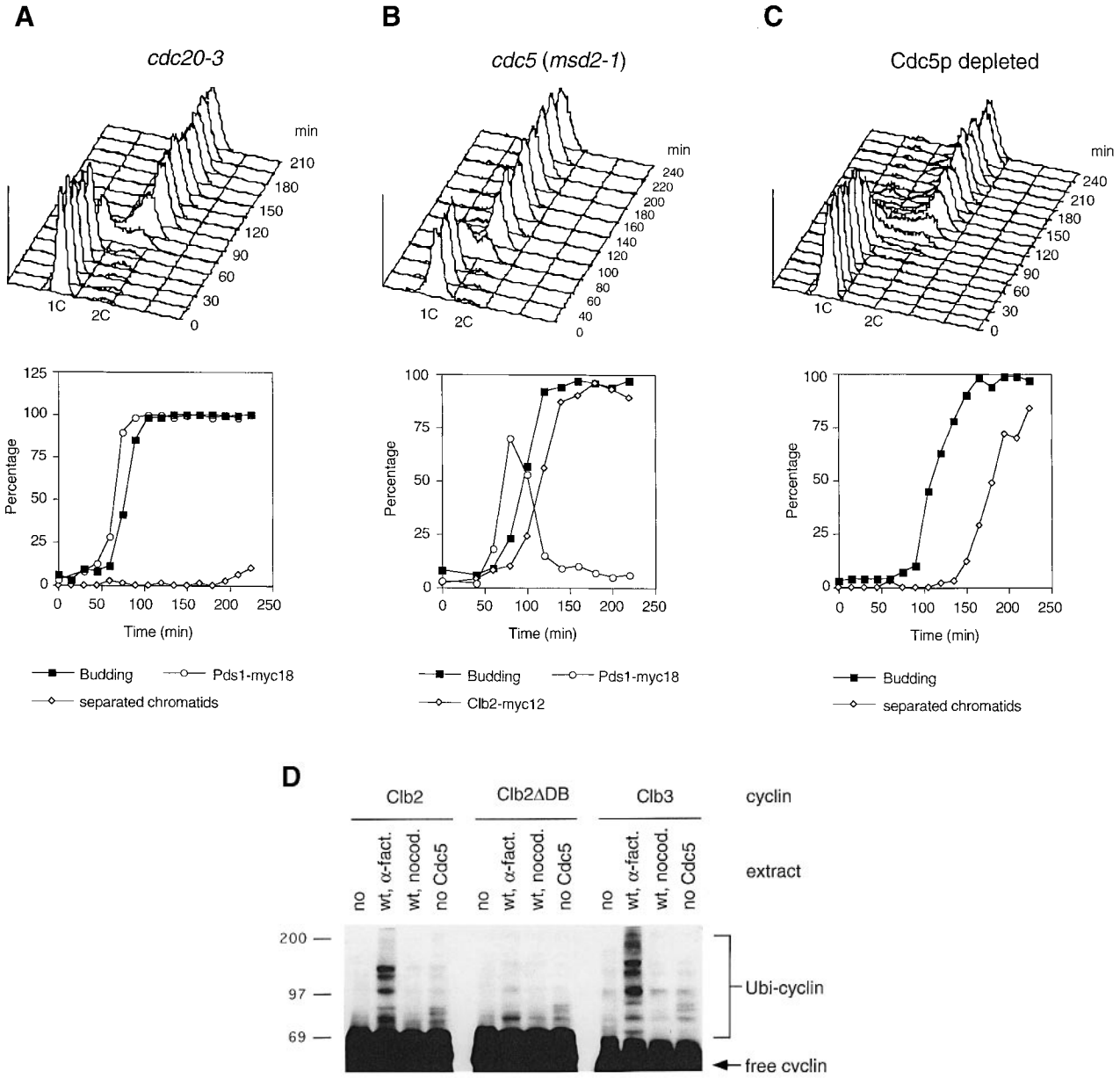
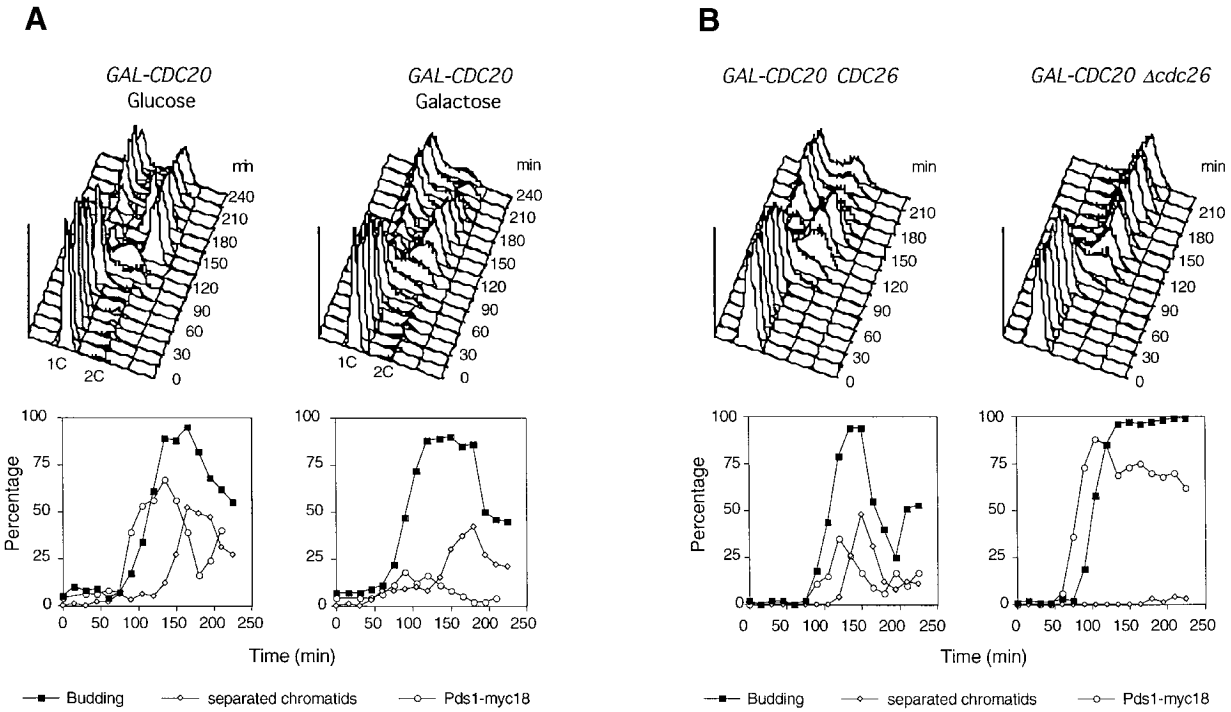
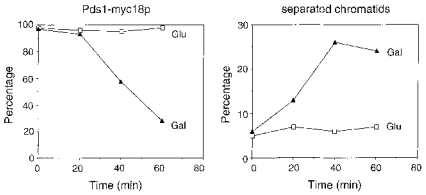
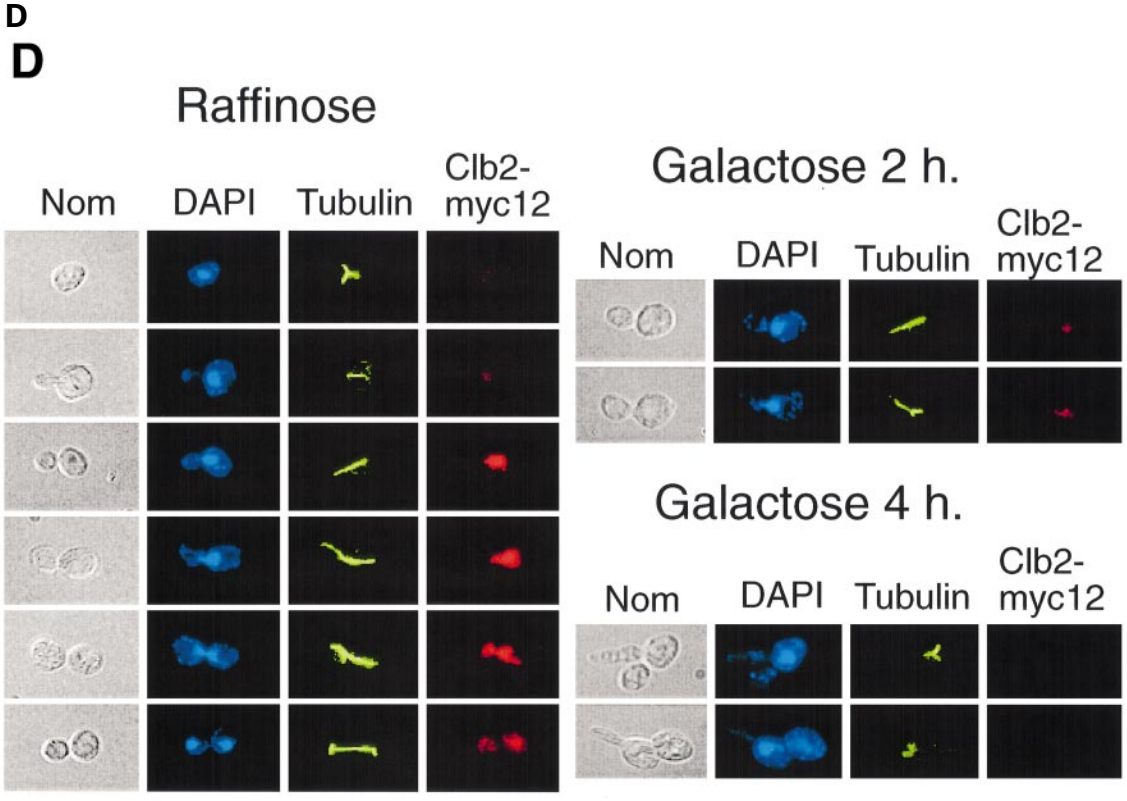


