The Polo-related kinase Cdc5 activates and is destroyed by the mitotic cyclin destruction machinery in *S. cerevisiae*

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Background: Following chromosome segregation in anaphase, ubiquitin-dependent degradation of mitotic cyclins contributes to the exit from mitosis. A key step in this process is catalyzed by a ubiquitin–protein ligase known as the anaphase-promoting complex (APC), the regulation of which is poorly understood. The Polo-related protein kinase Cdc5 in *Saccharomyces cerevisiae* might encode a regulator of the APC, because *cdc5* mutant cells arrest with a late mitotic phenotype similar to that observed in cells with defective cyclin destruction.

Results: We investigated the role of Cdc5 in the regulation of mitotic cyclin degradation. In *cdc5-1* mutant cells, we observed a defect in the destruction of cyclins and a reduction in the cyclin–ubiquitin ligase activity of the APC. Overexpression of *CDC5* resulted in increased APC activity and mitotic cyclin destruction in asynchronous cells or in cells arrested in metaphase. *CDC5* mutation or overexpression did not affect the degradation of the APC substrate Pds1, which is normally degraded at the metaphase-to-anaphase transition. Cyclin-specific APC activity in cells overexpressing *CDC5* was reduced in the absence of the APC regulatory proteins Hct1 and Cdc20. In G1, Cdc5 itself was degraded by an APC-dependent and Hct1-dependent mechanism.

Conclusions: We conclude that Cdc5 is a positive regulator of cyclin-specific APC activity in late mitosis. Degradation of Cdc5 in G1 might provide a feedback mechanism by which the APC destroys its activator at the onset of the next cell cycle.

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Background

The events of mitosis are governed by oscillations in the activity of cyclin-dependent protein kinases (Cdks), the activity of which requires association with mitotic cyclins [1–4]. Activation of mitotic Cdk–cyclin complexes, such as the Cdc2–cyclin B complex of vertebrates or the Cdc28–Clb complex of *Saccharomyces cerevisiae*, triggers the sequence of events that lead to chromosome segregation. Inactivation of mitotic Cdks is then required to allow progression from anaphase to G1. The major mechanism of Cdk inactivation in late mitosis is ubiquitin-dependent cyclin proteolysis by the 26S proteasome (reviewed in [5–7]). Overexpression of non-degradable mutant cyclin proteins results in a late anaphase arrest in a wide range of eukaryotes, suggesting that Cdk inactivation by cyclin degradation is a highly conserved mechanism controlling the exit from mitosis [8–14]. Complete cyclin destruction is not absolutely required for mitotic exit, however: additional Cdk inactivation mechanisms might contribute to mitotic exit under some conditions [15–19].

The ubiquitination of mitotic cyclins is a key regulatory step in their destruction. Cyclin ubiquitination, like that of other proteins, begins with the transfer of ubiquitin from the ubiquitin-activating enzyme (E1) to one of a family of ubiquitin-conjugating enzymes (E2) [20–22]. Ubiquitin is then transferred from E2 to the substrate by a ubiquitin–protein ligase (E3). For mitotic cyclins, the E3 is a large, multimeric enzyme known as the cyclosome or anaphase-promoting complex (APC) [21,23–27], the activity of which is low during interphase and high in late mitosis and G1 [21,23,28–31]. The mechanisms governing APC activity during the cell cycle are not well understood. In extracts of clam and frog cells, the mitotic Cdc2–cyclin B complex itself promotes activation of the APC [23,29,32]. On the other hand, in *S. cerevisiae* there is genetic evidence that APC-dependent cyclin destruction is inhibited by Cdc28–cyclin activity [33]. The cyclic AMP-dependent protein kinase might also contribute to regulation of the APC [34,35].

The APC is required not only for cyclin destruction but for the destruction of additional proteins, such as Pds1 and Cut2, that inhibit chromosome separation [36,37]. As a result, general defects in APC activity cause a metaphase arrest with unseparated chromosomes [10,24], not the late anaphase arrest that results from overexpression of non-degradable cyclins. Recent genetic evidence suggests that

the targeting of different substrates to the APC might be controlled by members of the Cdc20 protein family [17,18,38]. In *S. cerevisiae*, Cdc20 is required primarily for APC-mediated destruction of Pds1, whereas the related protein Hct1/Cdh1 is required for destruction of mitotic cyclins (for example, Clb2) and Ase1 [17,18]. Cdc20 might serve as a target for regulation in cells that are blocked in metaphase due to defects in spindle assembly. These cells arrest with high levels of Clb2, and recent evidence suggests that this checkpoint arrest is dependent on an inhibitory interaction between checkpoint signaling proteins and Cdc20 [39,40].

Whereas defects in APC subunits or Cdc20 cause a metaphase arrest before chromosome segregation, mutations in a distinct group of genes cause a late-anaphase arrest similar to that seen in cells overexpressing non-degradable mutant cyclin [41]. These genes encode potential regulatory proteins related to protein kinases (Cdc5, Cdc15, Dbf2), phosphatases (Cdc14), and GTPases (Tem1) [42–46], and an intriguing possibility is that these proteins are components of regulatory networks that promote Cdc28 inactivation by cyclin-specific APC activity or other inhibitory mechanisms.

Among the genes that function late in mitosis, *CDC5* is of particular interest because it encodes a member of the highly conserved family of Polo-related protein kinases [42]; the members of this family have been implicated in the control of mitosis in a wide range of eukaryotes [47]. In *Drosophila* and human cells, the related kinases Polo and Plk1, respectively, are required for normal maturation of centrosomes and the mitotic spindle [48,49]. The Polo-related kinases Plx1 in *Xenopus* [50] and Prk1 in human cells [51] might promote mitotic entry by activating the phosphatase Cdc25C, a key activator of Cdc2–cyclin B1. The Plo1 protein of *Schizosaccharomyces pombe* is required for both mitotic entry and exit: mutations in *plo1*$^+$ result in spindle defects as well as a failure to undergo septum formation and cytokinesis [52]. Unlike Polo-related kinases of other eukaryotes, the Cdc5 protein of *S. cerevisiae* does not appear to be required for mitotic entry: the late mitotic arrest of *cdc5* mutants suggests that mitosis and chromosome separation occur normally in the absence of *CDC5* function, but that progression beyond anaphase is blocked.

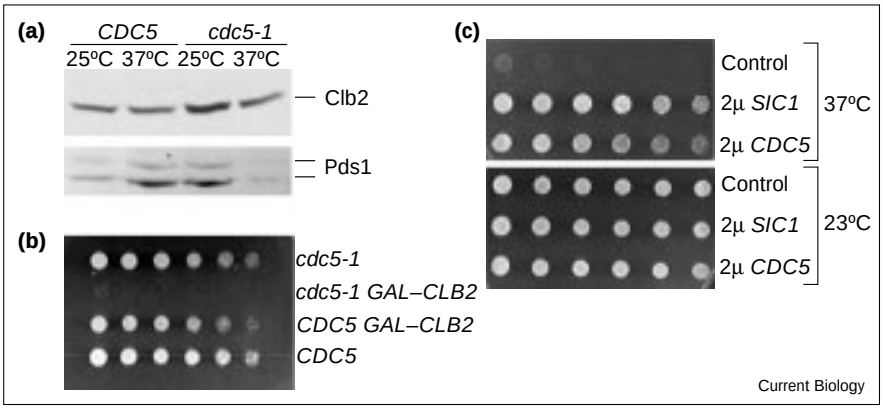
We tested the hypothesis that the late mitotic arrest of *cdc5* mutants reflects a requirement for Cdc5 in the inactivation of Cdc28 following chromosome segregation. We found that the cyclin-specific ubiquitination activity of the APC is reduced in the *cdc5-1* mutant and increased in cells overexpressing *CDC5*, suggesting that Cdc5 is a rate-limiting determinant of APC activity and cyclin destruction. Moreover, Cdc5 itself is degraded in G1 by an APC-dependent and Hct1-dependent pathway. By destroying its activator at the end of mitosis, the APC might set the stage for its own inactivation at the end of the next G1 phase.

