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# Ubiquitination of the $G_1$ cyclin Cln2p by a Cdc34p-dependent pathway

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**Recombinant $G_1$ cyclin Cln2p can bind to and stimulate the protein kinase activity of $p34^{CDC28}$ (Cdc28p) in an extract derived from cyclin-depleted and $G_1$-arrested *Saccharomyces cerevisiae* cells. Upon activating Cdc28p, Cln2p is extensively phosphorylated and conjugated with multiubiquitin chains. Ubiquitination of Cln2p *in vitro* requires the Cdc34p ubiquitin-conjugating enzyme, Cdc28p, protein phosphorylation and unidentified factors in yeast extract. Ubiquitination of Cln2p by Cdc34p contributes to the instability of Cln2p *in vivo*, as the rate of Cln2p degradation is reduced in $cdc34^{ts}$ cells. These results provide a molecular framework for $G_1$ cyclin instability and suggest that a multicomponent, regulated pathway specifies the selective ubiquitination of $G_1$ cyclins.**

*Key words*: CDC34/CLN/cyclin/ubiquitin/yeast

## Introduction

The transient and cyclical appearance of an array of cyclin/cyclin-dependent kinase (Cdk) activities is thought to drive cells through successive phases of the cell cycle. Thus, factors that positively or negatively modulate the activity of cyclin–Cdk complexes are likely to exert profound influence over progression through the cell division cycle. Three mechanisms for inhibiting the activity of cyclin–Cdk assemblages have been described. Kinase activity expressed by cyclin A–, D– or E–Cdk (Gu *et al.*, 1993; Harper *et al.*, 1993; Serrano *et al.*, 1993; Xiong *et al.*, 1993), Cln–Cdc28p (Peter *et al.*, 1993) and cyclin B–Cdc28p (Mendenhall, 1993) complexes can be specifically attenuated by physically associated inhibitor proteins. Conversely, the activity of cyclin B–$p34^{cdc2}$ complexes is constrained prior to mitosis by tyrosine phosphorylation of $p34^{cdc2}$ (reviewed in Solomon, 1993; Dunphy, 1994) and late in mitosis by rapid degradation of cyclin B.

Destruction of A and B cyclins (Murray *et al.*, 1989; Ghiara *et al.*, 1991; Glotzer *et al.*, 1991; Luca *et al.*, 1991; Gallant and Nigg, 1992; van der Velden and Lohka, 1993) is critical for exit from mitosis, and proteolysis of the yeast $G_1$ cyclin Cln3p may be required for correct regulation of $G_1$ phase (Tyers *et al.*, 1992; Cross and Blake, 1993). Cyclins A and B are slated for rapid destruction by the covalent attachment of ubiquitin (Glotzer *et al.*, 1991; Hershko *et al.*, 1991). Ubiquitin is a 76 amino acid polypeptide conserved among all eukaryotes. Attachment of ubiquitin to substrate proteins requires the coordinated actions of a group of enzymes (reviewed in Finley and Chau, 1991; Hershko and Ciechanover, 1992). Ubiquitin is first activated by adenylylation of its C-terminus by an enzyme referred to as E1, and activated ubiquitin is subsequently linked via a thiol ester to the cysteine residue of an enzyme referred to as E2. E2 activity is encoded by at least 10 genes in yeast (Chen *et al.*, 1993), and is thought to contribute significantly to the substrate specificity of the ubiquitination pathway. The charged E2 then transfers ubiquitin to a substrate protein by catalyzing the formation of an isopeptide bond between the C-terminus of ubiquitin and ε-N groups on lysine residues in the target protein. In some cases, the E2-dependent conjugation of ubiquitin to a target protein also requires the participation of a protein known as E3. Subsequent attachment of additional ubiquitin monomers to lysines on the substrate-tethered ubiquitin leads to the formation of long multiubiquitin chains, which specify rapid degradation of the appended protein. With the exception of the N-end rule (Varshavsky, 1992), little is known about how components of the ubiquitination pathway select specific substrates from the pool of available cellular proteins. Although a peptide segment that signals multiubiquitination has been identified in cyclin B (Glotzer *et al.*, 1991), the enzymes required for ubiquitination of cyclin B have not yet been directly identified (Hershko *et al.*, 1994).

In keeping with their names, other members of the cyclin family exhibit periodic accumulation during the cell cycle (Wittenberg *et al.*, 1990; Dulic *et al.*, 1992; Koff *et al.*, 1992; Matsushime *et al.*, 1992; Baldin *et al.*, 1993; Tyers *et al.*, 1993). Though periodic accumulation implies an unstable protein, the stability of most other cyclins has not been examined directly, and it remains unknown whether the degradation of other cyclins is constitutive or regulated and whether their destruction is required for proper regulation of the cell cycle. Despite our poor understanding of the control of cyclin stability, destruction of these regulatory molecules may be of general significance, as it provides a simple, irreversible means of resetting the activity of their cognate Cdk partners for successive cell cycles.

## Results

### *Cln2p produced by in vitro translation activates $Cdc28^{HA}p$*

We described previously an assay that measures the activation of a hemagglutinin epitope-tagged version of

