

The cyclin-dependent kinase Cdc28p regulates distinct modes of Cdc6p proteolysis during the budding yeast cell cycle

Lucy S. Drury, Gordon Perkins* and John F.X. Diffley

Background: Cdc28p, the major cyclin-dependent kinase in budding yeast, prevents re-replication within each cell cycle by preventing the reassembly of Cdc6p-dependent pre-replicative complexes (pre-RCs) once origins have fired. Cdc6p is a rapidly degraded protein that must be synthesised in each cell cycle and is present only during the G1 phase.

Results: We found that, at different times in the cell cycle, there are distinct modes of Cdc6p proteolysis. Before Start, Cdc6p proteolysis did not require either the anaphase-promoting complex (APC/C) or the SCF complex, which mediate the major cell cycle regulated ubiquitination pathways, nor did it require Cdc28p activity or any of the potential Cdc28p phosphorylation sites in Cdc6p. In fact, the activation of B cyclin (Clb)–Cdc28p kinase inactivated this pathway of Cdc6p degradation later in the cell cycle. Activation of the G1 cyclins (Clns) caused Cdc6p degradation to become extremely rapid. This degradation required the SCF^{CDC4} and Cdc28p consensus sites in Cdc6p, but did not require Clb5 and Clb6. Later in the cell cycle, SCF^{CDC4}-dependent Cdc6p proteolysis remained active but became less rapid.

Conclusions: Levels of Cdc6p are regulated in several ways by the Cdc28p cyclin-dependent kinase. The Cln-dependent elimination of Cdc6p, which does not require the S-phase-promoting cyclins Clb5 and Clb6, suggests that the ability to assemble pre-RCs is lost before, not concomitant with, origin firing.

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Received: 5 November 1999

Revised: 11 January 2000

Accepted: 11 January 2000

Published: 21 February 2000

Current Biology 2000, 10:231–240

0960-9822/00/\$ – see front matter

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Background

In eukaryotic cells, DNA replication initiates from large numbers of replication origins on multiple chromosomes throughout S phase of the cell cycle. Mechanisms ensuring that replication origins fire just once in each cell cycle have been intensively investigated in recent years [1,2]. One of the keys to preventing reinitiation of DNA replication involves the regulated assembly of pre-replicative complexes (pre-RCs) at replication origins [3–5]. During pre-RC assembly, the Mcm2–7 family of proteins are loaded onto DNA in a reaction that requires the origin-recognition complex (ORC) and Cdc6p [6–12].

Once an origin fires, the pre-RC is lost from the origin and does not reassemble until the end of the following mitosis [3]. Pre-RCs normally assemble during G1 phase and cannot assemble later in the cell cycle because the major cyclin-dependent kinase (Cdk) Cdc28p, together with B cyclin (Clb), prevents pre-RC formation [4,5,13,14]. As Clb–Cdc28p kinase is also essential for initiating DNA replication [15], pre-RC assembly and origin firing are mutually exclusive events. Pre-RC assembly is prevented from the onset of S phase until the end of mitosis.

Cdc6p appears to be a key player in preventing re-replication. Overexpression of Cdc18, the fission yeast Cdc6p

homologue, drives multiple rounds of DNA re-replication without mitosis [16–22]. Moreover, a budding yeast *cdc6* mutation that causes limited re-replication has been described [7]. In both budding and fission yeast, Cdc6p (and *cdc18*) are unstable proteins that must be synthesised in each cell cycle for DNA replication [16,23]. These proteins are present during G1 and disappear around the time of S-phase entry. In human cells, Cdc6p appears to be present throughout most of the cell cycle in proliferating cells but is lost when cells enter quiescence [24–26]. In budding yeast, Cdk phosphorylation of Cdc6p is required for ubiquitination *in vitro*, and mutation of Cdk consensus phosphorylation sites in Cdc6p stabilises it [27], suggesting a role for Cdk in Cdc6p degradation *in vivo*.

