with 10% heat-inactivated fetal calf serum (Sigma) and 5% penicillin/streptomycin (Gibco-BRL), were plated overnight on acid-washed glass cover slips (13 mm in diameter) in four-well plates at a density of 10^5 cells per milliliter in each well. Cells were serum-starved immediately before microinjection. Biotin dextran (2.5 mg/ml) (Molecular Probes, Eugene, OR) was injected alone or with eukaryotic expression vectors (0.1 mg/ml) encoding Myc-tagged GTPase constructs into the nucleus of at least 50 cells over a period of 10 min. Cells were returned to the incubator for 3 hours for optimal expression. RBCs were opsonized, and phagocytic assay and immunofluorescence were performed as described (*13*). The two modifications that we introduced were (i) the preactivation of J774 cells for 15 min at 37°C with phorbol 12-myristate 13-acetate (150 ng/ml) in serum-free medium before the phagocytic challenge with CR3 targets and (ii) the visualization of microinjected cells after staining with cascade blue–conjugated avidin (Molecular Probes). To block nonspecific binding of antibodies to the Fc receptor, we performed incubations with antibodies in the presence of excess human or murine IgGs. All injected (cascade blue positive) J774.A1 control cells or all Myc-expressing macrophages were assessed (that is, ≥50 cells per condition). Microinjection did not affect viability or morphology nor did it interfere with the cell's ability to bind targets. The percentage of phagocytosis-competent cells was similar in uninjected cells and cells that were injected with biotin dextran.

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Control of Cyclin Ubiquitination by CDK-Regulated Binding of Hct1 to the Anaphase Promoting Complex

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Proteolysis of mitotic cyclins depends on a multisubunit ubiquitin–protein ligase, the anaphase promoting complex (APC). Proteolysis commences during anaphase, persisting throughout G_1 until it is terminated by cyclin-dependent kinases (CDKs) as cells enter S phase. Proteolysis of mitotic cyclins in yeast was shown to require association of the APC with the substrate-specific activator Hct1 (also called Cdh1). Phosphorylation of Hct1 by CDKs blocked the Hct1-APC interaction. The mutual inhibition between APC and CDKs explains how cells suppress mitotic CDK activity during G_1 and then establish a period with elevated kinase activity from S phase until anaphase.

Entry into anaphase and exit from mitosis are promoted by APC-dependent proteolysis of regulatory proteins (*1*). Sister chromatid separation requires Pds1 degradation shortly before anaphase onset, whereas Cdk1 inactivation during late anaphase involves proteolysis of mitotic cyclins such as Clb2. How activity of the APC toward different substrates is regulated during the cell cycle is unclear. The APC itself might be regulated, because the cyclin ubiquitination activity associated with purified APC fluctuates during the cell cycle (*2, 3*). APC-dependent proteolysis requires two related proteins containing Trp-Asp repeats which function as substrate-specific activators. Cdc20 promotes degradation of "early" substrates such as Pds1 and Hct1 promotes degradation of "late" substrates such as Clb2 (*4–6*). In yeast, there is an inverse correlation between Cdk1 activity and degradation of mitotic cyclins (*7*). Ectopic inhibition of Cdk1 induces precocious cyclin degradation, suggesting a role for Cdk1 in the inhibition of cyclin proteolysis from S phase until anaphase (*8*). However, the relevant Cdk1 substrate has not been identified.

To test whether Hct1 is needed for cyclin ubiquitination, we incubated extracts from G_1-arrested wild-type and *hct1* mutant cells with Clb2 and Clb3 (*9*). Wild-type extracts supported destruction box–dependent cyclin ubiquitination, whereas *hct1* mutant extracts were as defective in this reaction as extracts from a *cdc16-123* mutant that contains a defective APC subunit (Fig. 1). Thus, Hct1 was required for APC-mediated cyclin ubiquitination.

To test whether Hct1 associated with the APC, we constructed *CDC16-HA3* strains containing Hct1 variants with Myc epitopes at the NH_2-terminus (Myc9-Hct1) or the COOH-terminus (Hct1-Myc9) (*10*). *HCT1-myc9* strains were defective in the degradation of Clb2 and Clb3, whereas Myc9-Hct1 was fully functional. Cdc16-HA3 coprecipitated with Myc9-Hct1 but not with Hct1-Myc9 in extracts prepared from cycling or G_1-arrested cells (Fig. 2A) (*11*). Another APC subunit, Cdc23-HA3, also coprecipitated with Myc9-Hct1 but not with Hct1-Myc9 (*12*). The correlation between Hct1 function and coprecipitation with APC subunits suggests that cyclin ubiquitination depends on an Hct1-APC interaction.