Results

Mutants in *CDC5* are defective in destruction of Clb2

The arrest phenotype of *cdc5-1* is reminiscent of cells overexpressing non-degradable Clb2, raising the possibility that this mutant is defective in cyclin destruction. Consistent with this possibility, we found that Clb2 levels were elevated in the *cdc5-1* mutant at the permissive temperature and remained high at the restrictive temperature (Figure 1a). We also analyzed levels of Pds1, an APC substrate normally degraded at the metaphase-to-anaphase transition. Pds1 levels were reduced in the *cdc5-1* mutant at the restrictive temperature, consistent with the anaphase arrest in these mutants (Figure 1a).

Figure 1



The *cdc5-1* mutant is specifically defective in Clb2 proteolysis. **(a)** Wild-type (JC243) and *cdc5-1* (JC247) strains were shifted from 25°C to 37°C for 3 h. Endogenous Clb2 and HA-epitope-tagged Pds1 were analyzed by western blotting with affinity-purified anti-Clb2 antibody and the anti-HA antibody 16B12, respectively. Pds1 normally migrates as a doublet. **(b)** Cultures of wild-type (JC95) and *cdc5-1* (JC94) strains containing a *GAL* promoter-driven *CLB2* construct, as well as control strains lacking the *CLB2* construct, were serially diluted and spotted to a plate containing 2% galactose at 23°C. **(c)** A *cdc5-1* strain (JC34) was transformed with a multicopy vector (pRS426) containing *SIC1*, *CDC5*, or no insert (control); overnight cultures were serially diluted and spotted to YPD plates at 23°C and 37°C.

We also examined the effect of *CLB2* overexpression on growth of the *cdc5-1* mutant (Figure 1b). Whereas the growth of a wild-type strain was not affected by *CLB2* overexpression, excess Clb2 was lethal in the *cdc5-1* mutant at the permissive temperature, suggesting that *cdc5-1* mutant cells are compromised in their ability to destroy mitotic cyclins. Furthermore, overproduction of Sic1, an inhibitor of Cdc28–Clb kinase activity [53,54], allowed growth of *cdc5-1* cells at 37°C (Figure 1c), as shown previously [15]. Thus, the primary defect in *cdc5-1* mutants is an inability to inactivate mitotic Cdc28 activity.

APC activity is compromised in *cdc5-1* mutants

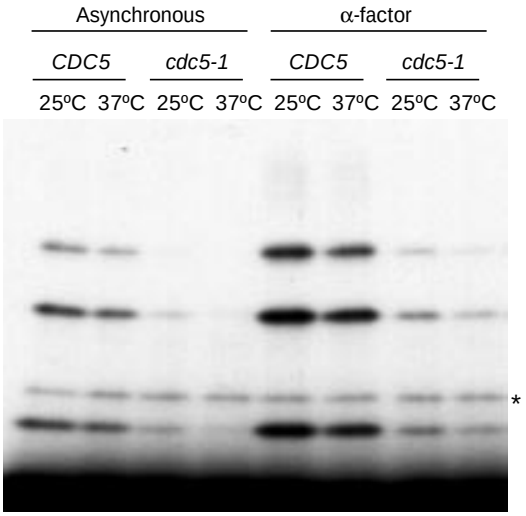
To test the possibility that impaired Clb2 degradation in the *cdc5-1* mutant is due to a defect in APC-mediated ubiquitination, we developed an assay to measure the cyclin–ubiquitin ligase activity of the APC *in vitro*. We isolated the APC from yeast extracts by immunoprecipitation with antibodies against APC subunits, including epitope-tagged Cdc16, epitope-tagged Cdc27, or endogenous Cdc26. Immunoprecipitates were incubated with excess purified yeast E1 (Uba1) and E2 (Ubc4), bovine ubiquitin, ATP, and ^{125}I-labeled sea urchin cyclin B amino terminus [10,55]. Analysis of reaction products by polyacrylamide gel electrophoresis revealed the formation of a series of ubiquitin-conjugated substrate molecules. Unlike previously described yeast APC assays in which activity is measured in crude cell lysates, activity measured by our method reflects the activity that is tightly associated with only the E3 component. Formation of ubiquitin–cyclin conjugates required ATP and all three ubiquitination enzymes, and activity increased linearly with increasing amounts of APC (data not shown). Activity was absent in the APC subunit mutants *cdc16-1* and *cdc23-1* (see below; data not shown).

We compared the APC activity in *cdc5-1* mutants to that in wild-type strains (Figure 2). APC from *cdc5-1* cells arrested at 37°C possessed negligible activity, suggesting that high Clb2 levels and the late-anaphase arrest in this mutant might be due to loss of APC activity. We also examined *cdc5-1* cells that had been arrested in G1 with mating pheromone (α-factor) and then shifted to the non-permissive temperature (Figure 2). APC activity from G1-arrested *cdc5-1* mutants at the permissive temperature was significantly lower than that in wild-type cells. Shift to the restrictive temperature caused a slight additional decrease in APC activity that was also seen in wild-type cells, suggesting that Cdc5 is not required to maintain APC activity in G1.

Peak Cdc5 kinase activity precedes activation of the APC in mitosis

Cdc5 mRNA is known to peak at the G2–M phase transition [42], and Cdc5 protein levels are low in G1, rise to a peak at G2–M, and decrease as cells enter the next G1 phase [56] (Figure 3c). The timing of Cdc5 activity in the yeast cell cycle, and particularly the relationship between Cdc5 activity and APC activity, are unknown. We therefore compared APC and Cdc5 activities in cells after release from a G1 arrest. APC activity was high in G1 (Figure 3a), dropped dramatically as cells passed Start, and remained low while Clb2 levels and Clb2-associated kinase activity increased. Shortly after the peak in Clb2-associated activity, APC activity increased abruptly, remained high as Clb2 levels declined, and then decreased as cells entered the next cell cycle (Figure 3). Cdc5-associated kinase activity and protein levels (Figure 3b,c) were undetectable in G1 cells, consistent with our observation that Cdc5 is not required for maintenance of APC activity. Cdc5 activity rose to a peak just before the increase in APC activity, consistent with the possibility that Cdc5 is a positive regulator of the APC.