In both budding and fission yeast, the SCF complex plays a role in Cdc6p (*cdc18*) degradation [5,28,29]. In budding yeast, SCF is a multisubunit protein complex required to target a number of key cell-cycle regulators for ubiquitin-mediated degradation [30]. SCF contains Skp1, the E2 ubiquitin ligase Cdc34 and the cullin Cdc53 along with one of a number of F-box-containing proteins. In the case of Cdc6p degradation, the F-box protein Cdc4p appears to be the most important component [5,29]; *cdc4* mutants, along with *cdc34* and *cdc53* mutants, are unable to degrade Cdc6p in cells arrested in mitosis. Cdc4 interacts with

Figure 1

Cdc6p degradation in G1 is not dependent on SCF, APC, active Clns or consensus phosphorylation sites. **(a)** Wild-type (WT, lanes 1–4), *cdc4* (lanes 5–8), *cdc34* (lanes 9–12) and *cdc53* (lanes 13–16) cells containing *GAL1,10-CDC6* were grown at 24°C in YP medium containing raffinose (YP-Raf) and blocked in G1 with α factor. The cultures were transferred to medium containing galactose at 37°C to induce Cdc6p. After 30 min, Cdc6p expression was repressed. Samples were taken at the time of repression and after, at the indicated times, to be processed for immunoblotting with a monoclonal raised against Cdc6p. LC, loading control (a section of the membrane between the 66 kDa and 45 kDa markers, stained with amido black after ECL detection of the proteins). **(b)** (i) A similar experiment was performed on temperature-sensitive *cdc16* (lanes 1–4) and *cdc23* (lanes 5–8) cells. (ii) The stability of Cdc6p and of a fusion protein between Clb2p and Myc (Clb2p-Myc), both expressed from the *GAL1,10* promoter, was examined in a wild-type or a *cdc16* mutant background as described above. The band below Clb2p-Myc (asterisk) is a non-specific band also seen in extracts from untagged strains (data not shown). **(c)** Logarithmically growing YLD38 cells (*CLN1-3 Δ* , *MET-CLN2*) were blocked in G1 by the addition of methionine to the medium. Cdc6p expression was induced and repressed as described above and samples were taken at the times indicated and processed for immunoblotting. **(d)** FACS analysis of a strain (YLD53) expressing *GAL1,10-CDC6-S/T(1–8)Ap* as the only copy of *CDC6*, compared with a congenic strain expressing only *GAL-CDC6* (YLD52). DNA content is indicated as haploid (1C) or diploid (2C). **(e,f)** Cdc6-S/T(1–8)Ap is unstable after transcriptional and translational repression in (e) α -factor-blocked cells (YLD35) but is (f) stabilised in the same cells blocked in nocodazole (NOC). In (a,b,c,e,f) the arrow indicates Cdc6p.

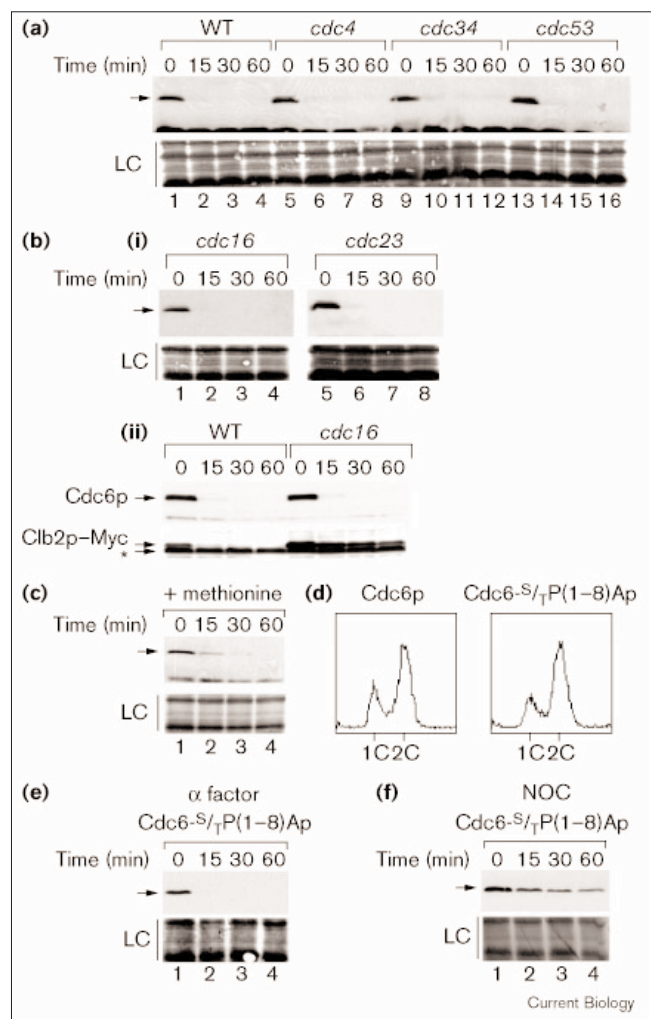
multiple elements in the amino-terminal 47 amino acids of Cdc6p. This amino-terminal domain is not essential for the biochemical activity of Cdc6p in loading of minichromosome-maintenance proteins (Mcms) but is required for Cdc6p degradation throughout the cell cycle [29].