Myc9-Hct1 was not associated with Cdc16-HA3 in extracts from cells arrested in S phase by hydroxyurea or in M phase by nocodazole (Fig. 2A). Cdc20, in contrast, was associated with APC subunits in both extracts (Fig. 2B). To test whether the Hct1-APC interaction was regulated during an unperturbed cell cycle, we measured the association between Myc18-Hct1 and Cdc16-HA3 in cells synchronized by centrifugal elutriation (Fig. 2C) (*13*). Hct1 was associated with Cdc16 during G_1 but not during the S, G_2, and M phases (*14*). Dissociation of Hct1 from the APC correlated with appearance of the S phase promoting Clb5-Cdk1 activity. Thus, the Hct1-APC interaction was cell cycle–regulated.

The Hct1-APC interaction occurred only

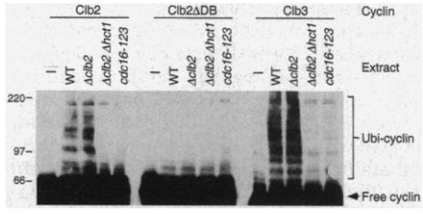

Fig. 1. Requirement of Hct1 for ubiquitination of mitotic cyclins. Strains (*MATa Δpep4 Δbar1*) were arrested in G_1 with α factor at 25°C and shifted to 37°C for 30 min. Protein extracts were incubated with adenosine 5′-triphosphate (ATP) and HA3-tagged cyclins (*9*). Clb2ΔDB lacks the destruction box. Cyclin-ubiquitin conjugates were detected by immunoblotting with an antibody to the HA epitope. Molecular sizes in kilodaltons are indicated on the left. *Δhct1* mutants are partially resistant to α factor. To allow complete arrest in G_1, *CLB2* was deleted (*4*).

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in cells lacking Cdk1 activity. To test whether Cdk1 might block the Hct1-APC interaction, we inactivated all Cdk1 kinases in nocodazole-arrested *Myc9-HCT1 CDC23-HA3* cells by overproduction of the B-type cyclin-Cdk1 (Clb-Cdk1) inhibitor Sic1. We added α factor pheromone to inhibit G_1 cyclin-Cdk1 (Cln-Cdk1). Cdk1 inactivation induced association of Myc9-Hct1 with Cdc23-HA3 and degradation of mitotic cyclins (Fig. 2D).