Fig. 6. Characterization of the mitotic arrest of *cdc5* and *cdc20* mutants. (**A**) Lack of Pds1p degradation and sister chromatid separation in *cdc20-3* mutants. Small, unbudded G_1 cells of a *cdc20-3* strain (K7108) containing *PDS1-myc18* and the tetR-GFP system (Michaelis *et al.*, 1997) were isolated by centrifugal elutriation and inoculated into fresh medium at 37°C. Samples were withdrawn every 15 min. The cellular DNA content was analysed by flow-cytometric analysis (top). Pds1-myc18p was detected by indirect immunofluorescence. Separated sister chromatids were determined by observing the signals from tetR-GFP in ethanol-fixed cells (bottom). (**B**) *cdc5(msd2-1)* cells are defective in Clb2p degradation but degrade Pds1p normally. Small, unbudded G_1 cells of *cdc5(msd2-1)* strains containing *PDS1-myc18* (K6451) or *CLB2-myc12* (K6876) were isolated by centrifugal elutriation and inoculated into fresh medium at 35°C. The fraction of cells which show staining from Pds1-myc18p or Clb2-myc12p was determined by indirect immunofluorescences. The time courses of cellular DNA content and budding were similar for K6451 and K6876; only results from strain K6451 are shown. (**C**) Sister chromatid separation in the absence of Cdc5p function. Strain K7004, whose sole *CDC5* gene is expressed from the *GAL* promoter, was grown in raffinose/galactose medium at 30°C. Cells were depleted of Cdc5 protein by incubation in raffinose medium for 90 min. Small, unbudded G_1 cells were isolated by centrifugal elutriation and inoculated into glucose medium. The fraction of cells with buds and with separated sister chromatids was determined from ethanol-fixed cells. (**D**) Lack of Clb2p ubiquitination in extracts from cells depleted of Cdc5p. Δ*cdc5 GAL-CDC5* cells (K7016) were grown in raffinose/galactose medium, harvested and incubated in glucose medium for 3 h causing depletion of Cdc5p and cell cycle arrest (no Cdc5). Uniform cell cycle arrest was confirmed by flow-cytometric DNA quantitation (data not shown). Wild-type cells arrested in G_1 by α-factor, or in M-phase by nocodazole, were used as controls. Extracts were prepared and incubated with the substrates Clb2–HA3p, Clb2ΔDB–HA3p and Clb3–HA3p, as described (Zachariae and Nasmyth, 1996). The Clb2ΔDB–HA3 protein lacks the destruction box. Reactions were analysed by immunoblot analysis.