Here, we have examined the role of Cdc28p in the degradation of Cdc6p *in vivo*. Cdc6p degradation was found to be surprisingly complex. Before the activation of Cdc28p at Start, Cdc6p was targeted for degradation by a pathway that did not involve SCF. This SCF-independent pathway was inactivated by Clb-Cdc28p later in the cell cycle. Late in G1, Cdc28p caused the rapid degradation of Cdc6p by SCF. The rapid degradation of Cdc6p was driven by the G1 cyclin (Cln) form of Cdc28p. Later in the cell cycle, Clb-Cdc28p also drove SCF-dependent degradation of Cdc6p, although at a slower rate.

Results

Cdc6p proteolysis before Start does not require SCF

We showed previously, using the yeast two-hybrid system, that Cdc6p interacts with Cdc4p, a component of SCF [29]. Moreover, the degradation of Cdc6p in nocodazole-arrested cells completely depends on the SCF components Cdc4, 34 and 53, and requires the amino-terminal 47 amino acids of Cdc6p which interact with Cdc4p *in vivo*. Surprisingly, however, Cdc6p is equally unstable in cells arrested with either α factor or nocodazole. This

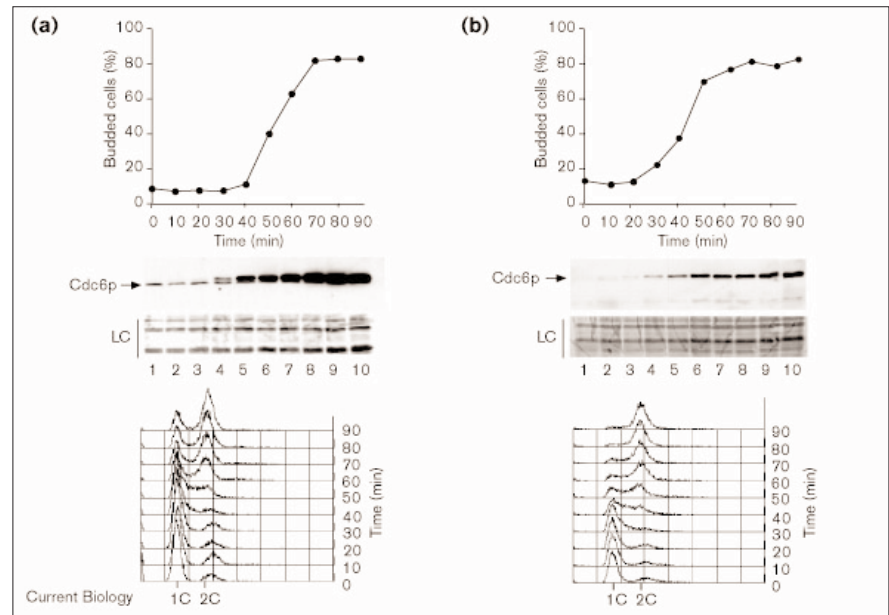


contrasts sharply with one of the best-characterised SCF substrates, Sic1p, which is stable in α -factor-arrested cells but is highly unstable in nocodazole-arrested cells [15].

We set out to investigate this paradox by examining the genetic requirements for Cdc6p degradation in α -factor-arrested cells. First, we examined the role of SCF in this degradation. In the experiment shown in Figure 1a, wild-type or SCF temperature-sensitive mutant cells (*cdc4*, *cdc34*, *cdc53*) containing an additional copy of the wild-type *CDC6* gene under the control of the *GAL1,10* promoter were first arrested with α factor in raffinose-containing medium at the permissive temperature (24°C). Cdc6p was then expressed and SCF was inactivated by transferring the cells to galactose-containing medium at the restrictive temperature (37°C) for 30 minutes. New Cdc6p synthesis was then blocked by addition of glucose to the medium (to repress transcription) and cycloheximide (to prevent translation of any remaining *CDC6* mRNA). Samples were taken at the indicated times and Cdc6p levels were determined by immunoblotting with a monoclonal antibody to Cdc6p. Figure 1a shows that, in α -factor-arrested cells, Cdc6p was rapidly degraded, not only in

