Inhibitory phosphorylation of the APC regulator Hct1 is controlled by the kinase Cdc28 and the phosphatase Cdc14
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**Background:** Exit from mitosis requires inactivation of mitotic cyclin-dependent kinases (CDKs). A key mechanism of CDK inactivation is ubiquitin-mediated cyclin proteolysis, which is triggered by the late mitotic activation of a ubiquitin ligase known as the anaphase-promoting complex (APC). Activation of the APC requires its association with substoichiometric activating subunits termed Cdc20 and Hct1 (also known as Cdh1). Here, we explore the molecular function and regulation of the APC regulatory subunit Hct1 in *Saccharomyces cerevisiae.*

**Results:** Recombinant Hct1 activated the cyclin–ubiquitin ligase activity of APC isolated from multiple cell cycle stages. APC isolated from cells arrested in G1, or in late mitosis due to the cdc4-1 mutation, was more responsive to Hct1 than APC isolated from other stages. We found that Hct1 was phosphorylated in *vivo* at multiple CDK consensus sites during cell cycle stages when activity of the cyclin-dependent kinase Cdc28 is high and APC activity is low. Purified Hct1 was phosphorylated in *vitro* at these sites by purified Cdc28–cyclin complexes, and phosphorylation abolished the ability of Hct1 to activate the APC in *vitro.* The phosphatase Cdc14, which is known to be required for APC activation in *vivo,* was able to reverse the effects of Cdc28 by catalyzing Hct1 dephosphorylation and activation.

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

**Background**
Exit from mitosis in *Saccharomyces cerevisiae* requires the ubiquitin-dependent proteolysis of multiple proteins, including the anaphase inhibitor Pds1 and the mitotic cyclins (Clbs) [1,2]. A key step in the ubiquitination of these proteins is catalyzed by a multi-subunit ubiquitin ligase known as the anaphase-promoting complex (APC; reviewed in [3,4]), the activity of which increases in late mitosis and remains high throughout G1 [5–7]. Mechanisms governing APC activation in late mitosis are poorly understood. In higher eukaryotes, phosphorylation of APC subunits is thought to promote APC activity [8–13]. In budding yeast, phosphorylation of the APC has not been reported, but there is evidence that the protein kinase Cdc28 inhibits APC-dependent cyclin proteolysis in *vivo* [14].

The function of the APC is regulated by the WD40-repeat proteins Cdc20 and Hct1 (also known as Cdh1). On the basis of genetic evidence from budding yeast, it is thought that Pds1 degradation at the metaphase–anaphase transition is promoted by Cdc20, after which Hct1 stimulates proteolysis of the cyclin Clb2 [15–18]. Recent biochemical evidence from vertebrates suggests that Cdc20 and Hct1 act as substoichiometric APC subunits that stimulate ubiquitin ligase activity and may also contribute to substrate recognition [19–22].

As key regulators of APC function, Cdc20 and Hct1 are likely to be critical targets for many mitotic regulatory pathways. Cdc20 appears to be regulated at multiple levels. It is an unstable protein whose concentration peaks in mitosis, after which APC-dependent proteolysis leads to decreased levels in G1 [16,19–21,23]. In cells arrested in metaphase by spindle damage, Cdc20 function is inhibited by association with checkpoint signaling proteins [21,22,24,25]. The regulation of Hct1 activity, however, is poorly understood. Hct1 levels do not change during the cell cycle, although in human cells the amount of Hct1 associated with the APC increases in G1 [19,21,23]. Thus, post-translational mechanisms probably regulate Hct1-dependent stimulation of APC activity.

Activation of the cyclin–ubiquitin ligase activity of the APC in late mitosis also requires the function of a large family of regulatory proteins, including the protein kinase Cdc5 [26,27], the Polo-like kinase Cdc5 [6,16,28], and
the protein phosphatase Cdc14 [29,30]. Cells lacking the function of these proteins arrest in late mitosis with high cyclin levels and profound defects in Hct1-dependent APC activity [6,27]. The mechanism by which the late mitotic regulatory proteins promote cyclin-specific APC activation is not clear.