Anaphase Promoting Complex (APC). To the three currently known APC substrates (mitotic B-type cyclins, Pds1p and Ase1p), we add two more: the Polo-like protein kinase Cdc5p and the WD40 protein Cdc20p. Both are degraded late during anaphase. Whether the mammalian homologues of these two proteins are also degraded by the APC is not known. Although *Drosophila* fizzy certainly does not behave like a cyclin and appears to be stable during exit from mitosis (Dawson *et al.*, 1995), the protein level of mammalian $p55^{Cdc}$ is cell cycle-regulated. $p55^{Cdc}$


A
GAL-CDC20
Glucose
GAL-CDC20
Galactose
min
240
210
180
150
120
90
60
30
0
1C
2C
Percentage
Time (min)
Budding
separated chromatids
Pds1-myc18
B
GAL-CDC20 CDC26
GAL-CDC20 Δcdc26


C
Pds1-myc18p
separated chromatids
Glu
Gal
Percentage
Time (min)


D
Raffinose
Nom
DAPI
Tubulin
Clb2-myc12
Galactose 2 h.
Galactose 4 h.

expression is initiated at the G_1/S transition, accumulates to the highest level at M phase and disappears in G_1. Inhibition of 26S proteasome prevents loss of $p55^{Cdc}$ at the M/G_1 transition, suggesting that degradation of $p55^{Cdc}$ is mediated by the cell cycle-regulated proteolytic pathway (Weinstein, 1997). Furthermore, mRNA and protein levels of mammalian Polo-like kinase, Plk1, are cell cycle-regulated in a similar manner to those of Cdc5, suggesting that Plk1 protein may also be subject to degradation by a similar type of proteolytic mechanism to those of cyclins A and B (Golsteyn *et al.*, 1995; Lee *et al.*, 1995).