Figure 2

SCF^{CDC4} is required for the rapid degradation of Cdc6p in late G1. **(a)** YCD4 (*cdc4-1*, *SIC1Δ*, *GAL-CDC6*) cells were blocked in G1 with α factor in YP-Raf at 24°C. The cells were then transferred to YP medium containing raffinose and galactose (YP-Raf,Gal) and α factor at 37°C. After 30 min, the cells were released into the cell cycle in YP-Raf,Gal at 37°C. Samples were taken at the indicated times for FACS analysis (bottom panels) and to determine the budding index (upper panels). Samples were also taken and processed for immunoblotting to detect Cdc6p (middle panels). LC, loading control (see legend to Figure 1). Lanes 1–10 correspond to the times at which the samples were taken, in 10 min intervals from 0 to 90 min. **(b)** A second, similar, experiment using YCD3 cells (*cdc4-1*, *SIC1Δ*) was performed. In this case, Cdc6p was expressed from its own promoter and the cells were kept in YP-Raf for the duration of the experiment.



wild-type cells (lanes 1–4), but also in *cdc4*, *cdc34* and *cdc53* mutants at the restrictive temperature (lanes 5–16). Under otherwise identical conditions in the same strains, Cdc6p became highly stabilised in the SCF mutants in nocodazole-arrested cells. Therefore, Cdc6p degradation during pre-Start G1, in contrast to mitosis, does not require SCF.

The anaphase-promoting complex, also known as the cyclosome (APC/C), mediates the second major pathway for the regulated proteolysis of key cell-cycle constituents. The APC/C is responsible for targeting a number of proteins including the B cyclins for ubiquitin-mediated degradation [30]. As the APC/C remains active in α -factor-arrested cells [31], we considered the possibility that it might be required for the pre-Start degradation of Cdc6p. Figure 1b(i) shows an experiment essentially identical to that in Figure 1a except that two APC/C mutants, *cdc16* and *cdc23*, were tested. Cdc6p remained as unstable in these APC/C mutants as it was in wild-type cells. These strains have previously been shown to be defective in degradation of the APC/C substrate Dbf4p [32]. To ensure that the APC/C is inactive, we constructed wild-type and *cdc16* mutant strains in which both Cdc6p and Clb2p were expressed from the *GALI,10* promoter. Figure 1b(ii) shows that, in wild-type cells arrested in α factor, both Cdc6p and Clb2p were highly unstable. In the *cdc16* mutant, Cdc6p was equally unstable, but Clb2 was significantly stabilised. From these experiments, we conclude that neither the APC/C nor SCF is required for Cdc6p proteolysis during pre-Start G1.

The proteolysis of Cdc6p seen in α -factor-arrested cells might be a response to mating pheromone that is unrelated

to the cell cycle. Alternatively, this degradation may be due to the mating-pheromone-induced inhibition of G1 Cln activity. To test this, we examined Cdc6p degradation in cells that have been arrested in pre-Start G1 by G1 Cln depletion. Cells in which two *CLN* genes (*CLN1* and *CLN3*) were deleted and the third (*CLN2*) placed under the repressible *MET3* promoter were arrested in G1 by addition of methionine to the medium. After arrest, Cdc6p was induced for 30 minutes and repressed as above. As in α -factor-arrested cells, Cdc6p was unstable in Cln-depleted cells (Figure 1c). Therefore, Cln-Cdc28p kinase is not required for degradation of Cdc6p in these pre-Start arrested cells.

The Cdc6p protein contains eight SP or TP motifs that might act *in vivo* as sites for phosphorylation by Cdks. As an alternative approach to examine the role of Cdc28p in Cdc6p degradation, we constructed a *CDC6* gene in which the phospho-accepting amino-acid residue (S or T, in the single-letter amino-acid code) in all eight sites was mutated to A (Cdc6-S/T-P(1–8)A). When we replaced the endogenous *CDC6* gene with this mutant by plasmid shuffle, we found that, when this was the only copy of Cdc6p, cells were viable, had apparently normal cell-cycle kinetics and showed no evidence of re-replication, as determined by flow cytometry, indicating that this mutant Cdc6p protein is functional for regulated DNA replication (Figure 1d). Both wild-type Cdc6p and Cdc6-S/T-P(1–8)A were degraded at the same rate in α -factor-arrested cells (Figure 1e). Therefore, neither Cdc28p nor any of the potential Cdk consensus sites in Cdc6p are required for degradation of Cdc6p before

Start. We designate this pre-Start degradation of Cdc6p as 'mode 1', which is independent of SCF and Cln-Cdc28p.