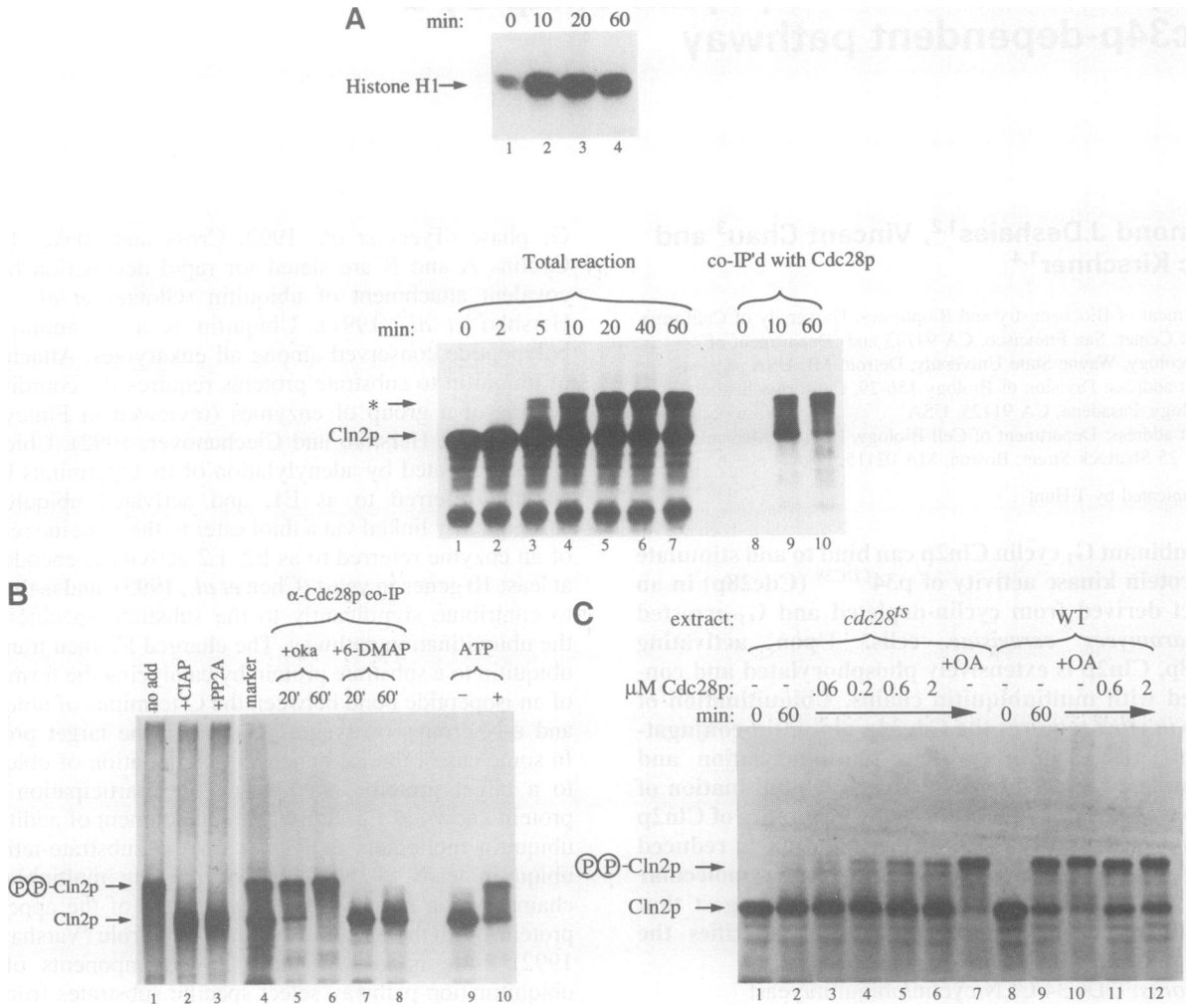


**Fig. 1.** Cln2p translated in reticulocyte extract activates Cdc28$^{HA}$p and is rapidly phosphorylated. (**A**) Cln2p synthesized *in vitro* in the presence of [$^{35}$S]methionine was incubated in 15 mg/ml RJD567 (*cln1,2,3*-Δ, *GAL–CLN3*, *CDC28$^{ha}$*) extract for 0–60 min at 24°C prior to immunoprecipitation of Cdc28$^{HA}$p with α-HA antibodies and histone H1 kinase assay (top panel) or SDS–PAGE and fluorography (bottom panel). Samples in lanes 8–10 (bottom panel) were subjected to immunoprecipitation with α-HA prior to SDS–PAGE. (*) refers to an 84 kDa form of Cln2p that appears upon incubation in yeast extract. (**B**) Cln2p synthesized *in vitro* in the presence of [$^{35}$S]methionine was incubated in 15 mg/ml RJD567 extract for 60 min at 24°C, and the portion assembled with Cdc28$^{HA}$p was retrieved by immunoprecipitation with α-HA antibodies. Washed immunoprecipitates were either mock-treated (lane 1) or incubated with 3 μl 10 U/μl calf intestinal alkaline phosphatase (lane 2) or 3 μl of a fraction enriched for protein phosphatase 2A (lane 3) for 30 min at 24°C prior to SDS–PAGE and fluorography. Lanes 5–8: Cln2p produced by translation *in vitro* in the presence of [$^{35}$S]methionine was incubated in 15 mg/ml RJD567 extract for 20 or 60 min in the presence of either 2 μM okadaic acid (lanes 5 and 6) or 5 mM 6-DMAP (lanes 7 and 8). Cln2p molecules assembled with Cdc28$^{HA}$p were retrieved by immunoprecipitation with α-HA antibodies prior to SDS–PAGE and fluorography. Lanes 9 and 10: washed immunoprecipitates prepared as described for lane 7 were incubated with (lane 10) or without (lane 9) 0.5 mM ATP in standard kinase assay buffer (Deshaies and Kirschner, 1995) for 10 min at 24°C. PP-Cln2p, hyperphosphorylated Cln2p. (**C**) Cln2p synthesized *in vitro* in the presence of [$^{35}$S]methionine was incubated in 18 mg/ml RJD564 (*cdc28$^{ts}$*) extract (lanes 1–7) or RJD567 extract (lanes 8–12) for 0–60 min at 24°C in the presence of either the indicated amounts of renatured Cdc28$^{HA}$p purified from *E.coli* (lanes 3–6 and 11 and 12) or 2 μM okadaic acid (lanes 7 and 10). PP-Cln2p, hyperphosphorylated Cln2p.

*Saccharomyces cerevisiae* p34$^{CDC28}$ (Cdc28$^{HA}$p) induced by adding $G_1$ or B cyclins to a crude yeast extract (Deshaies and Kirschner, 1995). To summarize, a concentrated extract prepared from *CLN*-depleted, $G_1$-arrested yeast cells was supplemented with both an ATP regenerating system and glutathione-*S*-transferase (GST)–cyclin chimeras purified from *Escherichia coli*. Cdc28$^{HA}$p activation was monitored by quantitating histone H1 kinase activity in anti-hemagglutinin (α-HA) immunoprecipitates. To investigate post-translational modifications that may occur to cyclin consequent to activation of its catalytic partner, we have modified the original assay by substituting $^{35}$S-radiolabeled Cln2p synthesized in a reticulocyte lysate for the GST–Cln2p used previously. As seen with GST–Cln2p, Cln2p produced by *in vitro* translation effectively activated quiescent Cdc28$^{HA}$p present in a *CLN*-depleted extract (Figure 1A, top panel).

### *Activated Cdc28$^{HA}$p promotes extensive phosphorylation of Cln2p*

To determine whether Cln2p was modified during incubation in yeast extract, we subjected samples from activation reactions to SDS–PAGE followed by autoradiography. Within 10 min of being added to yeast extract, ~50% of the Cln2p was converted from a 66 to an ~84 kDa form (Figure 1A, bottom panel, lanes 1–7). This dramatic shift

in molecular weight paralleled activation of $Cdc28^{HA}p$, and the 84 kDa species was enriched in $Cdc28^{HA}p$ immunoprecipitates (lanes 8–10). A similar, high molecular weight form of Cln2p was detected previously in yeast cell lysates (Wittenberg *et al.*, 1990; Tyers *et al.*, 1993). The immunoisolated 84 kDa form of Cln2p was restored to a low molecular weight form by digestion with either calf intestinal alkaline phosphatase or protein phosphatase 2A (Figure 1B, lanes 1–3), indicating that the 18 kDa molecular weight shift was principally due to phosphorylation of Cln2p.

Though hyperphosphorylated Cln2p was preferentially associated with $Cdc28^{HA}p$, extensive phosphorylation of Cln2p was not a prerequisite for stable binding to $Cdc28^{HA}p$. Cln2p was efficiently co-precipitated with $Cdc28^{HA}p$ from activation reactions supplemented with either the phosphatase inhibitor okadaic acid, which promotes Cln2p hyperphosphorylation, or the ATP analog 6-dimethylaminopurine (6-DMAP), which inhibits hyperphosphorylation (Figure 1B, lanes 4–8). Neither of these compounds influenced activation of $Cdc28^{HA}p$ by Cln2p (data not show).

To test whether hyperphosphorylation of Cln2p depended upon *CDC28* activity, we added Cln2p to a concentrated extract prepared from $G_1$-arrested $cdc28^{ts}$ cells. Both activation of Cdc28p histone H1 kinase (Figure 4A, bottom panel) and hyperphosphorylation of Cln2p (Figure 1C, lanes 1 and 2) were severely reduced in the $cdc28^{ts}$ extract. Purified, renatured $Cdc28^{HA}p$ (derived from *E.coli* inclusion bodies, see Materials and methods) fully complemented the $Cdc28^{HA}p$ activation defect of $cdc28^{ts}$ extract (Deshaies and Kirschner, 1995) and partially restored Cln2p hyperphosphorylation (Figure 1C, lanes 3–6). The inability to restore completely Cln2p phosphorylation by the addition of purified $Cdc28^{HA}p$ to $cdc28^{ts}$ extract may reflect the presence of elevated Cln2p phosphatase activity in this extract, as okadaic acid effectively promoted Cln2p hyperphosphorylation (Figure 1C, lane 7). Enhanced formation of hyperphosphorylated Cln2p under these conditions may reflect the stabilization of normally minor phosphorylations catalyzed by either residual Cdc28p activity or a distinct kinase with poor affinity for Cln2p substrate. Regardless, $Cdc28^{HA}p$ alone or a tightly bound kinase are sufficient to generate hyperphosphorylated Cln2p; Cln2p was extensively phosphorylated when ATP was added to α-HA precipitates containing $Cdc28^{HA}p$ assembled with underphosphorylated Cln2p (Figure 1B, lane 10; starting material in lanes 9 and 10 was derived from reactions supplemented with 6-DMAP as in lanes 7 and 8).