Either the APC or Hct1 might be regulated by Cdk1 activity. No differences

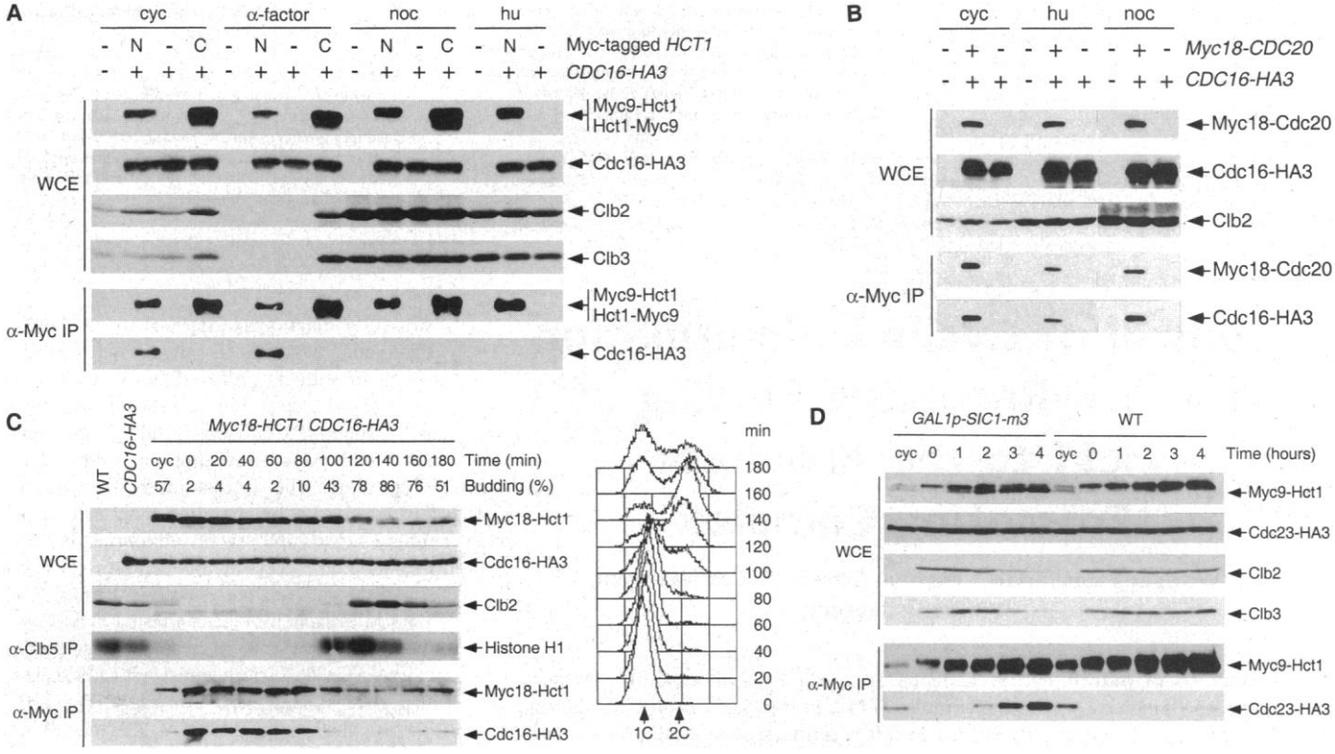

Fig. 2. Binding of Hct1 and Cdc20 to the APC during the cell cycle. Whole-cell extracts (WCE) and proteins immunoprecipitated with an antibody to Myc (α-Myc IP) were analyzed by immunoblotting. (**A**) Hct1-APC association at different cell cycle stages. Strains contained wild-type proteins (–) or Cdc16-HA3 (+) and Hct1 carrying a NH_2-terminal (N) or a COOH-terminal (C) Myc9 tag. Cells were grown at 25°C (cyc) and arrested with α factor, hydroxyurea (hu), or nocodazole (noc). (**B**) Cdc20-APC association in cells containing high Cdk1 activity. Strains containing wild-type genes (–) or *CDC16-HA3* (+) and *Myc18-CDC20* (+) were grown at 30°C (cyc) and arrested with hydroxyurea (hu) or nocodazole (noc). (**C**) Hct1-APC interaction during an unperturbed cell cycle. Small G_1 cells of a *Myc18-HCT1 CDC16-HA3 Δpep4* strain were released into glucose medium, and samples were withdrawn at the indicated times (*13*). Clb5-Cdk1 activity was measured with the substrate histone H1. Control strains (WT and *CDC16-HA3*) were grown in glucose medium. Graphs show cellular DNA content. (**D**) Induction of Hct1-APC association by Cdk1 inactivation. A *Myc9-HCT1 CDC23-HA3 Δpep4* strain (WT) and a congenic strain containing five copies of *GAL1p-SIC1-m3* (*24*) were grown in raffinose medium at 25°C (cyc) and arrested with nocodazole. Samples were taken at the indicated times after addition of galactose and α factor (5 μg/ml).