This contrasts with the SCF-dependent degradation of Cdc6p in nocodazole-arrested cells. Although the wild-type Cdc6p is rapidly degraded [29], Cdc6^{S/T}P(1–8)A was stabilised in these nocodazole-arrested cells (Figure 1f). Therefore, one or more of the potential Cdc28p phosphorylation sites is important for the degradation of Cdc6p during mitosis.

Degradation of Cdc6p in late G1 phase requires SCF

Although Cdc6p was degraded moderately rapidly in cells arrested in either α factor or nocodazole, we have previously shown several lines of evidence that Cdc6p is much more rapidly degraded during late G1/early S phase [29]. First, when Cdc6p is constitutively expressed from the *GAL1,10* promoter, it disappears soon after release from α factor and does not appear again until later in the cell cycle,

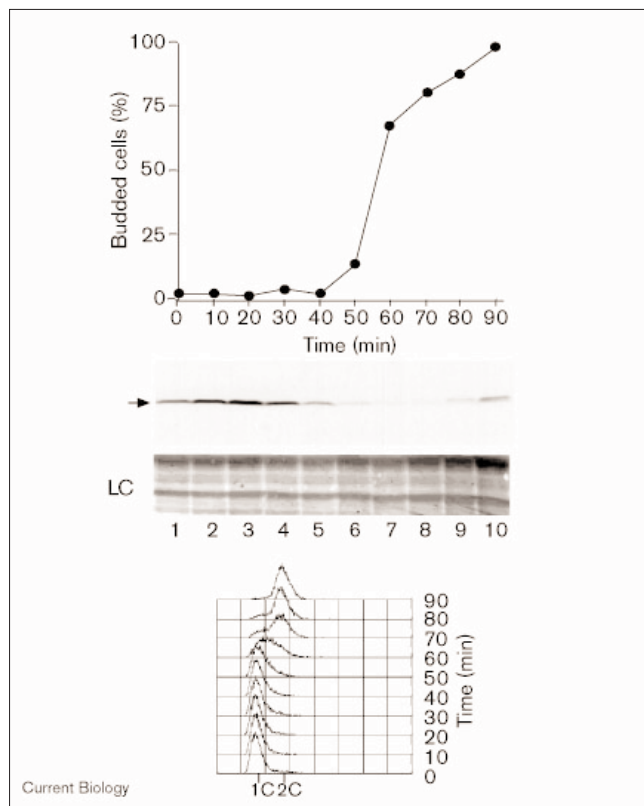
showing that Cdc6p cannot accumulate to high levels during S phase even when it is transcribed at high levels. Second, Cdc6p is so unstable in *cdc7*-arrested cells (late G1) that we have not been able to determine a half life. Finally, in hydroxyurea-arrested cells (early S phase), we are unable to detect Cdc6p even when expressed from the strong *GAL1,10* promoter. In the absence of SCF^{CDC4} function, however, Cdc6p is present at high levels and is stable in hydroxyurea-arrested cells. Together, these experiments indicate that Cdc6p is very rapidly degraded during late G1/early S phase by the SCF pathway. To examine the role of SCF in this late G1/early S phase degradation of Cdc6p in unblocked cells, we exploited the fact that Cdc4p is not essential for S-phase entry in cells in which *SIC1* has been deleted [15]. We arrested a culture of a *cdc4, SIC1 Δ* double mutant at 24°C and released the cells at 37°C to inactivate Cdc4p. In the absence of SCF^{CDC4} activity, the rapid degradation of Cdc6p in late G1 did not occur and Cdc6p continued to accumulate during the remainder of the cell cycle (Figure 2a). The lower levels of Cdc6p at the beginning of the experiment are likely to be due to the fact that mode 1 degradation is active in the α -factor-arrested cells and that induction of the *GAL1,10* promoter is less efficient at 37°C. Even when Cdc6p was expressed from its own promoter, it was not degraded before S-phase entry (Figure 2b) as it is in wild-type cells ([29] and see below). Taken with results previously published, this shows that the post-Start degradation of Cdc6p during late G1/early S phase is dependent on SCF^{CDC4}. We designate this rapid, SCF-dependent degradation of Cdc6p in late G1 as 'mode 2' and the less rapid SCF-dependent degradation seen in nocodazole-arrested cells as 'mode 3'.