### *Cln2p is ubiquitinated by Cdc34p in vitro*

Time-course experiments revealed that a new, heterogeneously modified form of Cln2p appeared following the hyperphosphorylated form (data not shown). This new species migrated as a high molecular weight (HMW) smear, as shown in Figure 2 (lane 2). Two results indicate that the HMW form of Cln2p arose from the covalent attachment of ubiquitin. First, purified ubiquitin (Ub) added to yeast extract stimulated the formation of HMW Cln2p (Figure 2, lanes 3 and 4). Second, HMW Cln2p was selectively precipitated by antibodies elicited against ubiquitin conjugates (α-Ub, lane 9), whereas all forms of

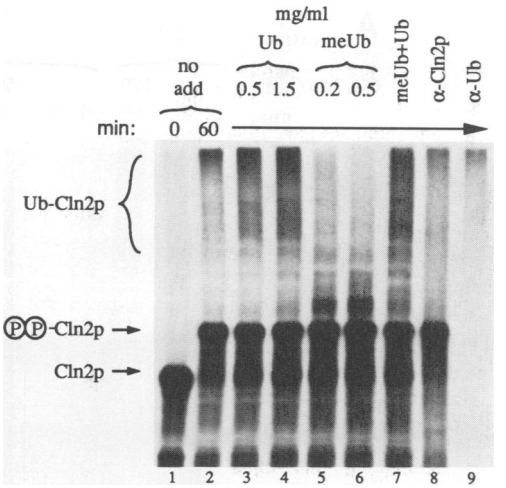


**Fig. 2.** Multiubiquitin chains are assembled on Cln2p *in vitro*. Cln2p synthesized *in vitro* in the presence of [$^{35}$S]methionine was incubated in 15 mg/ml RJD567 extract for 0 (lane 1) or 60 (remainder) min at 24°C in the presence of the indicated amounts of ubiquitin (Ub) or methyl ubiquitin (meUb). The reaction displayed in lane 7 contained 0.5 mg/ml meUb and 1.5 mg/ml ubiquitin. Aliquots of the reaction shown in lane 2 were immunoprecipitated with either 0.7 μg affinity-purified α-Cln2p (lane 8) or 3 μl α-ubiquitin conjugate (lane 9) antiserum prior to SDS–PAGE and fluorography. PP-Cln2p, hyperphosphorylated Cln2p; Ub-Cln2p, multiubiquitinated Cln2p.

Cln2p were precipitated by anti-Cln2p antibodies (α-Cln2p, lane 8). Two scenarios could account for the extensive ubiquitination of Cln2p observed here: either multiubiquitin chains were assembled on one or a small number of lysine residues, or many lysines were conjugated with short Ub chains. Methylation of the ε-N groups of lysines in Ub blocks multiubiquitin chain formation by preventing Ub–Ub ligation (Hershko and Heller, 1985). Reactions supplemented with methylated ubiquitin (meUb) yielded only low MW forms of ubiquitinated Cln2p (Figure 2, lanes 5 and 6), suggesting that multiubiquitin chains were typically assembled on a relatively small number of lysine residues in Cln2p.

During the mitotic cell cycle, the level of Cln2p diminishes at approximately the same time that *CDC34* activity is required for further progression (Wittenberg *et al.*, 1990; Tyers *et al.*, 1993). To test whether Cln2p might be a substrate for the Cdc34p ubiquitin-conjugating enzyme, we examined Cln2p modification in extracts from isogenic wild-type and $cdc34^{ts}$ cells arrested in $G_1$ phase with the mating pheromone α-factor. Whereas Cln2p activated $Cdc28^{HA}p$ (Figure 3B, lanes 3 and 4) and was hyperphosphorylated and ubiquitinated in $CDC34^{+}$ extract (Figure 3A, lanes 1 and 2), assembly of multiubiquitin chains on Cln2p was reduced in a $cdc34^{ts}$ extract (Figure 3A, lanes 4 and 5). The defect in the $cdc34^{ts}$ extract was confined to the formation of Ub–Cln2p, as $Cdc28^{HA}p$ activation (Figure 3B, lanes 1 and 2) and Cln2p hyperphosphorylation proceeded normally. The diminished ubiquitination of Cln2p in a $cdc34^{ts}$ extract was specifically due to a deficit of Cdc34p activity, as Cln2p ubiquitination was efficiently restored by the addition of purified Cdc34p (Figure 3A, lanes 6–9) but not by addition of the Ubc4p ubiquitin-conjugating enzyme (Seufert and Jentsch, 1990).

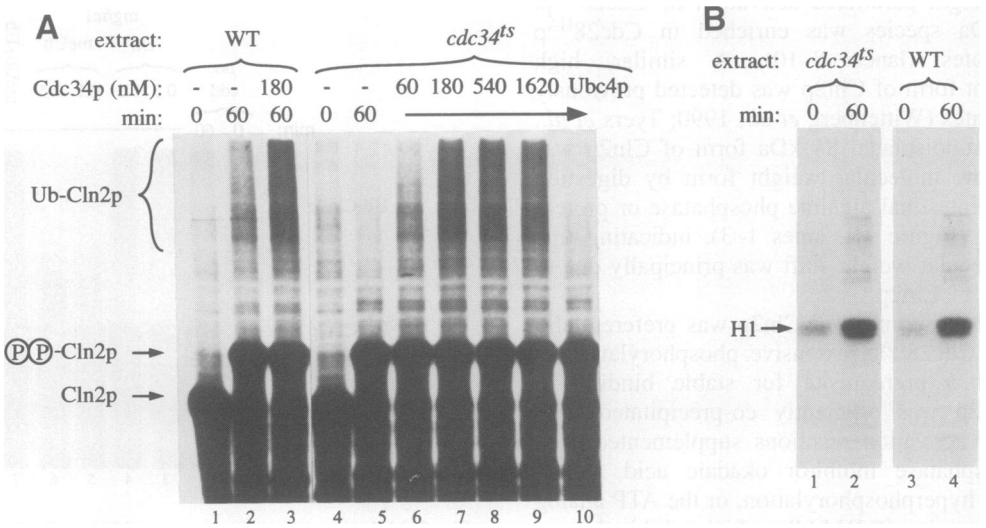


**Fig. 3.** Cdc34p is required for the assembly of multiubiquitin chains on Cln2p *in vitro*. (**A**) Cln2p synthesized *in vitro* in the presence of [$^{35}$S]methionine was incubated in 15 mg/ml RJD567 (lanes 1–3) or RJD670 (*cdc34$^{ts}$*, lanes 4–10) extract for 0 (lanes 1 and 4) or 60 (remainder) min at 24°C. Reactions were either mock-treated (lanes 1, 2, 4 and 5) or supplemented with the indicated amounts of Cdc34p or 1125 nM Ubc4p (lane 10), both purified from *E.coli*. Reactions were either directly evaluated by SDS–PAGE and fluorography (A), or Cdc28$^{HA}$p was immunoprecipitated with α-HA antibodies and incubated with [γ-$^{32}$P]ATP and histone H1 before SDS–PAGE (**B**). The kinase assays shown in (B) were performed with material from the reactions depicted in lanes 4, 5, 1 and 2, respectively, from (A). PP-Cln2p, hyperphosphorylated Cln2p; Ub-Cln2p, ubiquitinated Cln2p; H1, histone H1.

### *Cdc28p and protein phosphorylation promote Cln2p ubiquitination in vitro*

Since Cln2p bound to, activated and was hyperphosphorylated by Cdc28$^{HA}$p in yeast extract, we reasoned that Cdc28$^{HA}$p might influence the conjugation of ubiquitin to Cln2p. Extract derived from a *cdc28$^{ts}$* mutant failed to sustain efficient hyperphosphorylation (Figure 1C) or multiubiquitination (Figure 4A, top panel, lanes 1 and 2) of Cln2p (Figures 1C and 4A are different exposures of the same SDS–polyacrylamide gel). The inability of *cdc28$^{ts}$* extract to support hyperphosphorylation and multiubiquitination of Cln2p was at least partly due to a deficit of Cdc28p activity, as purified Cdc28$^{HA}$p partially rescued both modification defects (Figure 4A, top panel, lanes 3–6). Whereas Cdc28$^{HA}$p only partially restored Cln2p hyperphosphorylation and multiubiquitination in a *cdc28$^{ts}$* extract, histone H1 kinase activation was fully restored (Figure 4A, bottom panel). Okadaic acid efficiently restored both hyperphosphorylation and ubiquitination of Cln2p in *cdc28$^{ts}$* extract (Figure 4A, top panel, compare lane 7 with lane 9).