Figure 2

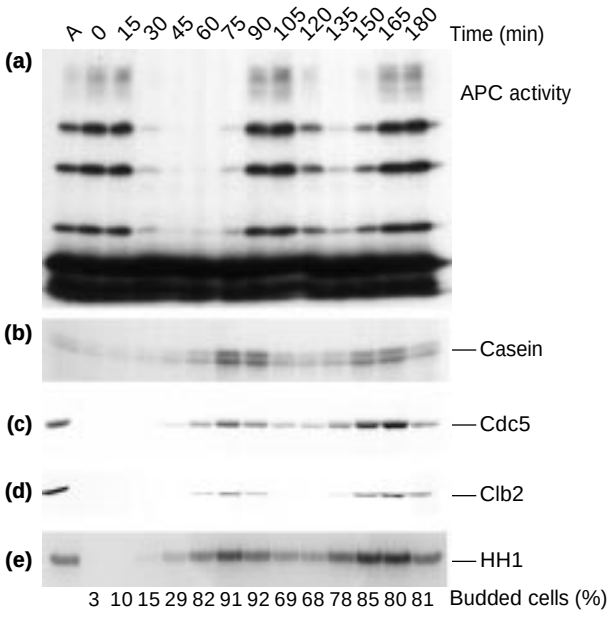


Cyclin–ubiquitin ligase activity of the APC is defective in *cdc5-1* mutants. Wild-type (AFS92) and *cdc5-1* (JC34) strains were transformed with a CEN–ARS plasmid carrying *CDC16HA* under the control of its own promoter. Asynchronous cultures were grown to mid-log phase at 25°C (lanes 1,3) or shifted to 37°C for 2 h (lanes 2,4). In lanes 5–8, cells were treated with 1 μg/ml α-factor until over 90% of cells displayed G1 morphology, then grown at either 25°C (lanes 5,7) or shifted to 37°C for 1 h (lanes 6,8). Cell lysates (500 μg) were prepared, and the APC was immunoprecipitated with the anti-HA antibody 12CA5. APC activity in immunoprecipitates was measured by the conjugation of ubiquitin to ^{125}I-labeled cyclin amino terminus as described in Materials and methods. A ladder of ubiquitin-conjugated substrate is apparent above the non-ubiquitinated substrate at the bottom of the figure. The asterisk indicates a background band present in the non-ubiquitinated substrate. The level of Cdc16 protein is not affected by the *cdc5-1* mutation (data not shown).

CDC5 overexpression decreases Clb2 levels but does not significantly affect Pds1

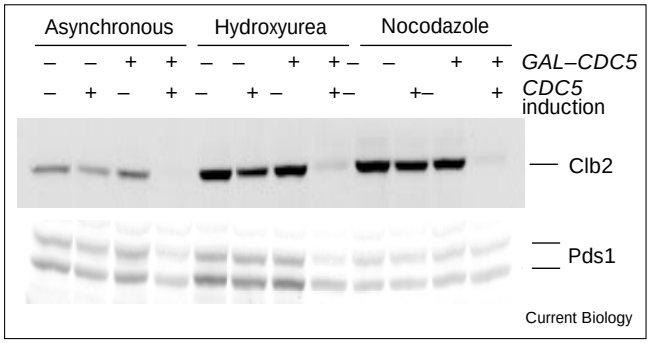
If Cdc5 is a limiting regulator of cyclin proteolysis, then increasing Cdc5 levels might decrease cyclin levels. We

Figure 3



Cell-cycle regulation of APC and Cdc5-dependent kinase activities. A *cdc5::CDC5^{HA3} cdc27::CDC27^{MBP}* strain (JC35) was synchronized in G1 by treatment with 1 μg/ml α-factor (over 96% of cells were in G1). Cells were released into YPD at 25°C and cell lysates were prepared from samples taken every 15 min. The lane labeled A represents a sample from asynchronous cells. (a) APC was immunoprecipitated from cell extracts (200 μg) with anti-MBP (maltose-binding protein) antibody, and cyclin–ubiquitin ligase activity was measured *in vitro* as described in Materials and methods. (b) Cdc5 was immunoprecipitated from 750 μg of cell extract with 12CA5, and Cdc5-dependent casein kinase activity was measured as described in Materials and methods. (c) Cdc5 was detected by western blotting with the 12CA5 antibody. (d) Clb2 was detected by western blotting with affinity-purified anti-Clb2 antibody. (e) Clb2-associated histone H1 kinase activity (HH1) was measured in anti-Clb2 immunoprecipitates from 100 μg cell extract. The percentage of budded cells in each sample is shown below the gels.

Figure 4



Cdc5 overproduction results in decreased Clb2 levels. A strain containing three integrated copies of *GAL–myc3–CDC5* (JC251) and a control strain lacking inducible *CDC5* (JC 243) were grown in YEP/raffinose to early log phase. Cultures were arrested either in S phase with 10 mg/ml hydroxyurea, in mitosis with 10 μg/ml nocodazole, or were left untreated as asynchronous cultures. Cultures were divided, and galactose or dextrose was added to 2% final concentration for 3 h to induce (+) or repress (–) *CDC5* expression, respectively. Clb2 and HA-epitope-tagged Pds1 were detected by western blotting of cell extracts with anti-Clb2 and 16B12 antibodies, respectively. In four separate experiments, no reproducible changes in Pds1 levels were observed in cells overexpressing *CDC5.*

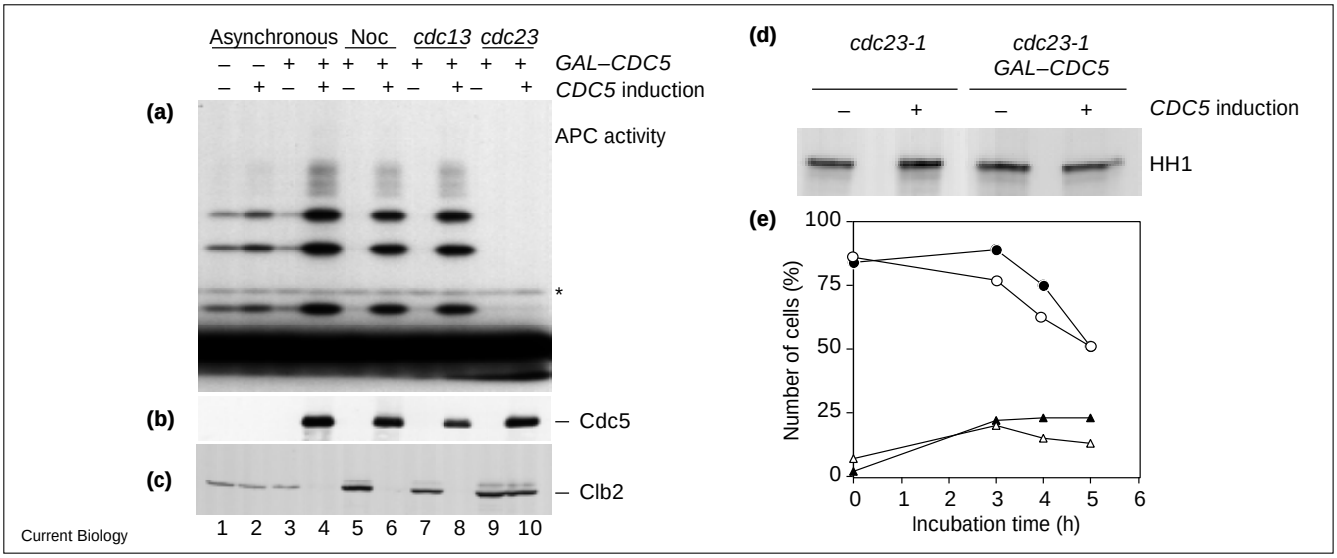
addressed this issue by testing the effects of *CDC5* overexpression. We constructed a strain containing three integrated copies of *myc3–CDC5* (which complements the *cdc5-1* defect) under the control of the *GAL* promoter. In an asynchronous population, induction of *CDC5* expression resulted in a dramatic drop in Clb2 levels (Figure 4). In four separate experiments, Pds1 levels did not change significantly after *CDC5* induction. In cells treated with hydroxyurea or nocodazole, which are arrested in S phase or mitosis respectively with high Clb2 levels, Cdc5 overproduction also triggered extensive Clb2 destruction. Once again, Pds1 levels were not reproducibly affected. Overexpression of *CDC5* in asynchronous cells did not significantly affect the cell-cycle profile during the 3 hour time course of these experiments, but continued overexpression of *CDC5* resulted in growth arrest with a non-uniform terminal phenotype (data not shown). As *CDC5* overexpression results in decreased Clb2 in cells arrested in S phase and mitosis, *CDC5* cannot be acting merely by causing a cell-cycle block at a stage when the APC is active. Instead, these results clearly show that Cdc5 can regulate Clb2 levels.