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

Results
Activation of the APC by Hct1 in vitro
We first set out to explore mechanisms of APC regulation by analyzing the ability of purified Hct1 to activate purified APC in vitro. Our initial step was to develop a conventional purification strategy to purify active APC from G1-arrested cells and inactive APC from cells that had been arrested in late mitosis by the cdc15-2 mutation (Figure 1). In both preparations, we observed approximately 12 co-purifying subunits that correspond to the 12 subunits observed in previous reports (Figure 1a; [31,32]). During the purification, the G1 APC maintained activity whereas the anaphase APC remained inactive (Figure 1b), suggesting that differences in activity were due to intrinsic subunits or modifications. In comparisons of several preparations from G1 and anaphase cells, however, we did not detect any differences in APC subunit composition or the electrophoretic mobility of any subunit on polyacrylamide gels (Figure 1a). Thus, APC activity in these experiments must be influenced by substoichiometric subunits or by modifications that do not affect gel mobility under these conditions.

Cyclin–ubiquitin ligase activity of the purified APC from a cdc15-2 mutant was potently stimulated by hexahistidine-tagged Hct1 (Hct1–His6) purified from baculovirus-infected insect cells (Figure 2a). Thus, Hct1 alone is sufficient to activate the cyclin–ubiquitin ligase activity of the APC. Hct1 was also able to activate the APC isolated by immunoprecipitation from several other late mitotic mutants (cdc5-1, cdc14-1, dbf2-2 and tem1-3; Figure 2b). In addition, Hct1 activated the APC from cells arrested at the DNA replication checkpoint following treatment with hydroxyurea (HU) and at the spindle assembly checkpoint following treatment with nocodazole, indicating that the APC inhibitory mechanisms acting at these checkpoint arrests do not completely block APC responsiveness to Hct1 (Figure 2b). Similarly, Hct1 activated the APC from a cdc20-1 mutant, indicating that no previous function of Cdc20 is required for Hct1 responsiveness in vitro (Figure 2b). Finally, we observed that Hct1 was able to hyperactivate the active APC isolated from cells arrested in G1 with the mating pheromone α-factor (Figure 2b). Thus, a major fraction of the APC from multiple cell cycle stages can be activated by Hct1.

Hct1 responsiveness of the APC increases in late mitosis
Analysis of APC activation over a range of Hct1 concentrations in vitro revealed that differences in Hct1 responsiveness exist at different cell cycle stages (Figure 2c–f). We analyzed activation of the APC from cells arrested in metaphase (nocodazole), anaphase (cdc15-2), or G1 (α-factor),
Activation of APC by recombinant Hct1. (a) Increasing amounts of purified Hct1–His₆ were added to approximately 6 nM APC purified from cdc15-2 cells, and cyclin–ubiquitin ligase activity was measured. The asterisk indicates a non-specific background band. (b) Cells were arrested at various cell-cycle stages with the indicated temperature-sensitive mutations or by treatment with α-factor (af), hydroxyurea (HU), or nocodazole (Noc). The hct1Δ strain was grown asynchronously at 37°C. APC was immunoprecipitated from 250 μg yeast lysate with anti-Cdc28 antibodies (a gift of L. Hwang), and incubated with either 15 nM Hct1–His₆ (+) or buffer (−), followed by measurement of cyclin–ubiquitin ligase activity. Control experiments were performed without added lysate (−APC). (c,d) APC was immunoprecipitated from lysates (70 μg) of wild-type cells treated with α-factor (af) or nocodazole (Noc), or from cdc15-2 cells arrested at 37°C (cdc15). Recombinant Hct1–His₆ was added in increasing amounts, and APC activity was measured. In (c), the average APC activity in four independent experiments is expressed as a percentage of maximum activity for that APC. In (d), the data have been linearized with a double-reciprocal plot. Standard errors are indicated, although in most cases they were smaller than the diameters of the symbols. We estimate that APC concentrations in all four experiments were in the nanomolar range. (e,f) APC was immunoprecipitated from lysates (70 μg) of cdc5-1, cdc15-2 and cdc14-1 cells arrested at 37°C. Following incubation with increasing amounts of Hct1, APC activity in a representative experiment was analyzed as in (c) and (d). Similar results were obtained in three independent experiments.

and in four independent experiments we found that the Hct1 concentration required for half-maximal activation of the G1 APC (5 nM) was consistently fourfold lower than that required for half-maximal activation of the APC from cells arrested by nocodazole or the cdc15-2 mutation (20 nM; Figure 2c,d).