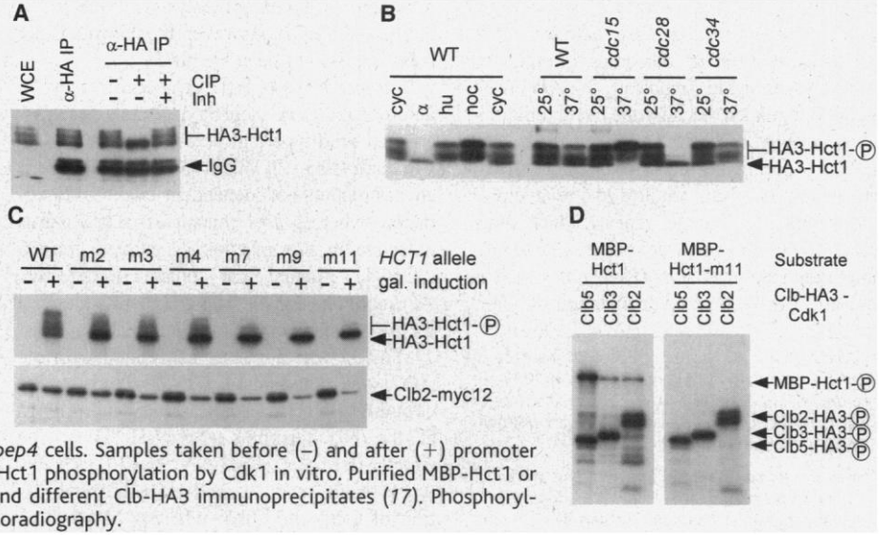

Fig. 3. Phosphorylation of Hct1 by Cdk1. (**A**) Modification of Hct1 by phosphorylation in vivo. HA3-Hct1 was detected by immunoblotting in whole-cell extracts (WCE) or anti-HA immunoprecipitates (α-HA IP) prepared from growing *HA3-HCT1* cells. Precipitates were incubated with (+) or without (–) alkaline phosphatase (CIP) and phosphatase inhibitors (Inh) (*15*). (**B**) Cell cycle regulation of Hct1 phosphorylation. HA3-Hct1 was detected in extracts from growing cells (cyc), from cells arrested with α factor (α), hydroxyurea (hu), nocodazole (noc) and from wild-type (WT) or mutant (*cdc15-2, cdc28-4, cdc34-1*) *HA3-HCT1* cells grown at 25°C and then shifted to 37°C for 3 hours. (**C**) Hct1 phosphorylation at Cdk1 consensus sites. *HA3-HCT1* (WT) and alleles lacking the indicated number of potential Cdk1 phosphorylation sites (*16*) were expressed for 2 hours from the *GALL* promoter in *CLB2-myc12 Δpep4* cells. Samples taken before (–) and after (+) promoter induction were analyzed by immunoblotting. (**D**) Hct1 phosphorylation by Cdk1 in vitro. Purified MBP-Hct1 or MBP-Hct1-m11 was incubated with [γ-^{32}P]ATP and different Clb-HA3 immunoprecipitates (*17*). Phosphorylated MBP-Hct1 and cyclins were detected by autoradiography.

could be detected in the mobility on SDS-polyacrylamide gels of APC subunits isolated from cycling cells and cells arrested in G_1 phase by α factor or in M phase by nocodazole (*12*). However, the mobility of Myc18-Hct1 varied during the cell cycle (Fig. 2C). To facilitate the analysis of this mobility shift, we tagged Hct1 with the smaller HA3 epitope (*10*). HA3-Hct1 from cycling cells migrated as multiple bands (Fig. 3A). Phosphatase treatment of HA3-Hct1 immunoprecipitates (*15*) eliminated the upper bands, demonstrating that Hct1 was phosphorylated in vivo.

Phosphorylation of Hct1 was then analyzed in extracts from cells arrested at different cell cycle stages (Fig. 3B). Hct1 was phosphorylated in *cdc34* mutants, which arrest before S phase with active Cln-Cdk1 but inactive Clb-Cdk1 kinases. Hct1 was also phosphorylated in cells arrested in S phase with hydroxyurea, in M phase with nocodazole, and in *cdc15* mutants, which arrest in late anaphase. All of these cell cycle arrests lead to the accumulation of active Clb-Cdk1 kinases. Hct1 was apparently unphosphorylated in cells arrested in G_1 by α factor or by a *cdc28* mutation. Thus, Hct1 might be phosphorylated in vivo by Cln- and Clb-Cdk1 kinases.