Ectopic expression of *CDC5* leads to increased APC activity

We tested the hypothesis that Cdc5 is an activator of the APC by measuring the activity of the APC isolated from cells overexpressing *CDC5*. In asynchronous cells or in cells arrested in mitosis with nocodazole, overproduction of Cdc5 resulted in a dramatic increase in the cyclin–ubiquitin ligase activity of the APC (Figure 5a). Similar results were obtained in *cdc13-1* mutant cells, which arrest in mitosis due to activation of the DNA-damage checkpoint. Increases in APC activity *in vitro* were accompanied by reduced Clb2 levels *in vivo* (Figure 5c), consistent with the possibility that the APC activity measured in our assay is a key determinant of cyclin levels in the cell. Furthermore, the decrease in Clb2 that accompanied *CDC5* overexpression was not observed in an APC mutant (*cdc23-1*), indicating that the effects of Cdc5 on Clb2 levels are mediated by the APC. There is evidence that Cdc28–Clb activity inhibits Clb2 proteolysis [33], and therefore *CDC5* overexpression could indirectly activate the APC by inhibiting Clb-associated kinase activity. We eliminated this possibility by showing that *CDC5* induction did not inhibit Clb2-associated kinase activity in *cdc23*-arrested cells, which contain stable Clb2 (Figure 5d).

CDC5 overexpression does not cause rebudding in nocodazole-arrested cells (data not shown), suggesting that the

Figure 5



Overproduction of Cdc5 results in increased APC-mediated Clb2 proteolysis. **(a)** A strain containing three copies of *GAL–HA3–CDC5* (JC256), or a control strain lacking inducible *CDC5* (AFS92), was grown to early log phase in YEP/raffinose (lanes 1–4), or was arrested in mitosis by treatment with 10 μg/ml nocodazole (Noc) after release from an S phase arrest as described in Materials and methods (lanes 5,6). Equivalent strains (JC291, JC282) carrying the *cdc13-1* (lanes 7,8) or *cdc23-1* (lanes 9,10) mutations were arrested by shifting to the non-permissive temperatures of 33°C or 37°C, respectively, for 3 h. Galactose or dextrose was then added for 3 h to induce (+) or repress (–) *CDC5* expression, respectively, and cell lysates were prepared. APC was immunoprecipitated from 200 μg cell extract with anti-Cdc26 antibodies, and cyclin–ubiquitin ligase activity *in vitro* was measured. The asterisk indicates a background band present in the non-ubiquitinated substrate. **(b)** Cdc5 and **(c)** Clb2 protein levels were analyzed as in Figure 3. **(d)** Cultures of *cdc23-1* cells, with and without inducible *CDC5*, were grown in YEP/raffinose to early log phase and then arrested in mitosis by growth at 37° for 3 h. Galactose or dextrose was added to 4% final concentration to induce (+) or repress (–) *CDC5* expression, respectively. Clb2-associated histone H1 kinase activity (HH1) was measured in anti-Clb2 immunoprecipitates from 100 μg cell extract. **(e)** Cultures of strain JC260 (see Table 1) in YEP/raffinose were arrested in 10 mg/ml hydroxyurea for 3 h and released into 10 μg/ml nocodazole. After 2.5 h, 0.25 mM $CuSO_4$ was added to induce expression of the GFP–LacI fusion protein. After a further 0.5 h, galactose (filled symbols) or dextrose (open symbols) was added to 2% final concentration to induce or repress *CDC5* expression, respectively. Sister-chromatid separation (triangles) was then assessed by microscopic analysis of fluorescently-labeled *lac* operator arrays on chromosome IV in 100 cells at the indicated times after *CDC5* induction or repression. Budding index (circles) was also measured in 100 cells.

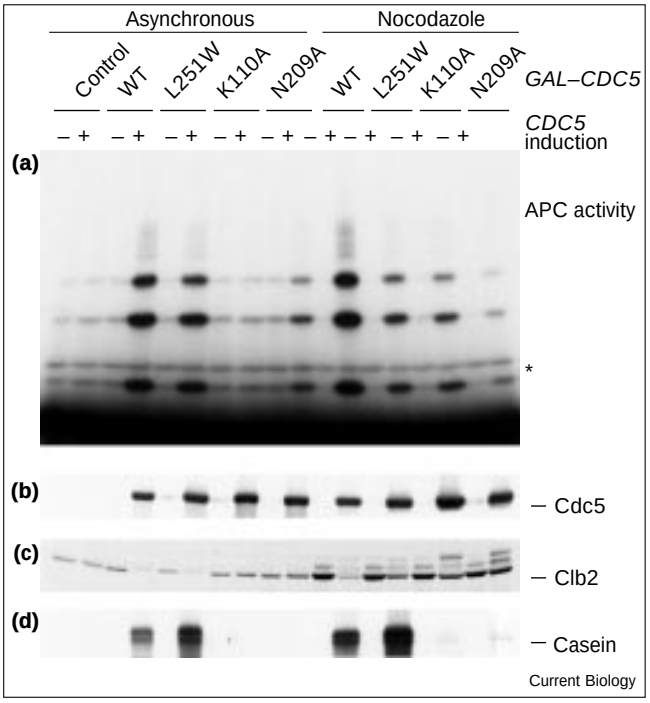
mitotic arrest is maintained. To pursue this possibility further, we examined sister-chromatid separation in these cells by the method described in Straight *et al.* [57], in which a locus on chromosome IV is marked with a *lac* operator array which can be visualized by the binding of a green fluorescent protein (GFP)–LacI fusion protein. *CDC5* overexpression had no significant effect on sister-chromatid separation or budding index (Figure 5e). As sister-chromatid separation is known to require Pds1 degradation [37], these results are consistent with our observation that Pds1 levels are not affected by *CDC5* overexpression (Figure 4b). Thus, although *CDC5* overexpression is able to activate APC-mediated Clb2 proteolysis in checkpoint-arrested cells, it does not allow cells to escape from this arrest.