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

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

**Hct1 is phosphorylated by Cdc28**

Our results indicate that the APC from multiple cell cycle stages is responsive to Hct1 in vitro, raising the question of why Hct1 does not activate these APCs in vivo. Hct1 levels
Hct1 phosphorylation in vivo at Cdc28 consensus sites. (a) Wild-type strains containing GAL–Hct1–HA or GAL–Hct1–28A–HA (a mutant in which the six Cdc28 consensus sites are changed to alanine) were grown in galactose for 2.5 h. Wild-type or mutant Hct1–HA proteins were immunoprecipitated using the anti-HA antibody from cell lysates (500 μg) and treated with phosphatase buffer plus phosphatase inhibitors (lanes 2,5), 100 U λ-phosphatase (lanes 3,6), or both λ-phosphatase and phosphatase inhibitors (lanes 4,7). Lane 1 is an immunoprecipitate from a lysate of cells lacking the tagged Hct1. Immunoprecipitates were immunoblotted with the anti-HA antibody 16B12. (b) The same strains as in (a) were arrested in c-factor (cf), hydroxyurea (HU), or nocodazole (Noc), or left untreated as asynchronous cultures (A). Galactose was then added to 2%, except in the uninduced asynchronous control (U). Anti-HA immunoprecipitates of cell lysates were then probed by immunoblotting with anti-HA antibody 16B12. Partial Hct1 degradation in this experiment resulted in the appearance of anti-HA-reactive bands below the main Hct1 bands. In (a,b), the positions of unphosphorylated (Hct1–HA) and phosphorylated (P-Hct1–HA) forms are marked.

are constant during the normal cell cycle [23], indicating that Hct1 activity in vivo may be regulated by post-translational modification. To explore potential Hct1 modifications, we constructed a strain containing an integrated copy of a gene encoding a hemagglutinin (HA) epitope-tagged form of Hct1 under the control of the GAL promoter. At least three electrophoretic mobility forms of Hct1–HA were apparent in lysates from these cells: a closely spaced doublet around 65 kDa and a heterogeneous series of bands migrating slightly slower than the doublet (Figure 3a, lane 2). The diffuse upper band, as well as the top band of the doublet, disappeared upon treatment of Hct1 immunoprecipitates with λ-phosphatase (Figure 3a), demonstrating that Hct1 is phosphorylated in vivo. The diffuse upper form of Hct1 was most prominent in cells arrested in S phase and mitosis, and was not detectable in cells arrested in G1 (Figure 3b). Thus, the presence of this form is inversely correlated with APC activity, suggesting that it may represent an inhibitory modification.

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

To further document a role for Cdc28 in Hct1 phosphorylation, we showed that purified, baculovirus-derived Hct1–His6 was efficiently phosphorylated in vitro by purified Cdc28–Clb2 complexes (Figure 4a), and that phosphorylation by Cdc28 led to a heterogeneous electrophoretic mobility shift similar to the Hct1 mobility shifts observed in vivo (Figure 4a,b). Quantitation of phosphate incorporation in these experiments indicated that approximately 3–5 phosphates were transferred to Hct1–His6 by Cdc28–Clb2.

We also analyzed phosphorylation of the mutant Hct1–28A protein by Cdc28–Clb2 in vitro (Figure 4a). Because the Hct1–28A protein could not be expressed at high levels in insect cells and was therefore less homogeneous upon purification, phosphorylation of the protein by a contaminating kinase was more pronounced; nevertheless, mutation of the six Cdc28 sites abolished over 80% of the Cdc28-dependent Hct1 phosphorylation at high Cdc28–Clb2 concentrations. On the basis of this result, as well as our evidence from intact cells, we conclude that Hct1 is phosphorylated by Cdc28 in vivo during S and M phases, and that the phosphorylation occurs at several cyclin-dependent kinase (CDK) consensus sites in the protein.

Five of the six CDK consensus sites in Hct1 are found in the amino-terminal half of the protein, whereas one site (Ser436) is located within the WD40 repeats of the carboxy-terminal half. We therefore constructed another Hct1 mutant in which only the five amino-terminal sites were changed to alanine. Mutation of these sites also abolished the diffuse upper forms of Hct1, suggesting that these five sites are the major sites of Cdc28-dependent phosphorylation in vivo (data not shown).