Cdc28$^{HA}$p may promote Cln2p ubiquitination by mechanisms independent of protein phosphorylation. To test directly whether Cln2p ubiquitination is modulated by protein phosphorylation, we mixed Cln2p and yeast extract in the presence of agents that promote (okadaic acid) or inhibit (protein phosphatase 2A, 6-DMAP) the accumulation of phosphorylated proteins (Figure 4B). Okadaic acid (lanes 4–6) stimulated the formation of both hyperphosphorylated and multiubiquitinated Cln2p, whereas both PP2A (lanes 7–9) and 6-DMAP (lanes 10–12) inhibited the accumulation of modified Cln2p. None of these agents influenced the assembly of active Cln2p–Cdc28$^{HA}$p complexes (data not shown).

Although rabbit reticulocyte lysate contains all of the enzymes required for the ubiquitin-mediated degradation of a variety of substrates (Ciechanover *et al.*, 1991), Cln2p was only poorly ubiquitinated upon translation. Rabbit reticulocyte extract supplemented with purified Cdc34p or Cdc28$^{HA}$p, either alone or in combination, failed to sustain hyperphosphorylation and multiubiquitination of Cln2p (Figure 5, lanes 1–5). In contrast, 10 mg/ml yeast extract promoted hyperphosphorylation and multiubiquitination of Cln2p. Thus, Cdc34p and Cdc28p by themselves could not catalyze multiubiquitination of Cln2p; some other component of yeast extract was required.

### *Cln2p is a substrate for Cdc34p in vivo*

The data presented above indicate that Cln2p was ubiquitinated *in vitro* in a reaction that required Cdc34p, Cdc28p, protein phosphorylation and some factor(s) resident in yeast extract. Since multiubiquitination of Cln2p by Cdc34p may serve to target Cln2p for rapid degradation by the 26S proteasome (Finley and Chau, 1991; Hershko and Ciechanover, 1992), we next addressed whether *CDC34* modulates the stability of Cln2p in living cells. Cells harboring a *cdc34$^{ts}$* mutation grew normally at 24°C and failed to grow at temperatures >35°C. Presumably, cells growing slowly at temperatures just below 35°C were sustained by a barely adequate level of Cdc34p activity. If Cln2p is a substrate of Cdc34p *in vivo*, overexpression of Cln2p at intermediate temperatures might titrate the nearly over-extended Cdc34p enzyme, thereby reducing the maximum temperature permissive for growth. *CLN2* expression from the galactokinase (*GAL1*) promoter (Johnston and Davis, 1984) reduced the permissive temperature of a *cdc34$^{ts}$* strain by nearly 5°C, but had little effect on the growth of a wild-type strain (Figure 6). Elevated expression of *CLN3* also reduced cell growth, but not as effectively as *CLN2*. A *CLN2* deletion mutant (*CLN2-Δ3*) that was unable to bind Cdc28$^{HA}$p

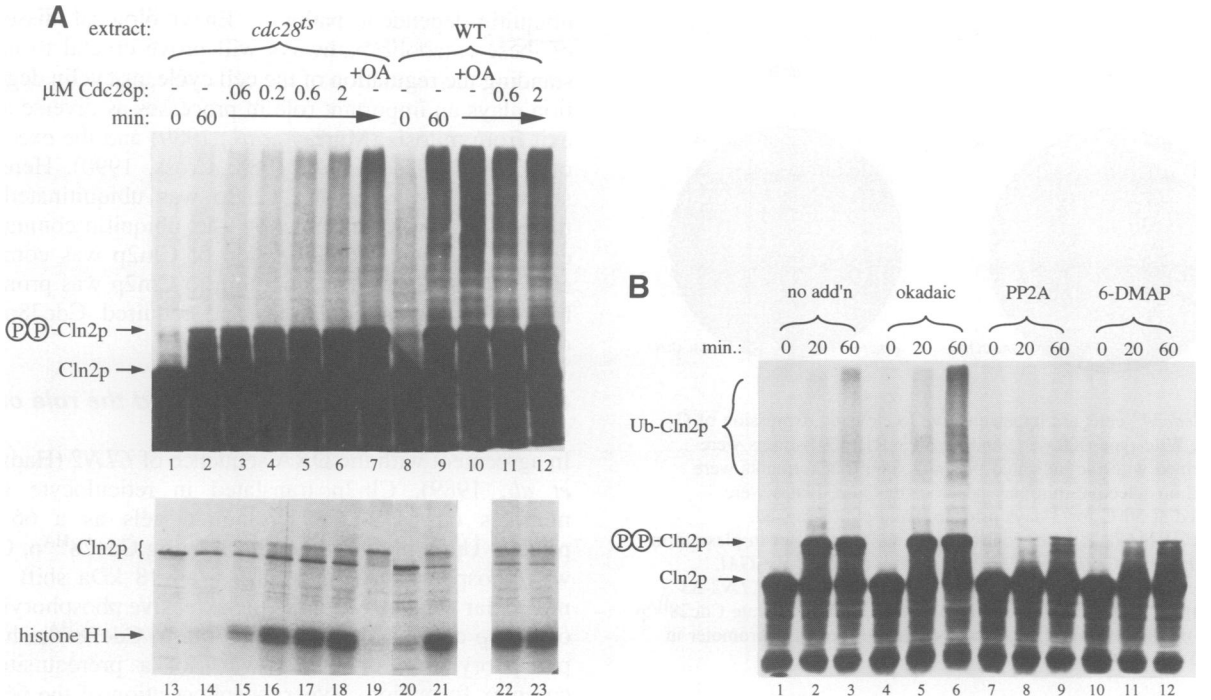


**Fig. 4.** Cdc28$^{HA}$p and protein phosphorylation promote the assembly of multiubiquitin chains on Cln2p *in vitro*. (**A**) Cln2p synthesized *in vitro* in the presence of [$^{35}$S]methionine was incubated in 18 mg/ml RJD564 (lanes 1–7) or RJD567 (lanes 8–12) extract for 0 (lanes 1 and 8) or 60 (remainder) min at 24°C. Reactions were either mock-treated (lanes 1, 2, 8 and 9), or supplemented with the indicated amounts of refolded Cdc28$^{HA}$p (lanes 3–6 and 11 and 12) purified from *E.coli* or 2 μM okadaic acid (lanes 7 and 10). Reactions were either evaluated directly by SDS−PAGE and fluorography (top panel), or Cln2p−Cdc28$^{HA}$p complexes were immunoprecipitated with α-Cln2p antibodies and incubated with [γ-$^{32}$P]ATP and histone H1 before SDS−PAGE (bottom panel). The kinase assays shown in the bottom panel were performed with material immunoprecipitated from the reactions represented in the corresponding lanes shown in the top panel. PP-Cln2p, hyperphosphorylated Cln2p; OA, okadaic acid. The top panel is a longer exposure of the same gel depicted in Figure 1C. (**B**) Cln2p synthesized *in vitro* in the presence of [$^{35}$S]methionine was incubated in 15 mg/ml RJD567 extract for 0, 20 or 60 min at 24°C with no further additions (lanes 1–3) or in the presence of either 2 μM okadaic acid (lanes 4–6), 1 μl of a fraction enriched for *Xenopus* protein phosphatase 2A (lanes 7–9) or 5 mM 6-DMAP (lanes 10–12). PP-Cln2p, hyperphosphorylated Cln2p; Ub-Cln2p, ubiquitinated Cln2p.

(unpublished results) failed to exaggerate the phenotype of the *cdc34$^{ts}$* mutant.