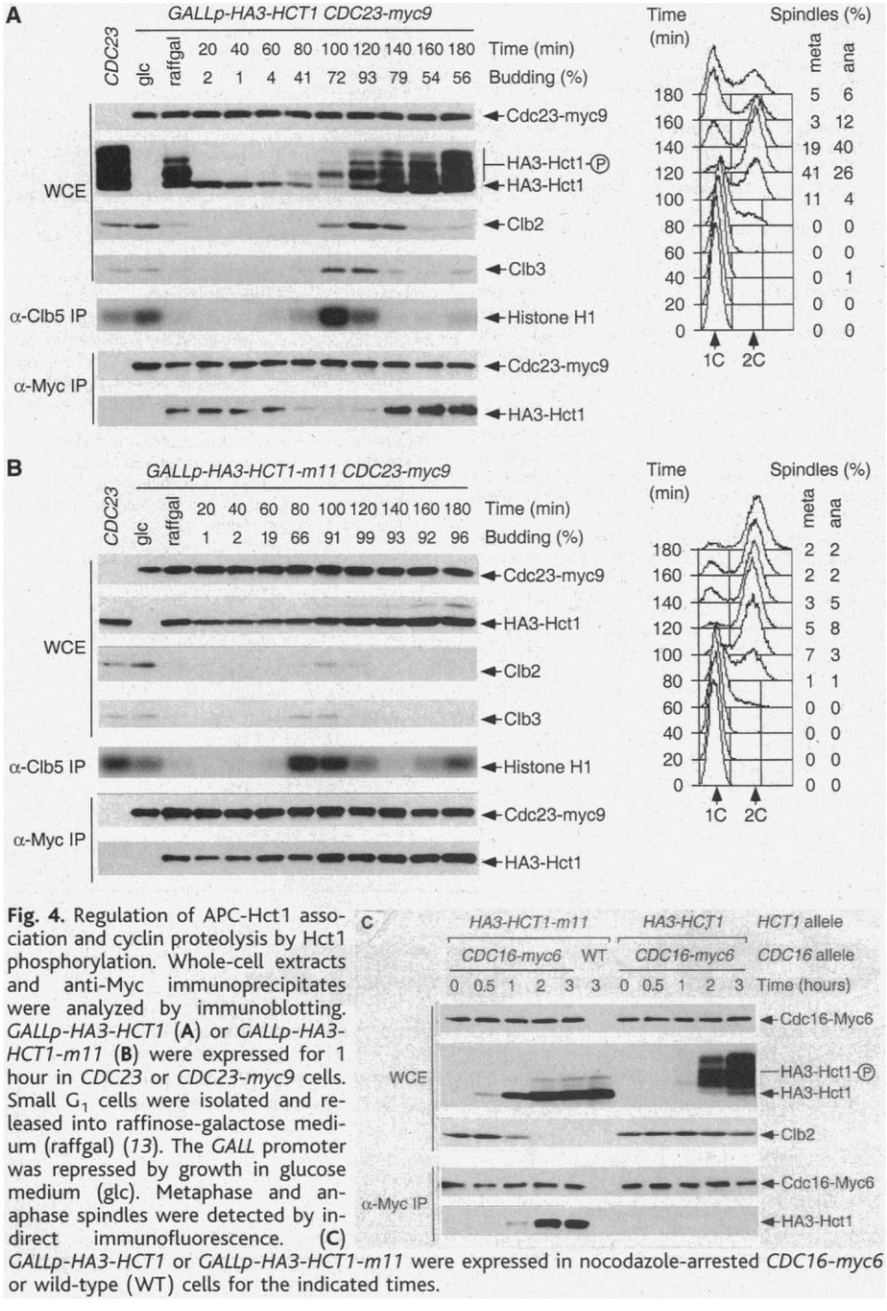

Fig. 4. Regulation of APC-Hct1 association and cyclin proteolysis by Hct1 phosphorylation. Whole-cell extracts and anti-Myc immunoprecipitates were analyzed by immunoblotting. *GALLp-HA3-HCT1* (**A**) or *GALLp-HA3-HCT1-m11* (**B**) were expressed for 1 hour in *CDC23* or *CDC23-myc9* cells. Small G_1 cells were isolated and released into raffinose-galactose medium (raffgal) (*13*). The *GALL* promoter was repressed by growth in glucose medium (glc). Metaphase and anaphase spindles were detected by indirect immunofluorescence. (**C**) *GALLp-HA3-HCT1* or *GALLp-HA3-HCT1-m11* were expressed in nocodazole-arrested *CDC16-myc6* or wild-type (WT) cells for the indicated times.