Cdc28-dependent phosphorylation inactivates Hct1

To assess the effects of Cdc28–Clb2-dependent phosphorylation on Hct1 activity, we developed methods that allowed us to treat Hct1–His6 with Cdc28–Clb2 and then re-purify it (Figure 4b). Phosphorylation of Hct1–His6 dramatically inhibited its ability to stimulate the cyclin–ubiquitin ligase activity of the purified APC from cdcl5 cells (Figure 4c). Treatment of the Hct1–28A mutant with Cdc28–Clb2 kinase had no effect on its ability to stimulate
Phosphorylation of Hct1 by Cdc28–Cib2 abolishes its ability to activate the APC. (a) Increasing amounts of Cdc28–Cib2 were tested for their ability to phosphorylate 150 ng purified Hct1–His6 or the Hct1-28A–His6 mutant. Quantitation of phosphate incorporation in these experiments indicated that approximately 4.4 mol phosphate was incorporated per mol wild-type Hct1–His6 at the highest Cdc28–Cib2 concentrations; in other experiments, phosphate incorporation varied from 3 to 5 mol/mol. Control lanes of samples with no added Hct1–His6 are indicated (−). (b) Purified Hct1–His6 (3 µg) was incubated with ATP and 10 µg Cdc28–Cib2 complex immobilized by immunoprecipitation on beads (lane 2). To control for contaminating kinases, Hct1–His6 was incubated in a reaction with ATP and beads alone (lane 1); to verify that the effects of Cdc28–Cib2 on Hct1–His6 were dependent on Cdc28–Cib2 kinase activity, ATP was omitted from the kinase reaction (lane 3). Following removal of the Cdc28–Cib2 complex, 25% of the reaction was analyzed by gel electrophoresis and Coomassie blue staining. The positions of unphosphorylated (Hct1–His6) and phosphorylated (P-Hct1–His6) forms are marked. (c) The three Hct1–His6 preparations shown in (b) were incubated in solution with 6 nM APC purified from cdc15-2 arrested cells, and cyclin–ubiquitin ligase activity in the reaction was measured. The quantitation of the APC activity as a percentage of maximum is shown below the gel. (d) Wild-type Hct1–His6 and mutant Hct1-28A–His6 (3 µg) were treated with Cdc28–Cib2 (10 µg), and activation of a purified cdc15-2 APC was tested as in (c). Because the mutant Hct1–His6 purified from insect cells appears unstable and is not as effective in APC activation as the wild-type protein, wild-type Hct1–His6 was diluted 1 in 30 after treatment with kinase and prior to incubation with the APC. Average APC activity in four independent experiments is shown as percentage of maximal activity achieved with untreated Hct1–His6.

APC activity (Figure 4d). We conclude that phosphorylation by Cdc28–Cib2 severely impairs stimulation of cyclin–ubiquitin ligase activity by Hct1.

To assess the role of Cdc28-dependent phosphorylation in Hct1 function in vivo, we analyzed the effects of moderate Hct1-28A expression on cell proliferation and cyclin levels. Expression of Hct1-28A, but not of wild-type Hct1, inhibited cell proliferation (Figure 5a) and caused a decrease in Cib2 levels in cells arrested in mitosis with nocodazole (Figure 5b). Expression of Hct1-28A was also able to partially suppress the growth defect of the late mitotic mutant cdc14-1 (Figure 5c), which is defective in the activation of cyclin-specific ubiquitin ligase activity [27]. These results are consistent with the possibility that the Cdc28 phosphorylation sites in Hct1 are involved in the negative regulation of APC activity.

The phosphatase Cdc14 dephosphorylates and activates Hct1
Our results raise the possibility that activation of cyclin destruction in late mitosis is triggered by dephosphorylation of inhibitory sites on Hct1. The phosphatase Cdc14 is an excellent candidate for the Hct1 phosphatase, as it is required for late mitotic APC activation and cyclin destruction [27], and is upregulated during mitosis [29]. The fact that the growth defect in cdc14-1 cells is rescued by Hct1-28A expression (Figure 5c) also supports the possibility that Hct1 is a key target of Cdc14.

We tested the ability of Cdc14 to dephosphorylate Hct1 by adding purified glutathione-S-transferase (GST)–Cdc14 to preparations of Hct1 that had been radioactively phosphorylated by purified Cdc28–Cib2 (Figure 6a). In these experiments, Hct1 was labeled by Cdc28 at approximately one site per molecule, resulting in only a partial shift in mobility on the gel. Under these conditions, small amounts of GST–Cdc14 rapidly catalyzed the removal of 50% of the Cdc28-dependent phosphorylation of Hct1, resulting in the collapse of the labeled band into the high mobility form (Figure 6a). We also assessed the Hct1 phosphatase activity of Cdc14 by measuring the radioactive phosphate released from Hct1 into solution. Here, Cdc14 again catalyzed the release of phosphate from Hct1 in a dose-dependent manner (Figure 6b).
Most importantly, treatment of Cdc28-phosphorylated Hct1 with Cdc14 partially restored the ability of Hct1 to activate the APC in vitro (Figure 6c). There was no effect when we added GST–Cdc14 proteins containing point mutations in the active site (C283S or C283S/R289A), which are known to abolish phosphorylation activity [30]. Thus, Cdc14 is able to dephosphorylate key Cdc28-dependent inhibitory sites on Hct1. Addition of purified GST–Cdc14 alone did not stimulate the inactive APC from dcl5-2 cells, and pre-incubation of inactive APC with GST–Cdc14 did not affect subsequent activation by purified Hct1 (data not shown). Thus, Cdc14 does not have a direct action on the APC itself in our experiments.