Proteolysis of Pds1p is necessary for sister chromatid separation, whereas that of mitotic cyclins is important for disassembly of the mitotic spindle and for cytokinesis in yeast. To appreciate why proteolysis of Cdc5p and Cdc20p might also be important, we must consider first the functions of these two proteins.

Functions of Cdc5p and Cdc20p

CDC20 encodes a WD40 protein whose closest relative in the yeast genome, Hct1/Cdh1p, has recently been shown to be required for APC-mediated proteolysis of Clb2p (Schwab *et al.*, 1997; Visintin *et al.*, 1997). Our data, along with those of Schwab *et al.* (1997) and Visintin *et al.* (1997), suggest that most if not all proteolysis mediated by the APC depends on WD40 protein cofactors. Destruction of 'early' proteins such as Pds1p depends on Cdc20p, whereas that of 'late' proteins such as Clb2p, Cdc5p and Ase1p depends on Hct1/Cdh1p (Figure 9).

Cdc20p accumulates within nuclei shortly before Pds1p is destroyed. Several data suggest that it plays a crucial role in promoting this event. First, ectopic expression of *CDC20* causes the precocious disappearance of Pds1p and this process is dependent on the APC. Second, *cdc20* mutants fail to destroy Pds1p and to separate sister chromatids. Furthermore, by showing that the kinetics of sister chromatid separation in *cdc20-3* Δ*pds1* mutants are identical to that in Δ*pds1* single disruptants, we demonstrated that the failure of *cdc20-3* mutants to separate sister chromatids is solely due to their failure to destroy Pds1p. The accumulation of Cdc20p during mitosis is therefore essential for Pds1p destruction and may indeed be one of the triggers for this process. We could not detect any Cdc20 protein in α-factor arrested cells. However, Pds1p was shown to be unstable in these cells (Cohen-Fix *et al.*, 1996). Moreover, Visintin *et al.* (1997) had recently shown that ectopically expressed Pds1p accumulates in α-factor arrested *cdc20-1* mutant cells, which suggests that Pds1p degradation in G_1 phase depends on Cdc20p. There are two possible explanations for these seemingly contradictory results: (i) an amount of Cdc20p, too small to be detectable in our immunoblots, escapes proteolysis in G_1 phase and remains functional; (ii) Cdc20p might only be required to activate APC-dependent proteolysis of Pds1p at the onset of anaphase, and not to maintain it during the subsequent G_1 phase. *cdc20-1* cells are already impaired in cell cycle progression at the permissive temperature. These cells might enter G_1 with an APC that was activated only partially at the previous anaphase.

Though the accumulation of Cdc20p during mitosis could play an important role in helping to trigger Pds1p proteolysis during undisturbed cell cycles, its accumulation is clearly not always sufficient for this process. We noticed that cells treated with nocodazole accumulate with high levels of both Pds1p and Cdc20p. Thus spindle damage detected via Mad and Bub proteins somehow blocks Pds1p proteolysis in the presence of high levels of Cdc20p. We found that induction of higher than normal levels of Cdc20p in nocodazole cells allowed most cells to commence Pds1p proteolysis in the presence of nocodazole. High levels of Cdc20p also allow cells to bypass cell cycle arrest due to DNA damage (Amon *et al.*, 1992; Lim and Surana, 1996) which is presumably due to proteolysis of Pds1p. These high levels may titrate out inhibitors induced by DNA damage and mitotic spindle surveillance mechanisms.

Unlike Cdc20p, the abundance of Hct1/Cdh1p remains roughly constant during the cell cycle (data not shown). What then switches on proteolysis of 'late' APC substrates? We show here that the Polo-like kinase encoded by *CDC5* plays a crucial role (Figure 9). *cdc5* mutants degrade Pds1p and separate sister chromatids on schedule, but they fail to commence with proteolysis of Clb2p. However, many other proteins, such as the protein phosphatase Cdc14p, the GTPase Tem1p and the kinases encoded by *DBF2* and *CDC15* are also involved in this process (Wan *et al.*, 1992; Surana *et al.*, 1993; Shirayama *et al.*, 1994; Toyn and Johnston, 1994). *CDC5* may have an especially important role, because expression of a non-degradable version of *CDC5* is capable, at least in the absence of the S phase cyclin Clb5p, of arresting cells in a state in which Clb2p is destroyed by the APC.