Hct1 contains 11 potential Cdk1 phosphorylation sites. To test whether these sites were phosphorylated in vivo, we replaced phospho-accepting serine and threonine residues with alanine residues and expressed different *HA3-HCT1* mutants from the weak, galactose-inducible *GALL* promoter (Fig. 3C) (*16*). The abundance of low-mobility Hct1 species decreased as more potential phospho-acceptor sites were removed. No cell cycle–regulated mobility shift was detected with the mutants m9 and m11, which lack 9 or all 11 potential Cdk1 phosphorylation sites, respectively. Thus, Hct1 was phosphorylated at multiple sites in vivo. There was an inverse correlation between the number of phosphorylatable residues and the ability of Hct1 variants to activate Clb2 degradation (Fig. 3C), suggesting that phosphorylation could inhibit Hct1 activity.

To test whether Hct1 was a Cdk1 substrate in vitro, we produced Hct1 and Hct1-m11 in *Escherichia coli* as fusion proteins with the maltose binding protein (MBP). MBP, MBP-Hct1, and MBP-Hct1-m11 were purified and incubated with Clb2-, Clb3-, and Clb5-Cdk1 kinases immunoprecipitated from strains expressing HA3-tagged cyclins (*17*). MBP-Hct1 but not MBP-Hct1-m11 (or MBP alone) was phosphorylated by all three kinases (Fig. 3D). MBP-Hct1 was also phosphorylated by Cln1- and Cln2-Cdk1 (*18*).

To analyze the role of Hct1 phosphorylation in vivo, we released small G_1 cells expressing *CDC23-myc9* and *GALLp-HA3-HCT1* or *GALLp-HA3-HCT1-m11* into a synchronous cell cycle (Fig. 4, A and B). HA3-Hct1 and HA3-Hct1-m11 were produced in similar amounts, and DNA replication and budding occurred normally in both strains. Unlike HA3-Hct1, the mutant protein was neither phosphorylated nor released from Cdc23-Myc9 as cells activated the Clb5-Cdk1 kinase. *HA3-HCT1-m11* cells failed to accumulate the mitotic cyclins Clb2 and Clb3, to form mitotic spindles, and to undergo cytokinesis (Fig. 4B). Thus, Hct1 phosphorylation was required for cell cycle events depending on mitotic CDKs.

GALLp-HA3-HCT1 and *GALLp-HA3-HCT1-m11* were also expressed in nocodazole-arrested *CDC16-myc6* cells, which contain high Clb-Cdk1 activity (Fig. 4C). Both proteins accumulated to similar levels, and HA3-Hct1 but not HA3-Hct1-m11 was phosphorylated. HA3-Hct1 neither coprecipitated with Cdc16-Myc6 nor induced Clb2 proteolysis, whereas HA3-Hct1-m11 both associated with Cdc16-Myc6 and triggered Clb2 proteolysis. Thus, Cdk1 blocked the Hct1-APC interaction by phosphorylation of Hct1.

Hct1 was essential for APC-mediated ubiquitination of mitotic cyclins in yeast. The Hct1 homologs of higher eukaryotes might

perform similar functions. *Drosophila* fizzy-related is required for cyclin removal during G_1 in vivo (*19*), and human Cdh1 binds to the APC and stimulates cyclin-B ubiquitination in vitro (*20*).

Hct1 provides a regulatory link between the two key regulators of cell cycle progression, CDKs and the APC. Hct1-dependent cyclin-B proteolysis during G_1 creates a state devoid of Clb-Cdk1 activity, which is required for the formation of replication-competent complexes at chromosomal origins (*21*). As cells reach a critical size in late G_1, Hct1 is inactivated by Cdk1 associated with Cln1, Cln2, or Clb5, which are refractory to the activity of Hct1. Cyclin-E–Cdk2 activity might have a similar role in animal cells (*19*). Once established, Cdk1 activity can be maintained by cyclins such as Clb2 and Clb3 that are susceptible to Hct1-dependent proteolysis.

Reactivation of Hct1 during anaphase coincides with stabilization of Sic1, and both events require dephosphorylation. These reactions might be catalyzed by the phosphatase Cdc14, which is essential for inactivation of mitotic CDKs (*22*) and whose overexpression causes cells to arrest with low Clb2 levels (*23*).