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

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

We next confirmed the role of Cdc14 in Hct1 dephosphorylation in vivo. Overexpression of CDC14 in mitotically arrested cells abolished Hct1 phosphorylation and triggered APC activation and Clb2 destruction (Figure 7c). Similar results were obtained in cells lacking the CDK inhibitor Sic1 (Figure 7c), the levels of which are known to increase in cells overexpressing CDC14 [33]. Thus, Cdc14 does not trigger Hct1 dephosphorylation indirectly by decreasing Cdc28 activity.

**Discussion**

We conclude that inhibitory phosphorylation of Hct1 is an important mechanism of APC regulation. Several lines of evidence suggest that Cdc28–cyclin complexes are responsible for catalyzing this phosphorylation. First, Hct1 undergoes phosphate-dependent mobility shifts during cell cycle stages when Cdc28 is known to be active, and these shifts are abolished when Cdc28 consensus phosphorylation sites in Hct1 are changed to non-phosphorylatable alanine residues. Second, purified Cdc28–Clb2 complexes catalyze phosphorylation of a large subset of these sites in vitro. Third, Cdc28-dependent phosphorylation inhibits Hct1 function in vitro, explaining previous observations that the activities of Cdc28 and APC are inversely correlated during the cell cycle [5], that inhibition of Cdc28 activity is sufficient to allow nocodazole-arrested cells to exit mitosis, and that artificial induction of Cdc28–Clb2 kinase activity in G1 cells leads to Clb2 stabilization [14].

While this manuscript was in preparation, Zachariae et al. [34] published evidence also suggesting that Hct1 phosphorylation by Cdc28 inhibits APC activity in vivo. They also observed phosphate-dependent Hct1 mobility shifts during cell cycle stages when Cdc28 activity is high, and
Cdc14 promotes Hct1 dephosphorylation and APC activation. (a) Hct1–His₉₄ (50 ng) was incubated with [γ-³²P]ATP and 20 ng Cdc28–Cib2 complexes immobilized on beads, or incubated with ATP in the presence of beads alone. The soluble Hct1–His₉₄ was incubated for 30 min at 30°C with purified GST or with the indicated amounts of purified GST–Cdc14. The percentage of Cdc28-dependent phosphate remaining on Hct1 is indicated below the figure. (b) The ³²P-phosphoribosylated form of Hct1–His₉₄ (final concentration 3 nM) was incubated for 5 min at 25°C with GST or the indicated amounts of GST–Cdc14. Proteins in the reaction were precipitated with acid, and radioactivity in the resulting supernatant was measured. Background phosphate release (in the presence of GST alone) has been subtracted. Because phosphorylated Hct1 is not readily prepared in large quantities, we could not use saturating substrate concentrations, and the rate of phosphate release in these experiments (0.1–0.3 nmol mg⁻¹ min⁻¹) is probably far below maximum. (c) Hct1–His₉₄ (100 ng) was incubated with 20 ng Cdc28–Cib2, either in the presence or absence of 1 mM ATP. We then added the indicated amounts of GST (lane G), GST–Cdc14 (lanes marked 14), or phosphatase-deficient mutant forms of GST–Cdc14 (C283S, lane C; C283S/R289A, lane C/R). After 30 min incubation at 30°C, treated Hct1 was tested for its ability to activate the cyclin–ubiquitin ligase activity of the APC in anti-Cdc28 immunoprecipitates from 400 µg lysates of cdc15-2 cells arrested at 37°C.