Positive regulation of APC activity by Cdc5 requires kinase activity

We investigated whether APC regulation by Cdc5 is dependent on Cdc5 kinase activity. Kinase-defective mutants of Cdc5 were constructed by mutating lysine 110 to alanine (Cdc5-K110A) or asparagine 209 to alanine (Cdc5-N209A) [56]. These mutants failed to complement the *cdc5-1* mutant (data not shown). In asynchronous populations, overproduction of kinase-defective mutants resulted in Cdc5 protein levels comparable to those of wild-type Cdc5, but had no significant effect on APC activity or Clb2 levels (Figure 6).

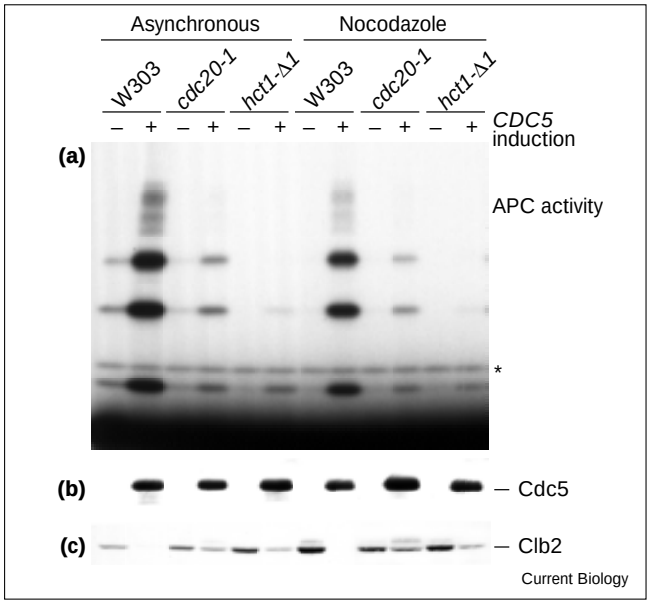
In nocodazole-arrested cells, kinase-defective Cdc5 mutants displayed low levels of kinase activity and induced minor increases in APC activity (Figure 6a,d), resulting in similarly moderate decreases in Clb2. Interestingly, the kinase activity of wild-type Cdc5 was also higher in nocodazole-arrested cells, despite similar levels of *CDC5* expression from the *GAL* promoter (Figure 6d). The correlation between kinase activity, APC activity and Clb2 levels suggests that the kinase activity of Cdc5 is required for the positive regulation of APC activity and Clb2 proteolysis.

Figure 6



The effect of *CDC5* overexpression on APC activity requires Cdc5-dependent kinase activity. Cells containing three integrated copies of wild-type *CDC5* (WT; JC256), *CDC5-K110A* (JC257), *CDC5-N209A* (JC278), or *CDC5-L251W* (JC279) under the control of the *GAL* promoter, or a control strain lacking inducible *CDC5*, were grown in YEP/raffinose as asynchronous cultures or were arrested in mitosis by treatment with 10 μg/ml nocodazole. Galactose or dextrose was then added to 2% final concentration for 3 h to induce (+) or repress (–) *CDC5* expression, respectively. (a) APC activity was measured in anti-Cdc26 immunoprecipitates from 200 μg cell extract. (b) Cdc5 and (c) Clb2 protein levels were analyzed as in Figure 3. (d) Cdc5-dependent casein kinase activity was measured in 12CA5 immunoprecipitates of 400 μg cell extract.

Figure 7



The effects of *CDC5* overexpression on APC activity require *HCT1* and *CDC20*. Wild-type (W303), *cdc20-1*, or *hct1-Δ1* strains containing three integrated copies of *GAL–HA3–CDC5* (JC256, 272, 276) were grown in YEP/raffinose at 25°C as asynchronous cultures or were arrested in mitosis by treatment with 10 μg/ml nocodazole. Galactose or dextrose was then added for 3 h to induce (+) or repress (–) *CDC5* expression, respectively, and cell lysates were prepared. (a) APC activity was measured in anti-Cdc26 immunoprecipitates from 200 μg cell extract. The asterisk indicates a background band present in the non-ubiquitinated substrate. (b) Cdc5 and (c) Clb2 protein levels were analyzed as in Figure 3.

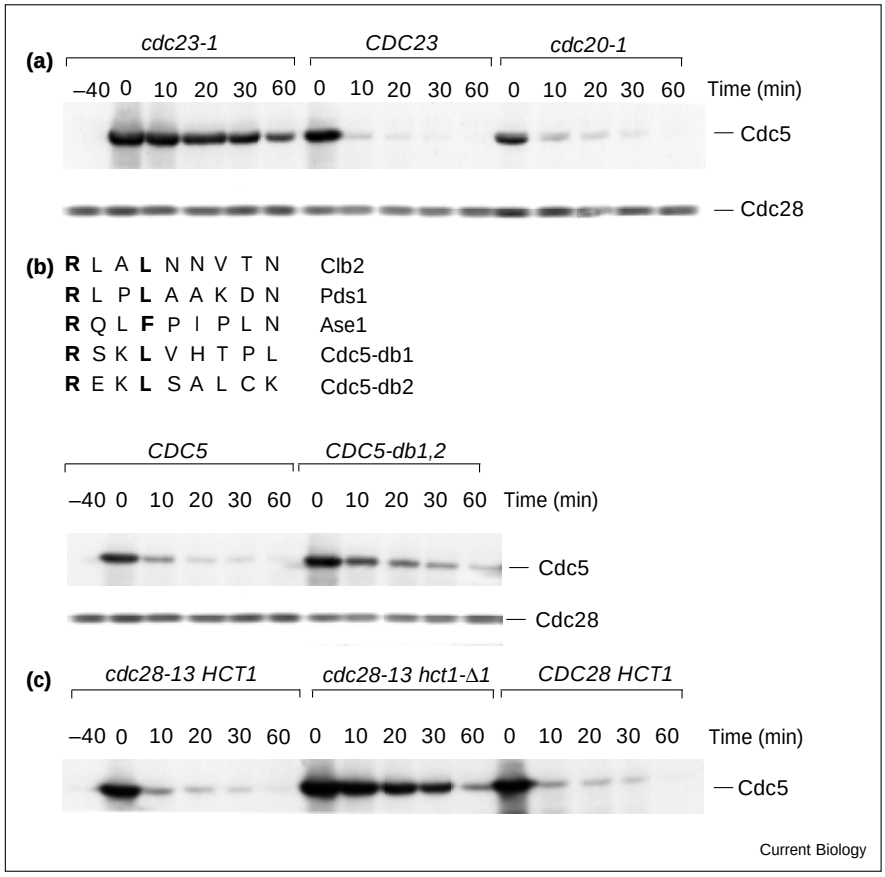
We also tested the effects of a Cdc5 mutant that is defective in the control of mitotic exit after DNA damage. Cells arrested in mitosis by DNA damage eventually adapt to the checkpoint arrest and exit mitosis in the presence of damage, and mutation of leucine 251 in Cdc5 to tryptophan (Cdc5-L251W; *cdc5-ad*) causes defects in this adaptation process, although normal cell-cycle progression is unaffected [58]. Overproduction of Cdc5-L251W in asynchronous cells resulted in an increase in APC activity and a decrease in Clb2 levels comparable to those caused by wild-type *CDC5* (Figure 6). In nocodazole-arrested cells (Figure 6d) and *cdc13* mutant cells (data not shown), the Cdc5-L251W mutant was significantly less effective in the stimulation of APC activity than wild-type Cdc5, however, even though it exhibited several-fold higher levels of kinase activity (Figure 6d). Our results suggest that *cdc5-ad* cells are unable to adapt to DNA damage due to a defect in their ability to activate Clb2 proteolysis. As suggested previously [58], it is possible that the Cdc5-L251W mutant has normal catalytic activity but is defective in the recognition of certain substrates.