Not all phenotypes of *cdc5* mutants can be explained by their failure to initiate Clb2p proteolysis. *cdc5* mutants separate sister chromatids but then fail to move the chromatids to opposite spindle poles of the cell. *apc* mutants fail to separate sister chromatids, but like *cdc20* mutants, manage to move them to the opposite ends of

Fig. 7. Consequences of overexpression of *CDC20* and *CDC5*. (**A**) Overexpression of *CDC20* prevents appearance of Pds1p. Strain K7112 containing *PDS1-myc18*, the tetR-GFP system and three copies of *GAL-CDC20* was grown in raffinose medium at 25°C, and small, unbudded G_1 cells were isolated by centrifugal elutriation. Half of the G_1 cells was inoculated into glucose medium (left panel) and the other half was inoculated into galactose medium (right panel) at 25°C. The percentage of cells with buds and separated sister chromatids was determined from ethanol-fixed cells. Pds1-myc18p was detected by immunofluorescence. (**B**) Constitutive degradation of Pds1p in cells overexpressing *CDC20* depends on the APC. A wild-type strain (K7112, 3×*GAL-CDC20 PDS1-myc18* tetR-GFP system) and a congenic Δ*cdc26* strain (K7148) were grown in raffinose medium at 25°C and small G_1 cells isolated by centrifugal elutriation were inoculated into galactose medium at 37°C. Samples were analysed as described in (A). (**C**) Overexpression of *CDC20* causes Pds1p degradation and sister chromatid separation in nocodazole-arrested cells. Strain K7112 was arrested with nocodazole in raffinose medium for 2.5 h. The culture was split in half and glucose or galactose was added. The percentage of cells containing Pds1-myc18 protein (left panel) and of cells with separated sister chromatids (right panel) was determined in samples taken every 20 min. (**D**) Overexpression of a non-degradable *CDC5* version prevents accumulation of Clb2p in Δ*clb5* cells. Strain K6848 (Δ*clb5 CLB2-myc12 GAL-CDC5ΔN70-HA3*) was grown in raffinose medium at 25°C (left panel; cells are ordered from G_1 at the top to telophase at the bottom). Expression of *GAL-CDC5ΔN70-HA3* was induced by addition of galactose (t = 0) and samples were taken after 2 and 4 h (right panel). Cells were fixed with formaldehyde and processed for immunofluorescence.