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Figure 7

Cdc14 promotes APC activation in crude cell extracts and in vivo. (a) The indicated amounts of GST, GST–Cdc14, or λ-phosphatase were added to cell lysates (1 mg) prepared from cdc14-1 cells arrested at 37°C and expressing Hct1–HA from the GAL promoter. Anti-HA immunoprecipitates were immunoblotted with anti-HA antibody 18B12 (upper panel), or cyclin–ubiquitin ligase activity was measured in anti-Cdc26 immunoprecipitates (lower panel). (b) The indicated amounts of GST (lane G), GST–Cdc14 (lanes marked 14), phosphatase-deficient GST–Cdc14 point mutants (lanes C and C/R), or λ-phosphatase were added to cell lysates (1 mg) prepared from cdc14-1 cells arrested at 37°C. Cyclin–ubiquitin ligase activity was then measured in anti-Cdc26 immunoprecipitates. (c) Wild-type (WT) or sic1Δ cells containing GAL–HCT1–HA alone or in combination with GAL–CDC14 were arrested in mitosis by treatment with 15 µg/mL nocodazole, followed by addition of galactose (4%) for 3 h. APC activity was measured in anti-Cdc26 immunoprecipitates from 600 µg cell extract (upper panel). Cib2 protein was detected by western blotting with anti-Cib2 antibody (middle panel). Hct1–HA protein was immunoprecipitated with anti-HA antibodies from 1 mg cell extract and analyzed by western blotting with anti-HA antibodies (lower panel). The positions of unphosphorylated (Hct1–HA) and phosphorylated (P-Hct1–HA) forms are indicated.
Figure 8

Model of the regulatory system governing Cdc28 inactivation in late mitosis. This scheme accounts for our evidence that the APC core undergoes a Cdc8-dependent and Cdc15-dependent increase in Hct1 sensitivity in late mitosis (indicated by an asterisk). Cdc14 is not required for this process but is required for the activation of APC by Hct1 dephosphorylation. It remains possible that Cdc5 and Cdc15 are also required for Hct1 dephosphorylation and for upregulation of Sic1 [8, 27].

found that Hct1 phosphorylation was inversely correlated with cyclin destruction in vivo. Overexpression of an Hct1 mutant lacking CDK consensus phosphorylation sites blocked cyclin accumulation in vivo. The mutant Hct1 protein was bound to the APC in vivo at cell cycle stages when Cdc28 activity is high, suggesting that Cdc28-dependent Hct1 phosphorylation blocks APC activation by inhibiting the Hct1–APC interaction.

We found that Cdc28-dependent Hct1 phosphorylation was prevented by mutation of five residues in Hct1, while Zachariae et al. [34] observed a complete loss of phosphorylation only when nine sites were mutated. This apparent discrepancy is readily explained. Whereas Zachariae et al. mutated all serines or threonines followed by a proline, we focused on the subset of sites that also have a basic residue two positions after the proline (as is often the case in CDK substrates). Zachariae et al. observed a reduction in Hct1 phosphorylation only when their mutations included one or more of the basic sites that we mutated.

Inhibitory Hct1 phosphorylation thus provides a mechanism by which Cdc28 suppresses cyclin-specific APC activity during S phase and early mitosis. It seems unlikely, however, that this is the only mechanism governing APC activity. Additional regulatory modifications may govern Hct1 function, as some Hct1 phosphorylation is observed in the Hct1-28A mutant and in G1 cells (Figure 3). In addition, cyclin destruction is probably controlled in part by modification of the APC core itself, as suggested by our observation (Figure 2c–f) that the APC from cdc14-arrested and G1-arrested cells is more responsive to Hct1 than the APC from other late mitotic mutants. These results are reminiscent of recent evidence in vertebrates that the affinity of the APC for Cdc20 varies during the cell cycle [19, 20], and are also consistent with previous evidence that APC activity in vertebrates is regulated by phosphorylation of core APC subunits [8–13].

On the basis of these considerations, we speculate that some modification of the APC in late mitosis results in enhanced affinity for Hct1 (Figure 8). Cdc14 is not required for this modification, as the APC from cdc14 cells, unlike that from cdc5 or cdc15 cells, displays the increased Hct1 sensitivity that is seen in G1 cells. In addition, the APC from cdc14-arrested cells has low but reproducibly higher activity than the completely inactive APC from cdc5 and cdc15 cells [27]. Finally, expression of the Hct1-28A mutant is able to partially suppress the late mitotic arrest of the cdc14-1 mutant (Figure 5c), but has no effect on the growth defects in cdc15-2 and cdc5-1 cells (data not shown). These lines of evidence all point to the possibility that the major restraint on APC activity in cdc14-arrested cells is the presence of inhibitory Hct1 phosphorylation, whereas additional APC defects prevent cyclin destruction in the cdc5 and cdc15 mutants (Figure 8).