Cdc20 also binds to the APC and presumably activates Pds1 ubiquitination. Association of Cdc20 occurred in the presence of high Cdk1 activity, which inhibits Hct1. Pds1 degradation, which allows sister chromatid separation, occurs while degradation of mitotic cyclins, which allows cytokinesis and DNA rereplication, is still inhibited (*6*). Thus, different properties of the APC activators Cdc20 and Hct1 help to ensure that anaphase, cytokinesis, and DNA replication occur in the right order.

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10. Strains were W303 derivatives. The genomic *HCT1* open reading frame was tagged with Myc epitopes as described (*6*). Hct1 is a rare protein. In growing cells, the amount of Myc9-Hct1 was approximately 20-fold lower than that of Cdc23-Myc9 (*12*). In Fig. 3, A and B, a *HA3-HCT1-TRP1* plasmid was integrated at the *HCT1* locus.
11. Extracts (5 mg) in buffer B70 (0.4 ml) were immunoprecipitated with monoclonal antibodies (5 μg) 12CA5 or 9E10 and protein A–agarose (30 μl) as described [W. Zachariae *et al.*, *Science* **279**, 1216 (1998)].
12. W. Zachariae and K. Nasmyth, unpublished results.
13. Strains were grown in raffinose medium at 25°C. Small G_1 cells were isolated by centrifugal elutriation and released at 30°C [E. Schwob and K. Nasmyth, *Genes Dev.* **7**, 1160 (1993)]. Flow cytometric DNA quantitation and indirect immunofluorescence were performed as described (*6*). Clb5-associated histone H1 kinase activity was measured with antiserum to Clb5 (*21*).
14. The drop in the amount of Cdc16 associated with Hct1 was not due to the modest reduction in Hct1 levels during the S and G_2 phases. In immunoprecipitates containing equal amounts of Myc18-Hct1, the amount of associated Cdc16-HA3 dropped 10-fold as cells entered S phase (*12*).
15. Immunoprecipitates were incubated with calf intestinal alkaline phosphatase (0.2 U/μl, Boehringer) and phosphatase inhibitors [S. Lanker, M. H. Valdivieso, C. Wittenberg, *Science* **271**, 1597 (1996)].
16. Ser-Pro and Thr-Pro sites were changed to Ala-Pro by site-directed mutagenesis at the following positions: Hct1-m2: 12,16; Hct1-m3: 12, 16, 42; Hct1-m4: 12, 16, 42, 157; Hct1-m7: 12, 16, 42, 157, 169, 173, 176; Hct1-m9: 12, 16, 42, 157, 169, 173, 176, 227, 239; Hct1-m11: 12, 16, 42, 157, 169, 173, 176, 227, 239, 418, 436. *HA3-HCT1* alleles were expressed from the *GALL* promoter on plasmid p416GALL [D. Mumberg, R. Mueller, M. Funk, *Nucleic Acids Res.* **22**, 5767 (1994)] (Fig. 3C) or on plasmids integrated at the *trp1* locus (Fig. 4).
17. *malE-HCT1* fusions were expressed from the rhamnose-inducible *rhaB* promoter [J.-N. Volff *et al.*, *Mol. Microbiol.* **21**, 1037 (1996)] in *E. coli* cells containing the $tRNA_{arg4}$ plasmid pUBS520 [U. Brinkmann, R. E. Mattes, P. Buckel, *Gene* **85**, 109 (1989)]. Cells were grown at 20°C in 2× TY (tryptone–yeast extract) medium with 2% ethanol and 0.2% rhamnose for 48 hours. MBP-Hct1 proteins were purified on amylose columns (New England Biolabs). Anti-HA precipitates prepared from *GAL1-CLB5-HA3*, *GAL1-CLB3-HA3*, and *GAL1-CLB2-HA3* strains were incubated with MBP-Hct1 or MBP-Hct1-m11 (0.1 μg) and [γ-^{32}P]ATP (5 μCi) at 30°C for 10 min as described [M. Tyers, G. Tokiwa, R. Nash, B. Futcher, *EMBO J.* **11**, 1773 (1992)].
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24. The nondegradable variant Sic1-m3 contains mutations in Cdk1 phosphorylation sites (Thr^5 → Val, Thr^{33} → Val, Ser^{76} → Ala) [E. Schwob, T. Böhm, K. Nasmyth, unpublished results; R. Verma *et al.*, *Science* **278**, 455 (1997)].

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