The effect of *CDC5* overexpression is attenuated in *hct1-Δ1* and *cdc20-1* mutants

Like Cdc5, Hct1 is thought to be a specific activator of APC-mediated Clb2 proteolysis [17,18]. It is therefore possible that Cdc5 regulates the APC by a mechanism that requires Hct1. On the other hand, the Hct1-related protein Cdc20 does not appear to be required for Clb2 proteolysis in G1 [18,30], and therefore might not be expected to play a role in the actions of Cdc5.

We addressed this issue by assessing the effects of Cdc5 overproduction in *hct1-Δ1* and *cdc20-1* mutants (Figure 7). Cyclin ubiquitination by the APC *in vitro* was negligible in *hct1-Δ1* cells (Figure 7a), and the stimulatory effects of *CDC5* expression on APC activity and Clb2 destruction were greatly attenuated in the *hct1-Δ1* mutant, in both asynchronous and nocodazole-arrested cultures (Figure 7a,c). In multiple experiments, asynchronous *cdc20-1* cells growing at the permissive temperature in the

Figure 8



Cdc5 degradation in G1 is dependent on the APC and Hct1. (a) The stability of Cdc5 in G1-arrested cells was examined by promoter turn-off. Wild-type, *cdc23-1,* and *cdc20-1* cells were grown in YEP/raffinose at 25°C, arrested with 1 μg/ml α-factor until over 90% of cells were in G1, and then shifted to 37°C for 1 h. *CDC5* was induced with 4% galactose for 40 min, followed by addition of dextrose (4%) and cycloheximide (10 μg/ml) to repress transcription and translation, respectively. Samples were taken at the indicated times after repression, and extracts were prepared in denaturing sample buffer. HA-epitope-tagged Cdc5 was analyzed by western blotting with 12CA5. As a loading control, Cdc28 was detected on western blots with anti-Cdc28 antibody. Cells remained arrested in G1 for the duration of the experiment. (b) Sequences of destruction boxes from known APC substrates (Clb2, Pds1 and Ase1) are aligned with the two putative destruction boxes at arginines 17 and 60 of Cdc5 (Cdc5-db1 and Cdc5-db2, respectively). Mutation of the indicated arginines and leucines in both of these two sequences resulted in the *CDC5-db1,2* mutant. Cdc5 stability in cells containing inducible wild-type *CDC5* (JC256) or *CDC5-db1,2* (JC309) was examined by promoter turn-off as in (a), except that the experiment was performed at 25°C, and sugar concentrations were 2%. (c) Mutant *cdc28-13 HCT1* (JC310) and *cdc28-13 hct1-Δ1* (JC311) cells were arrested in G1 by growth at 37°C for 3.5 h. Wild-type cells (JC256) were arrested in G1 with 1 μg/ml α-factor and shifted to 37°C for 1 h. In all cases, over 90% of cells displayed G1 morphology throughout the experiment. Cdc5 stability was measured by promoter turn-off as in (a).

absence of *CDC5* overexpression displayed only slightly decreased cyclin ubiquitination in our APC assay (Figure 7a). The *cdc20-1* mutation did reduce the stimulatory effects of Cdc5 overproduction on APC activity and Clb2 proteolysis, however (Figure 7). We could not examine the ability of *CDC5* to activate Clb2 proteolysis in *cdc20-1* cells arrested in mitosis at the restrictive temperature, because we were unable to induce *CDC5* expression under these conditions.

Our results suggest that Hct1 and Cdc20 both contribute to the effect of Cdc5 on the cyclin ubiquitination activity of the APC *in vitro*, despite previous indications that Cdc20 is dispensable for Clb2 proteolysis. The relative contributions of Hct1 and Cdc20 to Cdc5 function might not be equivalent, however, as we found that the *cdc5-1 hct1-Δ1* double mutant is viable, whereas the *cdc5-1 cdc20-1* double mutant is not (data not shown).

Cdc5 degradation in G1 is dependent on the APC and Hct1

Cdc5 protein levels closely parallel those of Clb2 throughout the cell cycle, and decrease immediately after APC activation (Figure 3). We have also observed that *CDC5* induction from the *GAL* promoter in α-factor-arrested cells, which contain fully active APC, results in less accumulation of Cdc5 than that seen in asynchronous cells (data not shown). Thus, we investigated the possibility that Cdc5 stability in G1, like that of Clb2, is regulated by the APC. We analyzed the stability of Cdc5 in α-factor-arrested cells after transient induction of *HA3–CDC5* from the *GAL* promoter. Cdc5 was rapidly destroyed in wild-type cells, with the bulk of Cdc5 protein disappearing within 10 minutes of repression of transcription and translation by treatment with dextrose and cycloheximide, respectively. In contrast, Cdc5 was stable in *cdc23-1* cells, confirming that Cdc5 degradation in G1 is dependent on the APC.

All APC substrates identified so far contain sequences known as destruction boxes, which were first identified in B-type vertebrate cyclins [55,59]. Mutation of a conserved arginine and leucine in the destruction box sequence prevents APC-mediated destruction [37,59,60]. To confirm that Cdc5 is an APC substrate, we also tested the effects of mutations in putative destruction box sequences in Cdc5. As the carboxy-terminal half of Cdc5 is completely stable in G1 (data not shown), we focused on destruction box sequences in the amino-terminal half of the protein. Only two of these sequences lie outside conserved regions of the kinase domain (Figure 8b). Mutation of conserved residues in either of these two sequences had no effect on Cdc5 stability in G1, but mutation of conserved residues in both sequences (resulting in the Cdc5-db1,2 mutant) caused partial Cdc5 stabilization (Figure 8b), suggesting that Cdc5 is a direct APC substrate in G1. The residual degradation of the Cdc5-db1,2 mutant is not observed in a *cdc23-1* mutant (data not shown), raising the possibility that the APC recognizes additional destruction box sequences in Cdc5.