The ability of Cdc28 and the APC to antagonize each other's activity leads to the potential for a regulatory loop that could enhance the abrupt, all-or-none kinetics of Cdc28 inactivation in late mitosis. The switch-like features of Cdc28 inactivation may also be enhanced by a similar antagonistic relationship between Cdc28 and its inhibitor Sic1, the synthesis and stability of which are inhibited by Cdc28 activity [35–37] (Figure 8). A key issue remains unresolved, however: what initiating event is responsible for reducing Cdc28 activity (or increasing APC or Sic1 activity) to some threshold where these regulatory relationships bring on rapid and complete Cdc28 inactivation? Our studies suggest that Cdc14 could help initiate this process by catalyzing Hct1 dephosphorylation. In addition, Visintin et al. [33] recently found that Cdc14 dephosphorylates Sic1 (leading to its stabilization) and the transcription factor Swi5, leading to an increase in SIC1 expression. We suspect that additional mechanisms, probably involving Cdc5 and Cdc15, also contribute to the late mitotic decline in Cdc28 activity.

Materials and methods

Yeast strains and protein methods

All strains were derived from W303. To construct strains for APC purification, CDC23-3HA (gift from P. Hieter) was integrated at the CDC23 locus of LH131 (Matα cdc23-1; a gift of L. Hwang) by one-step gene replacement. A His⁵ tag was inserted immediately before the stop codon of CDC16, and this construct was used to replace endogenous CDC16 of SLJ128 (MATα bar1 pep4 Δ::URA3) with CDC16-His⁵. These strains were crossed and sporulated to produce SLJ290 (MATα bar1; CDC23::CDC23-3HA CDC16::CDC16-His⁵::LEU2 pep4 Δ::URA3). Additional strains used for APC purification were derived from crosses to SLJ290. Wild-type and mutant HCT1 genes were cloned into a pRS304-based plasmid containing the GAL1/10 promoter and a single
carboxy-terminal HA tag [27]; these constructs were integrated at the TRP1 locus of a wild-type, an hct1Δ::LEU2 (gift of W. Seuffer), a cdc14C-1, or a sic1Δ::LEU2 (gift of A. Rudner) strain. For Cdc28 overproduction experiments, the Cdc14 gene was cloned into pDK20 [38] under control of the GAL11/10 promoter; this construct was integrated at the URA3 locus of wild-type and sic1Δ strains containing GAL-HCT1–HA integrated at the TRP1 locus. Yeast lysate preparation, immunoblotting, immunoprecipitation, and phosphatase treatment were as described [27,39]. Cyclic–ubiquitin ligase activity of the APC from yeast extracts was measured as described [6]. Ubiquitination activity was quantitated on a PhosphoImager using the ImageQuant program (Molecular Dynamics).

APC purification
A pellet from 5 × 10^10 log-phase cells was resuspended in 50 ml APC lysis buffer (50 mM Heps-NaOH pH 7.4, 75 mM KCl, 50 mM NaF, 1 mM MgCl2, 1 mM EGTA, 0.1% NP-40, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 4 μg/ml pepstatin) and cells were lysed by bead beating in a Mega Beater (Biospec) at 4°C. Lysates were clarified by centrifugation for 10 min at 9,000 × g at 4°C followed by ultracentrifugation (1 h, 80,000 × g, 4°C). The lysate was loaded onto a 5 ml HiTrap Chelating column (Pharmacia) charged with cobalt and equilibrated in APC Buffer A (50 mM Heps-NaOH pH 7.4, 200 mM KCl, 50 mM NaF, 0.1% NP-40, 10% glycerol). Following a wash with APC-Buffer A containing 10 mM imidazole, bound proteins were then eluted with a linear gradient (10–200 mM) of imidazole in APC-Buffer A. Fractions containing the APC were pooled, diluted to 50 mM KCl in APC Buffer B (20 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 10% glycerol, 1 mM dithiothreitol (DTT)), and loaded onto a 1 ml HiTrap SP column equilibrated in APC-Buffer B containing 50 mM KCl. Proteins were eluted with a 50–350 mM KCl gradient in APC-Buffer B. The APC fractions were pooled, diluted to 100 mM KCl with APC-Buffer C (APC Buffer B with 0.1% Tween-20), and loaded on to a 1 ml HiTrap Q column. Following a wash in APC-Buffer C plus 250 mM KCl, the APC was eluted from the column with a 250–750 mM KCl gradient in APC-Buffer C. Fractions containing the APC were pooled, diluted with an equal volume of APC-Buffer A, and repurified on a 1 ml HiTrap Chelating column charged with cobalt. APC-containing fractions were pooled and insulin (Sigma) was added to 0.1 mg/ml. The concentration of the purified APC was approximated based on the silver staining intensity of the Cdc16 band.