To evaluate directly the role of *CDC34* in Cln2p accumulation, we examined the level and stability of Cln2p in a *cdc34$^{ts}$* mutant. Wild-type and *cdc34$^{ts}$* strains grown at the permissive temperature were shifted to 37°C for 0–3 h, and extracts prepared at the indicated times were evaluated for their content of a hemagglutinin epitope-tagged form of Cln2p (Cln2$^{HA}$p; Tyers *et al.*, 1993) by immunoblotting with α-HA antibodies (Figure 7A, left panel). Cln2$^{HA}$p (present in both unmodified and multiply phosphorylated forms) accumulated in *cdc34$^{ts}$* cells shifted to 37°C, attaining a level ~3.5 times greater than that present in equivalently treated wild-type cells (lane 8 versus lane 4). To distinguish whether the elevated level of Cln2p in *cdc34$^{ts}$* strains resulted from increased synthesis or increased stability, we measured the half-life of Cln2p in a pulse−chase experiment. Wild-type and *cdc34$^{ts}$* strains containing a natural (non-tagged) *CLN2* allele were shifted to 37°C for 75 min, pulse-labeled with [$^{35}$S]methionine and chased in the presence of unlabeled methionine. An autoradiogram of α-Cln2p immunoprecipitates is displayed in the right-hand panel of Figure 7A. Cln2p was rapidly degraded in wild-type cells, but persisted far longer in *cdc34$^{ts}$* cells; quantitation of the radioactive gel revealed that the half-life of Cln2p was 5 min in wild-type cells and 20 min in *cdc34$^{ts}$* cells. Unfortunately, we were unable to measure Cln2p stability

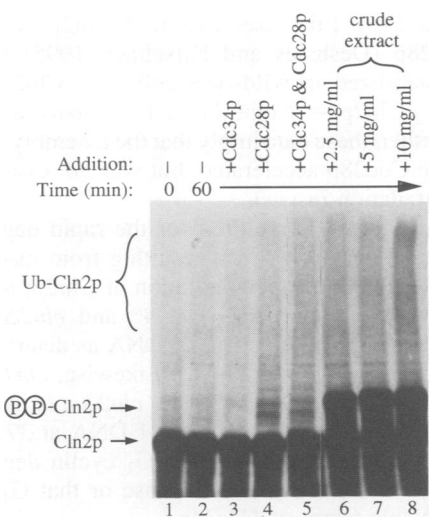


**Fig. 5.** Cdc34p and Cdc28$^{HA}$p are not sufficient to sustain ubiquitination of Cln2p. Cln2p produced by translation in a rabbit reticulocyte extract in the presence of [$^{35}$S]methionine was incubated for 0 (lane 1) or 60 (remainder) min at 24°C with either 0.8 μM purified Cdc34p (lane 3), 1.5 μM purified and renatured Cdc28$^{HA}$p (lane 4), both 0.8 μM Cdc34p and 1.5 μM Cdc28$^{HA}$p (lane 5) or 2.5–10.0 mg/ml RJD567 extract (lanes 6–8). PP-Cln2p, hyperphosphorylated Cln2p; Ub-Cln2p, ubiquitinated Cln2p.

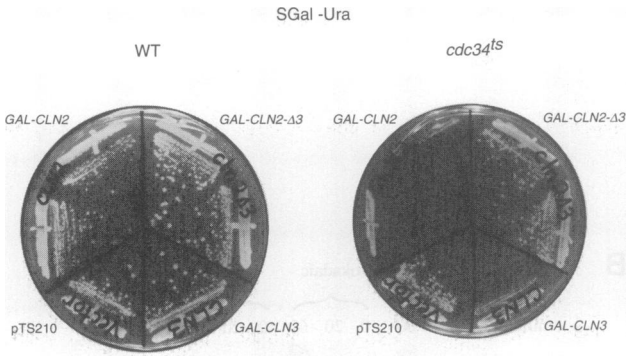


**Fig. 6.** *cdc34*$^{ts}$ cells are hypersensitive to elevated expression of $G_1$ cyclins. Wild-type (RJD494) and *cdc34*$^{ts}$ (RJD669) strains were transformed with the indicated plasmids, and transformants were streaked on selective minimal galactose medium. Plates were incubated at 30°C for 3 days prior to being photographed. *GAL–CLN2*, *CLN2* under control of the *GAL* promoter in plasmid in pTS210; *GAL–CLN2-Δ3*, *CLN2-Δ3* under control of the *GAL* promoter in plasmid in pTS210 (the protein encoded by *CLN2-Δ3* lacks amino acids 377–545 and is unable to bind or activate Cdc28$^{HA}$p *in vitro*); *GAL–CLN3*, *CLN3* under control of the *GAL* promoter in plasmid pW16 (Cross, 1990).

in *cdc34*$^{ts}$ cells immediately following a shift to 37°C, since Cln2p synthesis was transiently reduced upon shifting cells from 24 to 37°C. Thus, we cannot exclude the possibility that Cln2p was stabilized as an indirect consequence of cell cycle arrest at the *cdc34*$^{ts}$ block.

*In vitro*, efficient multiubiquitination of Cln2p required Cdc28$^{HA}$p. Pulse–chase radiolabeling/immunoprecipitation experiments indicated that wild-type Cln2p is only partially stabilized in *cdc28*$^{ts}$ cells, however (Figure 7B). Quantitation of the autoradiogram shown in Figure 7B indicated that the half-life of Cln2p was 8 min at 37°C in *cdc28*$^{ts}$ (lanes 9–12) compared with 4 min in wild-type cells (lanes 5–8). Likewise, a form of Cln2p that fails to bind Cdc28p (Deshaies and Kirschner, 1995) was also partially stabilized in wild-type cells ($t_{1/2}$ Cln2-Δbox = 12 min, $t_{1/2}$ Cln2p = 4 min; lanes 1–4 versus lanes 5–8). Taken together, these data imply that the assembly of Cln2p with active Cdc28p accelerated, but was not essential for, Cln2p degradation *in vivo*.

Whereas *CDC34* is required for the rapid degradation of Cln2p, the cell cycle arrest resulting from inactivation of *CDC34* is not due to accumulation of Cln2p; following a shift to 37°C, both *CLN2 cdc34*$^{ts}$ and *cln2Δ cdc34*$^{ts}$ strains arrest with a $G_1$ content of DNA as determined by flow cytometry (data not shown). Likewise, *cln1Δ cln2Δ cdc34*$^{ts}$, *cln1Δ cln3Δ cdc34*$^{ts}$ and *cln2Δ cln3Δ cdc34*$^{ts}$ strains arrested with a $G_1$ content of DNA at 37°C (data not shown), suggesting either that $G_1$ cyclin degradation is not required for entry into S phase or that $G_1$ cyclins are not the sole target of Cdc34p.

## Discussion

Cyclins are best known as unstable activators of Cdks. In the past few years, the basic features of the biochemical pathway by which cyclin B activates p34$^{cdc2}$ have been described (Solomon, 1993; Dunphy, 1994). In contrast, little is known as yet about the enzymes that earmark cyclin B, or any other cyclin, for rapid destruction by the ubiquitin-dependent pathway. Enzymological dissection of these catabolic pathways will prove crucial to understanding the regulation of the cell cycle, as cyclin degradation plays an important role in processes as diverse as the exit from mitosis (Murray *et al.*, 1989) and the execution of START (Nash *et al.*, 1988; Cross, 1990). Here, we show that the $G_1$ cyclin Cln2p was ubiquitinated and thereby rendered unstable by the ubiquitin-conjugating enzyme Cdc34p. Ubiquitination of Cln2p was complex; efficient conjugation of ubiquitin to Cln2p was promoted by protein phosphorylation, and required Cdc28p and other factors present in yeast extract.

### *Covalent modification of Cln2p, and the role of Cdc34p in Cln2p degradation*

In agreement with the DNA sequence of *CLN2* (Hadwiger *et al.*, 1989), Cln2p translated in reticulocyte lysate migrates on SDS–polyacrylamide gels as a 66 kDa protein. Upon binding to and activating Cdc28$^{HA}$p, Cln2p was phosphorylated, resulting in an 18 kDa shift in its molecular weight. Although the extensive phosphorylation of Cln2p correlated with its binding to Cdc28$^{HA}$p, hyperphosphorylation of Cln2p was not a prerequisite for complex formation. Hyperphosphorylation of the 66 kDa form of Cln2p readily occurred in immunopurified Cdc28$^{HA}$p complexes. Since the protein composition of these immunoprecipitates is not known, however, other co-precipitating kinases may contribute to the formation of hyperphosphorylated Cln2p.