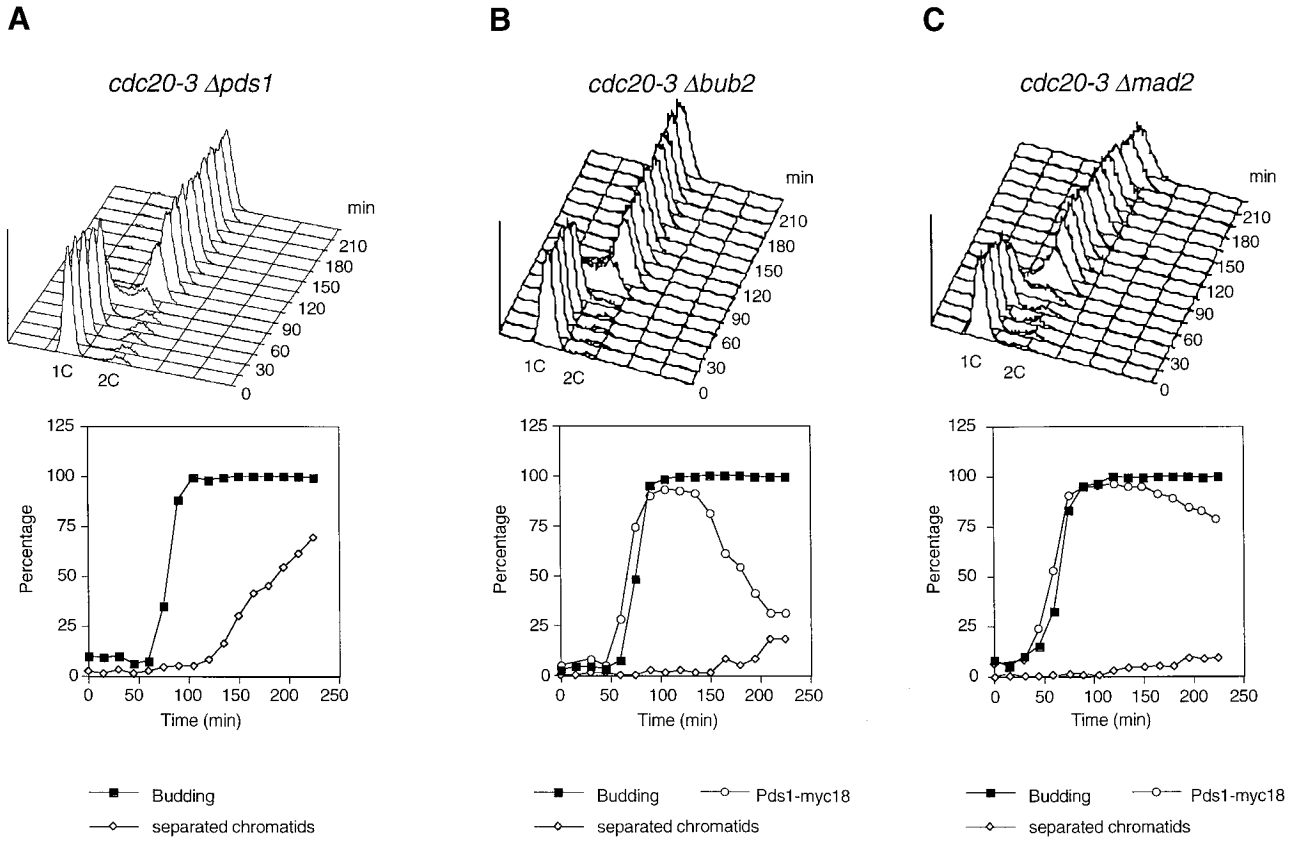


Fig. 8. Deletion of *PDS1* but not deletion of *MAD2* or *BUB2* causes *cdc20-3* mutant cells to separate sister chromatids. Strains containing the tetR-GFP system were grown at 24°C and small, unbudded G_1 cells isolated by centrifugal elutriation were inoculated into fresh medium at 37°C. The percentage of cells containing Pds1-myc18 protein and of cells containing separated sister chromatids was determined in samples taken every 15 min. All strains remained arrested as large budded cells up to 225 min after release into medium at 37°C. The following strains were analysed: (**A**) *cdc20-3 Δpds1* (K7109); (**B**) *cdc20-3 Δbub2 (*K7146); and (**C**) *cdc20-3 Δmad2* (K7174). In *cdc20-3 Δbub2* cells, the Pds1-myc18p staining decreased considerably but was still detectable in samples taken after 135 min. The residual Pds1-myc18p might be sufficient to prevent sister chromatid separation. The graph shows the percentage of cells containing high levels of Pds1-myc18 protein.

the cell if *PDS1* is deleted. The failure of *cdc5* mutants but not *pds1 apc* double mutants to execute anaphase B suggests that Cdc5p has an additional role in controlling spindle movements. Like the mammalian Polo kinase, which is associated with centrosomes (Golsteyn, 1995; Lee *et al.*, 1995), we found that Cdc5p associates with spindle pole bodies during mitosis. This raises the possibility that Cdc5p regulates the activity of both the mitotic spindle and astral microtubules.

Regulation of the APC

The dependence of Clb2p but not Pds1p proteolysis on proteins like Cdc5p emphasizes that the programme of proteolyis mediated by the APC during anaphase is a complex one. Different APC substrates are degraded with very different kinetics during metaphase, anaphase and even during G_1. Moreover, their proteolysis depends on different cofactors. The impression gained from studies with egg extracts has been of the APC switching from an inactive to active state during mitosis and then rapidly switching off again. This picture is clearly at odds with the picture emerging from yeast and with the observation that cyclin A is degraded well before cyclin B in animal cells (Hunt *et al.*, 1992).

One of the more interesting aspects of the APC-mediated proteolysis programme is its dependence on certain cofactors at certain cell cycle stages and its emancipation from these factors once cells have moved on through the programme. For example, *cdc20-3 Δpds1* mutants never initiate Clb2p proteolysis, even though they manage to separate sister chromatids to opposite spindle poles of the cell. Cdc20p is therefore essential for initiating Clb2p proteolysis. However, once cells have entered G_1, and Hct1/Cdh1p has acquired the ability to drive Clb2 proteolysis, Cdc20p is no longer required. Indeed, it is no longer present at appreciable levels in these cells. Likewise, Cdc5p is essential for switching on Clb2p proteolysis during anaphase, but it is neither present nor essential during early G_1. To explain this phenomenon, we propose that Cdc20p and Cdc5p might be required to overwhelm inhibitors of Clb2p ubiquitination. Once overwhelmed, these inhibitors may in turn be destroyed by the APC, which then allows Clb2p ubiquitination mediated by Hct1/Cdh1p and the APC to become independent of Cdc5p and Cdc20p.

Clb/Cdk1 kinases are candidates for such an inhibitor (in yeast but clearly not in animal egg extracts), because expression of a non-degradable version of *CLB2* from the *GAL* promoter prevents rapid proteolysis of a myc-tagged but otherwise wild-type Clb2p protein made from the endogenous *CLB2* gene (data not shown). It is interesting in this regard that proteolysis of Pds1p mediated by