Hct1 purification
HCT1 was amplified from genomic DNA by PCR and cloned into pFB-BHA at the NcoI site to create pFB-Hct1-H6. The Cdc28 phosphatase mutant mCdc28-Ha128, a myosephate-mediated mutagenesis of pFB-Hct1H6 [40] to change serine 16, 42, 227, 239 and 436 and threonine 176 to alanine. Baculoviruses encoding wild-type or mutant Hct1–His6 tags were generated using the Bac-to-Tac expression system (Gibco BRL). Wild-type and mutant Hct1–His6 were purified from baculovirus-infected Sf9 cells by metal-affinity chromatography as described [41]. Tween-20 (0.1%) and insulin (0.1 mg/ml) were added prior to storage.

Cdc28–Clb2 purification
To produce an active Cdc28–Clb2 kinase, lysate was prepared from insect cells co-infected with baculoviruses encoding Cdc28–HA and CaK1–HA3 (a gift of A. Farrell), and combined with a lysate of bacteria expressing a Clb2–maltose binding protein (MBP) fusion protein (a gift of R. Deshaies). The Cdc28–Clb2–MBP complex was then purified on an amylose column (NEB), followed by cation-exchange chromatography (Pharmacia SP Sepharose Fast Flow). Approximately equimolar amounts of Cdc28 and Clb2 were present in the purified complex.

Cdc14 purification
CDC14 was amplified from genomic DNA by PCR and cloned into the BamHI site of pGEX-3X (Pharmacia). Bacteria transformed with this construct were grown to an OD600 of 0.8, and expression of GST–Cdc14 was induced with IPTG (0.1 mM) for 16 h at 23°C. The recombinant protein was purified on a glutathione–sepharose 4B column (Pharmacia), followed by anion-exchange chromatography (Pharmacia HiTrap Q). Point mutants in the Cdc14 active site were generated by subcloning CDC14 into pBSKII+ (Stratagene) for oligonucleotide-directed mutagenesis.

Phosphorylation of Hct1 by Cdc28–Clb2
Purified Cdc28–Clb2–MBP was incubated for 20 min at 23°C in a 20 μl reaction mixture containing 100 μM ATP, 150 ng Hct1–His6, and 2.5 μCi [γ-32P]ATP (3000 Ci/mmol) in kinase buffer (50 mM Heps-NaOH pH 7.4, 10 mM MgCl2, and 1 mM DTT). Reaction products were analyzed by 8% SDS–PAGE followed by autoradiography. To determine the effect of Cdc28–Clb2 phosphorylation on Hct1–His6, the Cdc28–Clb2 complex was immobilized on protein-A–sepharose beads (Sigma) by immunoprecipitation with anti-Clb2 [42] and anti-MBP (NEB) polyclonal antibodies. Immobilized Cdc28–Clb2 complexes were used to phosphorylate Hct1–His6 in reactions containing 1 mM ATP. Following a 30 min incubation at 25°C, Hct1–His6 was separated from the Cdc28–Clb2 by removal of the beads.

Hct1 phosphate release assay
Hct1–His6 (2.7 μg) was radiolabeled by 20 min incubation at 25°C with 1.1 μg Cdc28–Clb2 and 100 μCi [γ-32P]ATP (3,000 Ci/mmol) in a 560 μl reaction. Free ATP was removed by gel filtration on a 4 ml Sephadex G25 column equilibrated in HBS (25 mM Heps-NaOH pH 7.4, 150 mM NaCl, 1 mM DTT), followed by addition of glycerol (10% v/v) and insulin (0.1 mg/ml). Aliquots of labeled Hct1 (90 ng; 200,000 cpm) were incubated for 5 min at 25°C in 20 μl reactions with GST or GST–Cdc14, followed by addition of 10 μl BSA (10 mg/ml) and 180 μl ice-cold 20% trichloroacetic acid. Following incubation on ice for 30 min, the mixture was centrifuged (16,000 × g, 10 min, 4°C), and 100 μl of the supernatant was removed for quantitation by scintillation counting.

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