We also investigated whether Cdc5 degradation in G1 is dependent on Hct1 or Cdc20. Like that of Clb2, the stability of Cdc5 in G1 was unaffected in the *cdc20-1* mutant at the restrictive temperature (Figure 8a). We also tested the requirement for *HCT1* in Cdc5 degradation. Because *hct1-Δ1* mutants treated with α-factor re-replicate their DNA and thus do not exhibit a normal G1 arrest [17], we instead arrested cells in G1 with the *cdc28-13* mutation (APC activity in this mutant is comparable to that in α-factor-arrested cells; S.L.J., unpublished observations). As in α-factor-arrested cells, Cdc5 was quickly destroyed in *cdc28-13*-arrested cells (Figure 8c). In *cdc28-13 hct1-Δ1* double mutants, however, Cdc5 was greatly stabilized. We conclude that Cdc5 degradation in G1 is dependent on both the APC and Hct1.

Discussion

Cdc5 is an activator of cyclin-specific APC activity

Our observations argue that Cdc5 is a key regulator of APC activity and cyclin destruction. Decreased Cdc5 activity, as in the *cdc5-1* mutant, results in decreased cyclin ubiquitination by the APC *in vitro*, decreased cyclin destruction *in vivo*, and increased sensitivity to cyclin overexpression. Increased Cdc5 activity results in greater APC activity and cyclin destruction.

Cdc5 does not appear to stimulate APC activity towards all substrates. Cells expressing the *cdc5-1* mutant are defective in the destruction of cyclins but not that of Pds1; similarly, *CDC5* overexpression promotes cyclin destruction but has relatively modest effects on the levels of Pds1. These effects of Cdc5 are similar to those of Hct1: *hct1-Δ1* cells display a specific defect in cyclin destruction, and *HCT1* overexpression in asynchronous cells triggers the destruction of cyclins but not Pds1. These findings, combined with our observation that *CDC5* overexpression has little effect on APC activity in *hct1-Δ1* cells, suggest that the actions of Cdc5 overlap with those of Hct1. Cdc5 might act upstream of Hct1 in the specific control of cyclin destruction, or Hct1 may simply be an essential APC subunit required for cyclin–ubiquitin ligase activity.

Although the functions of Cdc5 and Hct1 might overlap, there are significant differences in the phenotypes of *CDC5* and *HCT1* overexpression. Whereas *HCT1* overexpression causes accumulation of G2 cells with elongated buds [17], *CDC5* overexpression is toxic but does not cause a uniform cell-cycle arrest. Moreover, the toxicity of *CDC5* overexpression is not completely alleviated by mutation of APC subunits (J.F.C., unpublished observations), whereas mutation of *CDC23* allows growth of cells overproducing *HCT1* [17].

Despite previous evidence that Cdc20 is required primarily for the destruction of Pds1 and not that of Clb2 [18], we found that *cdc20-1* mutants were defective in *CDC5*-stimulated cyclin destruction. Thus, the substrate specificity of Cdc20 might overlap with that of Hct1 under these conditions, as suggested by previous observations that the overexpression of either *HCT1* or *CDC20* can induce the destruction of both Pds1 and Clb2 in nocodazole-arrested cells [18].

CDC5 overexpression in nocodazole-arrested cells does not trigger separation of sister chromatids, presumably due to its inability to induce Pds1 destruction. This seems inconsistent with previous work showing that chromosome separation can be achieved in nocodazole-arrested cells when Sic1 is overproduced [33], suggesting that decreased Cdc28–Clb activity can trigger Pds1 destruction. Unlike our experiments, these studies of Sic1 overproduction were performed in the presence of α-factor, which was included to prevent the increase in G1 cyclin levels that occurs when mitotic Clb levels decrease. Perhaps an increase in G1 cyclin levels in our experiments prevented the activation of APC activity toward Pds1, despite the striking increase in APC activity toward cyclins. Alternatively, *CDC5* overexpression might not trigger the destruction of all mitotic cyclins, such that some Cdc28 activity remains to suppress Pds1 destruction.

Does Cdc5 have other functions in addition to the promotion of cyclin destruction?

Our observation that Sic1 overexpression allows the *cdc5-1* mutant to exit mitosis suggests that the key defect in this mutant is an inability to inactivate Cdc28 in late mitosis. Although our results suggest that this defect is due at least in part to a reduction in cyclin-specific APC activity, it remains possible that *cdc5-1* cells are also defective in other Cdc28 inactivation mechanisms, such as the production of

Sic1. In *hct1-Δ1* cells, which exit mitosis despite a specific defect in cyclin destruction, Sic1 has been implicated in the inactivation of Cdc28 [17,18].

Previous work has shown that the *cdc5-1* mutant has a plasmid maintenance defect and is synthetically lethal with *orc2-1*, a mutant in a subunit of the origin recognition complex; in addition, there is evidence that Cdc5 interacts with Dbf4, a protein implicated in the control of replication initiation [57]. Although these results could indicate a direct role for Cdc5 in DNA replication, our results provide an alternative explanation. The kinase activity of mitotic Cdk–cyclin complexes is thought to prevent the establishment of pre-replicative complexes, which are required for the initiation of replication [61–63]. The high levels of Clb2 in *cdc5-1* mutant cells may interfere with the normal timing of pre-replicative complex formation.

Regulating the regulator: Cdc5 degradation

APC activity in *cdc5-1* mutant cells is very low even at the permissive temperature, resulting in an increased sensitivity to cyclin overexpression. Low APC activity is also seen in *cdc5-1* cells arrested in G1 by pheromone treatment, presumably because full activation is not achieved in the preceding mitosis. A shift of G1-arrested *cdc5-1* cells to the restrictive temperature does not cause a further significant decrease in APC activity, however, suggesting that Cdc5 is required for the initiation of APC activation in late mitosis, but not for the maintenance of APC activity in G1. This possibility is consistent with the low levels of Cdc5 protein and Cdc5 activity in G1 cells, and with our observation that Cdc5 is subject to APC-dependent degradation in G1. These mechanisms might ensure that when the APC is eventually inactivated at the end of G1, a key activator is no longer present to initiate premature APC activation.

Material and methods

Plasmid and strain construction

All strains (Table 1) are derivatives of W303 *MAT*a *ade2-1*, *can1-100*, *his3-11,15*, *leu2-3*, *trp1-1*, *ura3-1*, *ssd1-d*, or backcrossed to W303 at least four times. JC94 and JC95 are congenic strains derived by crossing JC34 to W303 *MATα ura3::GAL-CLB2-URA3* (gift of A. Rudner). The 2μ *CDC5* construct contained the *CDC5* open reading frame plus 300 bp 5′ sequence and 500 bp 3′ sequence in pRS426. High-copy *SIC1* expression was achieved with pRS426 containing *SIC1*. In strains JC243 and JC247, the *PDS1* gene was replaced with a version of the *PDS1* gene encoding the Pds1 protein tagged at the carboxyl terminus with a triple hemagglutinin (HA3) tag; this was generated by the PCR epitope-tagging method of Schneider *et al.* [64]. In strain JC35, *CDC5* was replaced with a version of the gene that encoded an amino-terminally HA3-epitope-tagged Cdc5 protein, and *CDC27* was replaced with a gene encoding Cdc27 tagged with MBP by a 'pop-in, pop-out' strategy [65]. Strain JC251 was constructed by three copy integration of pJC59, which contained a tagged version of the *CDC5* gene (encoding Cdc5 tagged at the amino terminus with a triple Myc epitope tag) under the control of the *GAL1,10* promoter in pDK20 (kindly provided by D. Kellogg), at the *ura3* locus of JC243. Southern blot analysis with a 450 bp *Sal*I–*Hpa*I internal fragment of *CDC5* was used to identify a three-copy integration event. Similarly, strain JC256 contains three copies of pJC57, which is identical to pJC59 except that an HA3 epitope tag replaces the Myc tag. Overexpression of *CDC5* in this strain results in Cdc5 protein levels being approximately 20–50-fold higher than the endogenous level observed in asynchronous cells. Oligonucleotide-directed mutagenesis was used to introduce the point mutations K110A, N209A and L251W into pJC57. Strains JC257, JC278 and JC279 contain three copies of *CDC5-K110A*, *CDC5-N209A,* or CDC5-*L251W,* respectively, integrated at *ura3* and identified by Southern analysis. Strains JC260, JC272, JC276, JC282 and JC291 were derived from JC256 by crossing into the desired background. W320 *MATα hct1-Δ1* was a gift of W. Seufert [17]; SB213 *MATα his3::CUP-GFP LacI-HIS3 trp1::LacO* was a gift of S. Biggins. Strain JC309 contains pJC88, containing *CDC5-db1,2* in which the mutations R17A, L20A, R60A, and L63A were introduced by oligonucleotide-directed mutagenesis, integrated at the *ura3* locus. Strains JC310 and JC311 were derived by crossing JC276 to *MATα cdc28-13*.