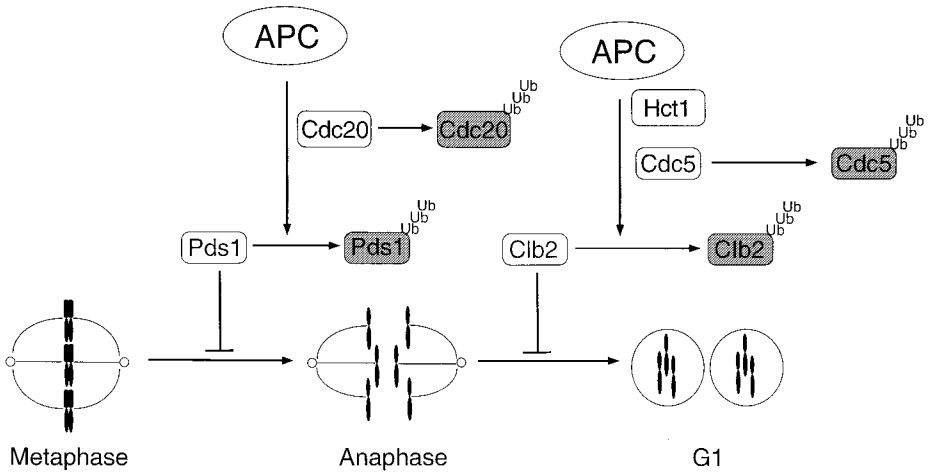


Fig. 9. Model for the role of the Polo kinase Cdc5p and the WD40 repeat proteins Cdc20p and Hct1/Cdh1p in APC-mediated ubiquitination. Cdc20p is required for ubiquitination by the APC of the 'early' substrate Pds1p, which functions as an inhibitor of anaphase. Cdc5p is required to activate Hct1/Cdh1p-dependent ubiquitination of 'late' substrates such as Clb2p, whose degradation is important for exit from mitosis. Cdc5p and Cdc20p are themselves substrates of the APC.

Cdc20p/APC, which takes place shortly before separation of sister chromatids, clearly occurs in the presence of active Clb/Cdk1 kinases. Furthermore, expression of non-degradable *CLB2* does not block Pds1p proteolysis. Thus, though Clb/Cdk1 kinases might inhibit 'late' Hct1/Cdh1p/APC mediated proteolyis (Clb2p, Cdc5p and Ase1p), they do not inhibit the earlier proteolysis of Pds1p mediated by Cdc20p. Ubiquitination mediated by Cdc20p might be less sensitive, or not at all sensitive, to Clb kinases than that mediated by Hct1/Cdh1p. It might therefore play a crucial role in starting the Clb2p proteolysis process, which is only later taken over by Hct1/Cdh1p when Clb kinases have become less active.

We finally turn to the issue of why Cdc5p and Cdc20p are destroyed by the APC during anaphase. Both proteins seem to have a role in switching on proteolysis of mitotic cyclins and their absence during early G_1 may facilitate the down regulation of cyclin and Pds1p proteolysis, which occurs as cells start the next cycle. Cdc5p barely changes, if at all, during the cell cycle of *HCT1/CDH1* disruptants (data not shown). Nevertheless, expression of a nondegradable version of Cdc5p is clearly deleterious to cells, as is ectopic expression of *CDC20*. The proteolysis of these two proteins presumably contributes to the high fidelity with which cells duplicate their constituents and segregate them to both daughter cells.

Materials and methods

Strains and media

All yeast strains were derivatives of W303 (also called K699: *HML*a *HMR***a** *ho ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11, 15 ura3*). Strains were grown in YEP medium supplemented with 2% glucose (Glu), or raffinose (Raf), or raffinose plus galactose (RafGal). Media were prepared as described by Sherman (1991). For the ubiquitination assay, strains were grown in YEP medium containing 50 mM sodium phosphate (pH 6.5, $YEPP_i$ medium).