Subsequent to the appearance of the 84 kDa form, Cln2p was extensively conjugated with ubiquitin. Cln2p was predominantly ubiquitinated on only one or a few lysines, as the heterogeneous collection of ubiquitinated Cln2p molecules was converted to a discrete, low molecular weight form(s) when the chain-terminating inhibitor methyl ubiquitin was present during ubiquitination reactions. Two aspects of Cln2p ubiquitination in yeast extract deserve further comment. First, only a small fraction (10–20%) of Cln2p accumulated as Ub–Cln2p. Whereas Cln2p may be a poor substrate for ubiquitin-conjugating enzymes, the low level of accumulated conjugates probably reflects a dynamic interplay between ubiquitin-conjugating and -deconjugating enzymes in yeast (Baker *et al.*, 1992; Papa and Hochstrasser, 1993). Second, little degradation of Cln2p was observed during a 60 min incubation *in vitro*. We suspect that yeast extract contains an inhibitor of the proteasome, as addition of yeast extract inhibits the degradation of cyclin B in *Xenopus* extract (data not shown).

Despite the low efficiency of Cln2p ubiquitination and degradation *in vitro*, we believe that this reaction is physiologically related to the documented instability of Cln2p for three reasons: (i) ubiquitination of Cln2p depended upon the activity of its catalytic partner Cdc28p; (ii) ubiquitination of Cln2p was positively regulated by the phosphorylation of serine or threonine residues. There is no reported requirement for either serine/threonine phosphorylation or Cdc28p in the degradation of other substrates by the ubiquitin-dependent pathway; and (iii) ubiquitination of Cln2p *in vitro* was dependent upon a protein, Cdc34p, that was also required for its rapid destruction in living cells.

Although the half-life of Cln2p was 4-fold longer in

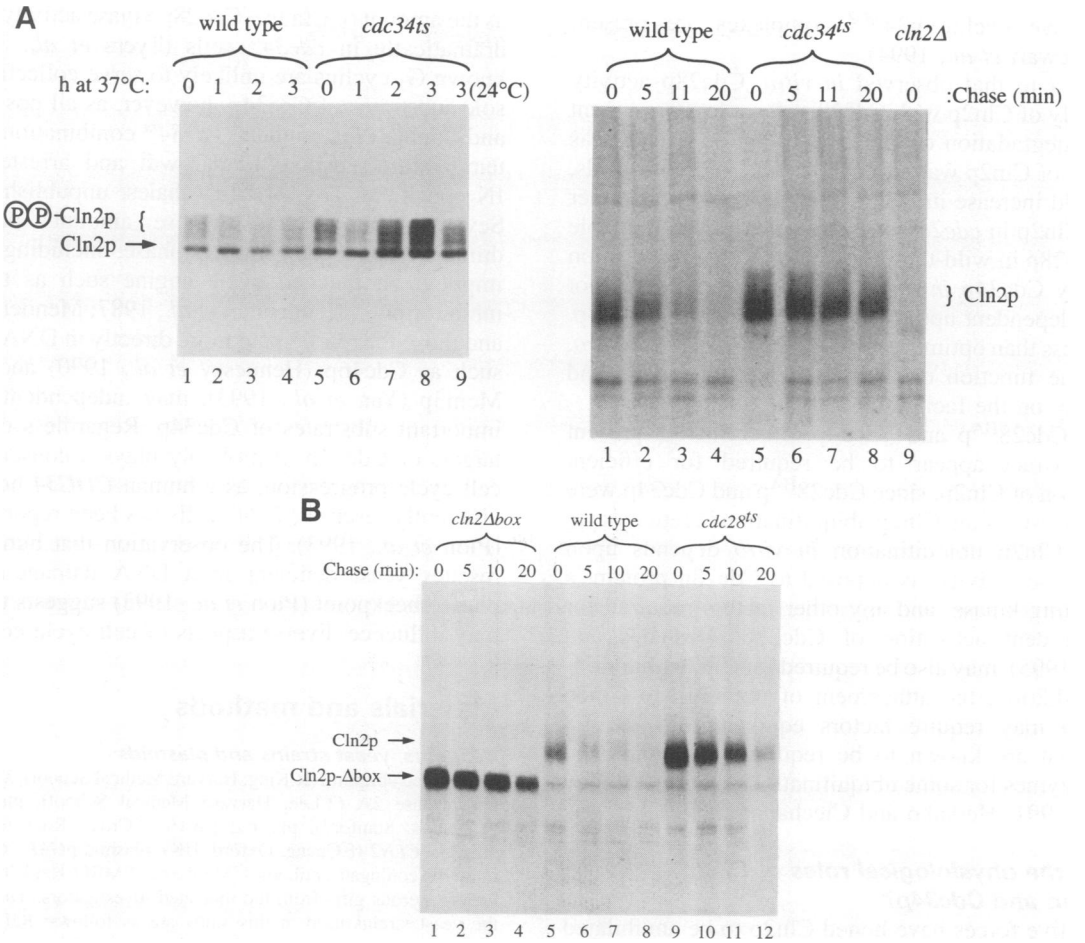


**Fig. 7.** Cln2p accumulation and stability in *cdc34*$^{ts}$ and *cdc28*$^{ts}$ cells. (**A**) Left panel: wild-type (RJD699) and *cdc34*$^{ts}$ (RJD707) strains expressing *CLN2* tagged with a triple hemagglutinin epitope (Tyers *et al.*, 1993) were grown at 24°C in YEPD medium, shifted to 37°C for 0–3 h, and then lysed by agitation with glass beads in the presence of SDS. In all, 20 μg of protein from each sample were subjected to SDS−PAGE and transferred to nitrocellulose. Antigens were detected by successive incubation with α-HA antibodies, HRP-conjugated goat anti-rabbit antibodies, and ECL reagents. Right panel: wild-type (RJD494), *cdc34*$^{ts}$ (RJD795) and *cln2*Δ (RJD721) cells grown at 24°C were shifted to 37°C for 75 min, pulse-radiolabeled for 5 min with [$^{35}$S]methionine and chased with cold methionine for 0–20 min as indicated. Labeled cells were lysed by agitation with glass beads, and the level of Cln2p antigen was evaluated by immunoprecipitation with affinity-purified Cln2p antibodies followed by SDS−PAGE and fluorography. (**B**) Wild-type (RJD699), *cdc28*$^{ts}$ (RJD705) and *cln2-Δbox* (RJD752) cells expressing *CLN2* tagged with a triple hemagglutinin epitope were grown at 24°C, shifted to 37°C for 75 min, pulse-radiolabeled for 5 min with [$^{35}$S]methionine and chased with cold methionine for 0–20 min as indicated. Labeled cells were lysed by agitation with glass beads, and the level of Cln2p antigen was evaluated by immunoprecipitation with affinity-purified Cln2p antibodies followed by SDS−PAGE and fluorography.

*cdc34*$^{ts}$ compared with wild-type cells, Cln2p was still degraded rapidly compared with bulk yeast proteins (Seufert and Jentsch, 1990). Thus, either the *cdc34*$^{ts}$ allele possesses weak activity at 37°C, or Cln2p is metabolized by multiple pathways. Stabilization of the transcriptional regulatory protein MATα2 requires simultaneous deletion of four genes that each encode a distinct ubiquitin-conjugating enzyme (Chen *et al.*, 1993). In addition, the degradation of a B-type cyclin ectopically expressed in $G_1$ cells proceeds by destruction box-dependent and -independent pathways (Amon *et al.*, 1994). The high degree of genetic redundancy observed among the 10 known ubiquitin-conjugating enzyme genes in *S.cerevisiae* (only two are essential for growth; Chen *et al.*, 1993) suggests that destruction pathways collaborate to delimit the half-lives of extremely short-lived proteins.