Yeast methods

Standard protocols for transformation, mating, sporulation, tetrad dissection, α-factor and hydroxyurea arrests were used [65]. For galactose induction of epitope-tagged *CDC5*, cultures were grown to early log phase in YEP/2% raffinose. Asynchronous cultures were induced or repressed by the addition of 2% (final concentration) galactose or dextrose, respectively, for 3 h. For galactose induction in nocodazole-arrested cells, cultures were pre-arrested in 10 mg/ml hydroxyurea for 3 h, released into 10 μg/ml nocodazole for 2.5 h, after which galactose was added (2%) in the continued presence of nocodazole (10 μg/ml). For cell-cycle arrests plus induction, cultures were pre-arrested at the non-permissive temperature for 3 h, followed by induction/repression with 2% (33°C cultures) or 4% (37°C cultures) galactose/raffinose. For sister-chromatid separation experiments, GFP-tagged sister chromatids were examined in 100 cells at each time point following the method described in Straight *et al.* [57].

Protein analysis

Cell lysates were prepared by lysis with a BioSpec Beadbeater 8 in LLB (50 mM Hepes-NaOH, pH 7.4, 75 mM KCl, 0.1% NP40, 5 mM NaF, 5 mM β-glycerophosphate, 1 mM $MgCl_2$, 1 mM DTT) plus 1 mM PMSF

Table 1

Yeast strains used in these studies.

Name	Relevant genotype
JC34	*MAT*a *bar1 cdc5-1*
JC94	*MAT*a *cdc5-1 ura3::GAL–CLB2–URA3*
JC95	*MAT*a *ura3::GAL–CLB2–URA3*
JC35	*MAT*a *bar1 cdc5::CDC5^{HA3} cdc27::CDC27^{MBP}*
AFS92	*MAT*a *bar1*
JC256	*MAT*a *bar1 ura3::3X(GAL–CDC5^{HA3}–URA3)*
JC291	*MAT*a *cdc13-1 ura3::3X(GAL–CDC5^{HA3}–URA3)*
JC282	*MAT*a *cdc23-1 bar1 ura3::3X(GAL–CDC5^{HA3}–URA3)*
JC305	*MAT*a *cdc23-1*
JC272	*MAT*a *cdc20-1 bar1 ura3::3X(GAL–CDC5^{HA3}–URA3)*
JC276	*MAT*a *hct1-Δ1::LEU2 ura3::3X(GAL–CDC5^{HA3}–URA3)*
JC257	*MAT*a *ura3::3X(GAL–CDC5-K110A^{HA3}–URA3)*
JC278	*MAT*a *bar1 ura3::3X(GAL–CDC5-N209A^{HA3}–URA3)*
JC279	*MAT*a *ura3::3X(GAL–CDC5-L251W^{HA3}–URA3)*
JC260	*MAT*a *his3::CUP1–GFP–LacI–HIS3 trp1::LacO–TRP1 ura3::3X(GAL–CDC5^{HA3}–URA3)*
JC243	*MAT*a *pds1::PDS1^{HA3}*
JC251	*MAT*a *pds1::PDS1^{HA3} ura3::3X(GAL–CDC5^{MYC3}–URA3)*
JC247	*MAT*a *cdc5-1 pds1::PDS1^{HA3}*
JC309	*MAT*a *bar1 ura3::GAL–CDC5-db1,2^{HA3}–URA3*
JC310	*MAT*a *cdc28-13 ura3::3X(GAL–CDC5^{HA3}–URA3)*
JC311	*MAT*a *cdc28-13 hct1-Δ1::LEU2 ura3::3X(GAL–CDC5^{HA3}–URA3)*

and 1 μg/ml each of leupeptin, aprotinin and pepstatin. Clb2 western blots and Clb2-associated kinase assays were performed as described [66]. HA-epitope-tagged Cdc5 and HA-epitope-tagged Pds1 were detected on western blots with mouse monoclonal antibodies 12CA5 or 16B12, respectively. HA3–Cdc5 kinase activity was measured in 12CA5 immunoprecipitates washed sequentially in LLB, high-salt QA (20 mM Tris-HCl, pH 7.6, 250 mM KCl, 1 mM $MgCl_2$, 1 mM DTT), and 5KB (50 mM Hepes-NaOH, pH 7.4, 200 mM KAc, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT). Kinase assays (30 μl) were performed in 50 mM Hepes-NaOH, pH 7.4, 60 mM KAc, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 50 μM ATP, plus 5 μg casein and 2.5 μCi [^{32}P]ATP.

In vitro *ubiquitination assay*

The APC was immunoprecipitated with either polyclonal antibodies to Cdc26 [27], the monoclonal antibody 12CA5 for strains containing a plasmid bearing *CDC16HA* under its own promoter [67] or polyclonal antibodies to MBP (New England BioLabs) for strains containing *CDC27–MBP* replacing endogenous *CDC27*. Similar results were obtained with all antibodies. After washing immunoprecipitates in LLB, high salt QA, and QA (20 mM Tris-HCl, pH 7.6, 100 mM KCl, 1 mM $MgCl_2$, 1 mM DTT), ligase activity was measured by adding 15 μl reaction mix containing 3.5 pmol Uba1, 47 pmol Ubc4, 1 nmol bovine ubiquitin (Sigma), 1 mM ATP, and 0.25 μl ^{125}I-labeled sea urchin cyclin B (amino acids 12–91) [10,55]. After 15 min at 25°C, ubiquitin conjugates were resolved by electrophoresis on a 7.5–15% gradient gel and visualized by autoradiography with the BioMaxMS system (Kodak). Uba1–His_6 was purified by metal-affinity chromatography from a yeast strain expressing Uba1 under the control of the *CUP1* promoter, kindly provided by J. Dohmen. Ubc4 was purified from a bacterial expression vector [68] following the protocol of H-F. Elin (personal communication).

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