Plasmid and strain constructions

Standard genetic techniques were used to manipulate yeast strains (Sherman, 1991) and standard protocols were used for DNA manipulation (Maniatis *et al.*, 1982). For epitope-tagging of *CDC5* at the C-terminus, sequences from +1081 to +2337 (ATG = +1; Kitada *et al.*, 1993) were cloned into YIplac211. The YIplac series of integrative vectors was described by Gietz and Sugino (1988). Cassettes encoding several HA or myc epitope tags were inserted into an *Xba*I site created in front of the stop codon. The resulting plasmids were cut with *Sal*I and integrated at the *CDC5* locus, giving strains K6019 (*CDC5-HA3*) and K6142 (*CDC5-myc15*). To tag the *PDS1* gene, sequences from +525 to +1544 (Yamamoto *et al.*, 1996a) were cloned into YIplac128. After insertion of myc tags into an *Xba*I site in front of the stop codon, the plasmid was cut with *Hin*dIII and integrated into the *PDS1* locus giving strain K6445 (*PDS1-myc18*). The *CDC20* gene was tagged at the N-terminus. Sequences from –412 to +757 (Sethi *et al.*, 1991) were cloned into YIplac204, and myc-cassettes were inserted into an *Spe*I site created at position +13. The resulting plasmid was cut with *Mlu*I for integration at the *CDC20* locus, to give strain K7143 (*CDC20-myc18*). All strains grew normally at 37°C, demonstrating that the epitope-tagged proteins were fully functional. To manipulate expression of *CDC5-HA3* and *CDC20-myc18*, the genes were fused to the *GAL1-10* promoter and cloned into the integrative vectors YIplac211 and YIplac128, respectively. The *GAL-CDC5ΔN70-HA3* fusion was constructed by deleting *CDC5* sequences from +4 to +207 using PCR. *CDC20* sequences from +198 to +224 and +49 to +75 were deleted from the *GAL-CDC20-myc18* plasmid to construct the *GAL-CDC20ΔDB1-myc18* fusion and *GAL-CDC20ΔDB2-myc18* fusion, respectively. Both sequences were deleted from the *GAL-CDC20-myc18* plasmid to construct *GAL-CDC20ΔDB3-myc18* fusion. The *GAL-CDC5* and *GAL-CDC20* constructs were integrated into the yeast genome at the *ura3* and the *leu2* loci, respectively. The *CDC26*, *BUB2* and *MAD2* genes were disrupted by the PCR-targeting method. Cells were transformed with a cassette containing the *Schizosaccharomyces pombe his5*$^+$ which complements the *his3* mutation of W303. The cassette was amplified by PCR from pFA6a-HIS3MX6 (Wach *et al.*, 1997) with target gene-specific primers.

Analysis of RNA and protein

The methods described by Cross and Tinkelenberg (1991) and Price *et al.* (1991) were used for RNA isolation and Northern blot analysis, respectively. Immunoblot analysis was performed as described by Zachariae and Nasmyth (1996). Clb2p, Cdc28p and Swi6p were detected using polyclonal rabbit antisera. The monoclonal antibodies 12CA5 and 9E10 were used to detect HA- and myc-tagged proteins, respectively.

Ubiquitination assay

The *in vitro* ubiquitination assay was carried out according to Zachariae and Nasmyth (1996). Strains were grown in 0.3 l medium to a density of 7×10^6 cells/ml and then arrested as described in the figure legends. Cells were converted to spheroplasts which were resuspended in 0.33 volumes (0.2 ml) of buffer A (150 mM HEPES–KOH pH 7.3, 180 mM potassium acetate, 15 mM Mg acetate, 30% glycerol, 3 mM DTT, 0.3 mM PMSF, 0.3 μg/ml pepstatin) and lysed by shaking with glass beads. The lysate was centrifuged for 5 min in an Eppendorf microcentrifuge and the supernatant was used for the ubiquitination reaction. Prior to the assay, extracts were normalized to a protein concentration

of 30 mg/ml with 0.33×buffer A. Extracts containing HA-tagged Cdc5 proteins used as substrates were prepared from strains K6280 [*MAT***a** *GAL-CDC5-HA3* (several copies)] and K6612 [*MAT***a** *GAL-CDC5*Δ*N70-HA3* (one copy)], which express equivalent amounts of Cdc5 proteins. Strains were grown at 30°C in 0.4 l of YEPP_iRaf medium to 7×10^6 cells/ml and Cdc5p expression was induced by addition of 2% galactose for 160 min. Cells were converted to spheroplasts, which were broken in 0.25 ml of buffer A. The lysate was first centrifuged for 5 min in an Eppendorf micro-centrifuge and then for 20 min at 9000 *g*. Extracts were frozen in liquid nitrogen and stored at –80°C. The protein concentration of the substrate extracts was 35 mg/ml. Reactions containing 32 µl extract, 4 µl of 10×ATP regenerating system and 1.6 µl Cdc5–HA3p or Cdc5ΔN70–HA3p extract in a total volume of 40 µl were assembled and processed essentially as described. Reactions were separated in 7.2% SDS–polyacrylamide gels and Cdc5p and Cdc5p–ubiquitin conjugates were detected by immunoblotting using the antibody 12CA5.

Other techniques

To arrest cells permanently in G_1, 0.5 µg/ml of α-factor was added to a culture of *bar1* cells. For arrest/release experiments, *BAR1* strains were arrested in G_1 with 1 µg/ml of α-factor for 2.5 h at 25°C. Cells were harvested by filtration, washed and released into fresh medium lacking α-factor. Cells were arrested in mitosis by addition of nocodazole to 15 µg/ml. The isolation of small, unbudded G_1 cells by centrifugal elutriation was carried out as described by Schwob and Nasmyth (1993). The DNA content of cells stained with propidium iodide was measured on a Becton Dickinson FACScan (San Jose, CA) as described by Epstein and Cross (1992). Indirect immunofluorescence and photomicroscopy were performed as described by Piatti *et al.* (1996). Sister chromatids were observed in strains carrying the tetR-GFP system essentially as described recently (Michaelis *et al.*, 1997).

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