### *The pathway of Cln2p ubiquitination*

Efficient ubiquitination of Cln2p *in vitro* required both protein phosphorylation and Cdc28p activity. However, it is not clear how the requirement for protein phosphorylation is related to the requirement for Cdc28p. Protein phosphorylation may promote ubiquitination of Cln2p by several mechanisms. Although the phosphorylation of Cln2p kinetically precedes and correlates with its ubiquitination, a causative role for Cln2p phosphorylation has not been established. Protein phosphorylation promotes the rapid degradation of RAG2 (Lin and Desiderio, 1993) and c-Fos (Papavassiliou *et al.*, 1992), suggesting that it may play a broad role in signaling selective protein turnover. Besides Cln2p, a factor required for ubiquitination of Cln2p may be a target of phosphoregulation. Cdc34p itself has at least two potential Cdc28p phosphorylation sites (Goebl *et al.*, 1988), and is phosphorylated on serine *in vivo* (Goebl *et al.*, 1994). Whereas Cdc28p may promote Cln2p ubiquitination *in vitro* by acting as a protein kinase, Cdc28p may also induce Cln2p to adopt a specific conformation that can then be recognized by Cdc34p. Rapid degradation of cyclin A and B2 in frog extract requires their affiliation with p34$^{cdc2}$,

even if active cyclin−p34$^{cdc2}$ complexes are present *in trans* (Stewart *et al.*, 1994).

In contrast to that observed *in vitro*, Cdc28p activity and assembly of Cln2p with Cdc28p play a less prominent role in the degradation of Cln2p in living cells. Whereas the half-life of Cln2p was extended 4-fold in *cdc34$^{ts}$* cells, only a 2-fold increase in half-life was observed for either wild-type Cln2p in *cdc28$^{ts}$* cells or a Cln2p mutant unable to bind Cdc28p in wild-type cells. Perhaps the recognition of Cln2p by Cdc34p *in vivo* is facilitated by, but is not absolutely dependent upon, assembly with active Cdc28p. Under the less than optimal conditions encountered *in vitro*, however, the function of Cdc34p may come to depend more strictly on the facilitating role of Cdc28p.

Besides Cdc28$^{HA}$p and Cdc34p, other factors present in yeast extract appear to be required for efficient ubiquitination of Cln2p, since Cdc28$^{HA}$p and Cdc34p were not sufficient to sustain Cln2p ubiquitination in reticulocyte extract. If Cln2p ubiquitination *in vitro* depends upon Cdc28p kinase activity, as opposed to Cdc28p protein, a Cdk-activating kinase, and any other factors required for Cln2p-dependent activation of Cdc28p (Deshaies and Kirschner, 1995), may also be required for Cln2p ubiquitination. In addition, the attachment of ubiquitin to Cln2p by Cdc34p may require factors equivalent to the E3 enzymes that are known to be required in conjunction with E2 enzymes for some ubiquitination reactions (Finley and Chau, 1991; Hershko and Ciechanover, 1992).

### What are the physiological roles of Cln2p destruction and Cdc34p?

What selective forces have honed Cln2p to be annihilated with such dispatch? Degradation of cyclins A and B is required to restore p34$^{cdc2}$ to the interphase state, thereby allowing the cell to exit mitosis (Murray *et al.*, 1989; Luca *et al.*, 1991; Gallant and Nigg, 1992; van der Velden and Lohka, 1993). Perhaps progression through a preceding cell cycle gateway likewise depends upon reducing the activity of G1 cyclins via proteolysis. Whereas overexpression of *CLN2* is normally not toxic, overexpression of *CLN2* in the W303 strain background severely reduces growth, and overexpression of C-terminally truncated forms of Cln2p (in multiple genetic backgrounds) causes cells to arrest with a $G_2$/M content of DNA (R.J.Deshaies, unpublished results). Thus, degradation of Cln2p may facilitate the successful negotiation of either $G_2$ or M phase, or both. A straightforward expectation is that rapid degradation of Cln2p resets the $G_1$ form of Cdc28p kinase to ground state prior to subsequent G1 phases. In the absence of other post-translational mechanisms to restrain Cln2p−Cdc28p activity, the accumulation of Cln2p would compromise a cell's ability to regulate START. Indeed, *CLN2-1* renders cells unable to arrest in $G_1$ upon nutrient deprivation (Hadwiger *et al.*, 1989). Although this large deletion stabilized Cln2p partially, degradation still proceeded rather briskly (R.J.Deshaies, unpublished results). An unequivocal answer to the role of Cln2p degradation awaits the engineering of a thoroughly stabilized Cln2p.

Which substrates of Cdc34p cause the cell cycle engine to stall in $G_1$ upon their accumulation in *cdc34$^{ts}$* cells? Besides Cln2p, Cln3p itself or a positive regulator of Cln3p−Cdc28p complexes may be a substrate of Cdc34p, as the amount of Cln3p−Cdc28p kinase activity is elevated dramatically in *cdc34$^{ts}$* cells (Tyers *et al.*, 1992). The known $G_1$ cyclins are unlikely to serve collectively as the sole substrates of Cdc34p, however, as all possible single and double *clnΔ* mutant−*cdc34$^{ts}$* combinations remained temperature-sensitive for growth and arrested with an IN content of DNA (R.J.Deshaies, unpublished results). Several other proteins whose amounts or activities diminish upon entry into S phase, including those that impinge on the cell cycle engine such as the Cdc28p inhibitor p40 (Mendenhall *et al.*, 1987; Mendenhall, 1993) and those that participate more directly in DNA replication such as Cdc46p (Hennessy *et al.*, 1990) and Mcm2p−Mcm3p (Yan *et al.*, 1993), may independently serve as important substrates of Cdc34p. Regardless of the exact targets of Cdc34p, it probably plays a conserved role in cell cycle progression, as a human *CDC34* homolog that efficiently rescues *cdc34$^{ts}$* cells has been reported recently (Plon *et al.*, 1993). The observation that human *CDC34* rescues yeast deficient in a DNA damage-induced $G_2$ phase checkpoint (Plon *et al.*, 1993) suggests that Cdc34p may influence diverse aspects of cell cycle control.

## Materials and methods

### Reagents, yeast strains and plasmids

Methylated ubiquitin (R.King, Harvard Medical School), *Xenopus* protein phosphatase 2A (T.Lee, Harvard Medical School), plasmid pTS210 (T.Stearns, Stanford), plasmid pW16 (F.Cross, Rockefeller), plasmid YEp13−*CLN2* (F.Chang, Oxford, UK), plasmid p*GAL*−*CLB2* and anti-ubiquitin conjugate antibodies (M.Glotzer, EMBO, Heidelberg, Germany) were generous gifts from the indicated investigators. The genotypes of the yeast strains used in this study are as follows: RJD567 is *cln1Δ*, *cln2::LEU2*, *cln3Δ*, *trp1*, *leu2*, *ura3*, *pep4::LEU2*, transformed with a *GAL-CLN3*, *URA3*, *CEN ARS* plasmid; RJD494 is *leu2*, *ura3*, *trp1*, *pep4::TRP1*, *MATa*; RJD699 is RJD494 containing *CLN2$^{3\times HA}$::LEU2* in place of *CLN2*; RJD564 is *cdc28-4*, *ura3*, *leu2*, *pep4::TRP1*, *bar1::LEU2*, *MATa*; RJD705 is *leu2*, *ura3*, *cdc28-4*, *cln2::CLN2$^{3\times HA}$::LEU2*, *MATa*; RJD669 is *cdc34-2*, *leu2*, *ura3*, *pep4::TRP1*, *MATa*; RJD670 is *cdc34-2*, *leu2*, *ura3*, *bar1::LEU2*, *pep4::TRP1*, *MATa*; RJD707 is RJD670 containing *CLN2$^{3\times HA}$::LEU2* in place of *CLN2*; RJD795 is *cdc34-2*, *ura3*, *leu2*, *trp1*, *MATa*; RJD721 is *ura3*, *leu2*, *trp1*, *his3*, *cln2::LEU2*, *MATa*; and RJD752 is *leu2*, *ura3*, *trp1*, *pep4::TRP1*, *cln2-Δbox$^{3\times HA}$::LEU2*.

Expression of *CLN2 in vitro* was achieved by inserting a *Bam*HI fragment (derived from YCpG2CLN2; Wittenberg *et al.*, 1990) containing the *CLN2* gene into the *Bam*HI site of pGEM2 (yielding pRD68), such that the 5′ end of *CLN2* was adjacent to the T7 promoter.

Galactose-dependent expression of *CLN2* and *CLN2-Δ3 in vivo* was achieved by inserting a *Bam*HI fragment containing the *CLN2* gene into the *Bam*HI site of pTS210 (*CEN*, *ARS*, *URA3*), yielding pRD98 (*CLN2*) and pRD102 (*CLN2-Δ3*). The *CLN2* allele in each plasmid was flanked by the *GAL1* promoter and the *ACT1* terminator on its 5′ and 3′ ends, respectively. The *Bam*HI fragment used to construct pRD98 and pRD102 was derived by PCR with the oligo pairs given below. The *Bam*HI sites and the translation initiation (RDO6) and termination (RDO7,10) codons are in bold. Both sets of oligos amplify the *CLN2* coding sequence with two codons (TCT AGA) appended at the 3′ end. The endpoint for *CLN2-Δ3* is amino acid 241. *CLN2*: RDO6, 5′-GC GCG TTA ATA CGA CTC ACT ATA **GGA TCC** ACC **ATG** GCT AGT GCT GAA CC-3′, and RDO7, 5′-CG **GGA TCC CTA** TAT TAC TTG GGT ATT GCC-3′. *CLN2-Δ3*: RDO6 and RDO10, 5′-CG **GGA TCC CTA** TCT AGA GGC ATC ACT ATC CTG GG-3′. A plasmid containing *CLN2* isolated from a genomic library (YEp13−*CLN2*) was used as template in the PCR. Three independent clones of pRD98 and pRD102 were analyzed by transformation into yeast strains RJD494 and RJD669 to compensate for the possible introduction of mutations during PCR. All three clones yielded results similar to those presented in Figure 6.

### In vitro transcription and translation

pRD68 was linearized by digestion with *Eco*RI and transcribed with T7 RNA polymerase as per the supplier's instructions. Following

transcription, the mRNA was extracted sequentially with phenol/chloroform and chloroform, precipitated with ethanol, and resuspended in 50 µl water. Messenger RNAs (~0.5 µl per 10 µl reaction) were translated for 60 min at 30°C in rabbit reticulocyte extract according to the supplier's instructions. Translation reactions were terminated by adding cold methionine to 1 mM, incubating for 5 min at 30°C, followed by adding cycloheximide to 100 µg/ml.

### *In vitro Cdc28$^{HA}$p activation reactions*

Activation reactions were conducted as described previously (Deshaies and Kirschner, 1995), except that rabbit reticulocyte translation reactions (0–3 µl) containing [$^{35}$S]methionine-labeled Cln2p were used in place of GST−Cln2p purified from *E.coli*. Cdc28$^{HA}$p-associated histone H1 kinase activity was measured following immunoprecipitation of Cdc28$^{HA}$p as described previously (R.J.Deshaies and M.Kirschner, manuscript submitted). The physical state of Cln2p was evaluated as follows. Reactions were terminated by mixing 4 µl of activation reaction with 16 µl of SDS−PAGE sample buffer, heated for 4 min at 95°C and applied (5 µl of sample/lane) to SDS−8% polyacrylamide gels. Following electrophoresis, gels were stained with Coomassie blue, destained and treated with the fluorographic enhancer Amplify according to the supplier's instructions.

### *Preparation of affinity-purified Cln2p antibodies*

A plasmid (pRD71) that directs synthesis of GST−Cln2p in *E.coli* was described previously (Deshaies and Kirschner, 1995). GST−Cln2p was purified from the insoluble fraction of *E.coli* lysate as described previously (Deshaies and Schekman, 1990). Purified GST−Cln2p was injected into rabbits (BAbCo, Richmond, CA), and the resulting sera were affinity-purified on a matrix containing immobilized TrpE−Cln2p essentially as described (Deshaies and Schekman, 1990).

### *Immunoprecipitations and immunoblots*

Wild-type and *cdc34$^{ts}$* cells grown at 24°C were shifted to 37°C for 75 min to impose the *cdc34$^{ts}$* block. Cells were then harvested, resuspended in prewarmed growth medium and supplemented with 30 µCi/$OD_{600}$ tran-$^{35}$S-label (ICN). Following a 5 min pulse-label at 37°C, cells were rapidly harvested and resuspended in prewarmed growth medium supplemented with 20 µg/ml cysteine, 20 µg/ml methionine and 1 mM ammonium sulfate. At the indicated times, aliquots of the culture were diluted in ice-cold 10 mM $NaN_3$, 50 mM NaF. Washed cell pellets were lysed by agitation with glass beads, lysates were supplemented with affinity-purified anti-Cln2p antibodies and immune complexes were recovered, washed and processed for SDS−PAGE and fluorography essentially as described (Rothblatt *et al.*, 1989), except that only a single round of immunoprecipitation was performed. Radioactivity in individual protein bands was quantitated as described (Chen *et al.*, 1993).

Steady state levels of Cln2p in different yeast strains were assessed by immunoblotting as follows. Log-phase yeast cultures were shifted to 37°C, and at the indicated time points cells were harvested, washed with ice-cold 10 mM $NaN_3$, 50 mM NaF and resuspended in 25 mM Tris−HCl pH 7.2, 20 mM NaF, 1 mM EDTA, 1% SDS, 0.1 µM okadaic acid, 0.5 mM PMSF and 10 µg/ml leupeptin and pepstatin. Samples were immediately immersed in a boiling water bath for 3 min, supplemented with an equal volume of glass beads, vortexed for 90 s, reboiled for 2 min and microfuged. Equal amounts of protein from each extract were then supplemented with 0.5 vol 3× SDS−PAGE sample buffer and loaded onto a 7.5% SDS−polyacrylamide gel, electrophoresed, transferred to nitrocellulose and immunoblotted with α-HA ascites fluid (1/1000 dilution). Filter-bound antibodies were probed with horseradish peroxidase-conjugated goat anti-mouse antibodies, and decorated proteins were visualized by enhanced chemiluminescence (ECL).

### *Protein purification*

Cdc34p and Ubc4p were purified as described previously (Banerjee *et al.*, 1993). Cdc28$^{HA}$p was purified from *E.coli* as follows. 6 l of LB supplemented with 100 µg/ml ampicillin and 30 µg/ml chloramphenicol were inoculated with 60 ml of a saturated culture of *E.coli* strain BL21(DE3)/pLysS transformed with pRD88 (pRD88 contains CDC28$^{HA}$ inserted downstream of the T7 promoter in pET11; Deshaies and Kirschner, 1995). Cells were harvested, washed and lysed, as described for the preparation of GST−Cln2p (Deshaies and Kirschner, 1995), except that the incubation with lysozyme was omitted and DTT was included in the lysis buffer at 2 mM. Cdc28$^{HA}$p-containing inclusion bodies were nuclease-treated and washed as described (Deshaies and Schekman, 1990). DTT was included in all wash buffers at 2 mM. Purified inclusion bodies were dispersed in 10 ml 8 M urea, 5 mM DTT, 5 mM EDTA, and incubated at 24°C for 1 h. Insoluble material was pelleted by centrifugation for 10 min at 14 000 *g* (Eppendorf microfuge), and the supernatant was diluted 5-fold with USB (6 M urea, 25 mM MES pH 6.0, 5 mM DTT), and then loaded onto a 40 ml column of S Sepharose equilibrated with the same buffer. The column was washed with two volumes of USB and developed with a 300 ml gradient of 0.0–0.5 M sodium chloride in USB. Fractions containing Cdc28$^{HA}$p were pooled (0.83 mg/ml), mixed with an equal volume of 7 M urea, dialyzed with rapid stirring against 100 volumes of 2 M urea in CRB (CRB: 20 mM HEPES pH 7.6, 5 mM DTT, 0.5 mM EDTA), dialyzed slowly (no stirring) against two changes of 100 volumes of CRB and dialyzed rapidly against CRB plus 15% glycerol. Aliquots of Cdc28$^{HA}$p (0.26 mg/ml) were frozen in liquid nitrogen and stored at −80°C. In all, 60% of S Sepharose-purified Cdc28$^{HA}$p was recovered in a soluble form following the removal of urea. Following renaturation, Cdc28$^{HA}$p precipitated in buffers with >50 mM sodium chloride. Despite its salt sensitivity, renatured Cdc28$^{HA}$p was activated efficiently when added to yeast extract containing $G_1$ or B cyclins (Figure 5B; Booher *et al.*, 1993).

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