Mode 2 proteolysis requires Cln but not Clb5,6 activity

As this rapid Cdc6p destruction occurred after α -factor-release and is, therefore, downstream of Start, we were interested in testing the requirement for Cdc28p. To begin to examine this, we arrested a population of cells expressing Cdc6p from the *GAL1,10* promoter in pre-Start G1 by Cln depletion as above (Figure 1c). Cdc6p could be readily detected in these arrested cells (Figure 3, lane 1), consistent with the experiment in Figure 1c. The culture was then released from the G1 arrest by transferring to methionine-free medium to induce Cln expression. This medium also contained galactose to ensure continued Cdc6p expression. After release and concomitant with budding, Cdc6p disappeared and reappeared later in the cell cycle (Figure 3, lanes 1–9). Therefore, activation of Cln2–Cdc28p is sufficient to activate the rapid degradation of Cdc6p in late G1/early S phase.

SCF substrates, such as Sic1p, must be phosphorylated in order to be recognised for polyubiquitination. Therefore, we determined whether the potential Cdk phosphorylation sites on Cdc6p were required for the rapid, SCF-dependent degradation of Cdc6p downstream of Start. Cells containing

Figure 3



Cdc6p degradation in late G1 requires Cln activity. A logarithmically growing culture of YLD38 cells (*CLN1-3 Δ , MET-CLN2, GAL-CDC6*) was arrested in G1 by the addition of methionine. Cdc6p (arrow) was induced for 45 min, following which the cells were released into the cell cycle in methionine-free medium containing galactose. Samples (lanes 1–10) were taken at the indicated times to determine the budding index (upper panel), for FACS analysis (bottom panel) and to be processed for immunoblotting (middle panel).

GAL-Cdc6-S₇P(1-8)A were arrested in α factor and released into galactose-containing medium. Figure 4 shows that, although the Sic1 protein was rapidly degraded as buds emerged in cells expressing either wild-type Cdc6p or Cdc6-S₇P(1-8)Ap, Cdc6-S₇P(1-8)Ap was not rapidly degraded as cells entered S phase. Thus, at least a subset of Cdk sites is required for the rapid degradation of Cdc6p.

Clns drive S phase by promoting the accumulation of B cyclin (Clb)–Cdc28p. This is accomplished in three ways: by targeting the Clb–Cdc28p inhibitor Sic1p for degradation by the SCF^{CDC4} pathway; by inactivating the APC/C, thus stabilising the B cyclins; and by inducing transcription of the first set of Clbs, Clb5 and Clb6. Thus, Clns might trigger rapid Cdc6p degradation either directly or indirectly, by activating Clb5 and Clb6, which in turn might phosphorylate Cdc6p, targeting it for degradation. These two possibilities can be distinguished because Clb5 and Clb6, which normally trigger the initiation of DNA replication, are non-essential. In their absence, other Clbs can support DNA replication. Nevertheless, because there is a delay before other Clbs are activated, the onset of DNA replication is significantly delayed in strains lacking Clb5 and Clb6 (*clb5,6Δ*). If rapid Cdc6p degradation is triggered by Clbs, then, in the absence of Clb5 and Clb6, rapid degradation should either be alleviated or delayed until the other Clbs are expressed. Alternatively, if Clns directly target Cdc6p for degradation, rapid degradation should not be delayed in the *clb5,6Δ* strain. In Figure 5a, cells expressing wild-type Cdc6p from the *GAL1,10* promoter in either a wild-type or a *clb5,6Δ* mutant background were arrested

in G1 with α factor and released. Flow cytometry showed that, as expected, entry into S phase was delayed by approximately 45 minutes in the *clb5,6Δ* mutant whereas budding (a Cln-, not Clb-, dependent process) occurred at approximately the same time in the two strains. Immunoblot analysis showed that rapid Cdc6p degradation, like budding, occurred at the same time in the two strains. Moreover, Cdc6p degradation occurred at the same time as Cln-dependent Sic1p degradation. Taken together with the experiment shown in Figure 3, these results demonstrate that the rapid degradation of Cdc6p at the end of G1 phase is dependent on Clns but is not dependent on Clb5 and Clb6.

The experiments described above used Cdc6p expressed from the *GAL1,10* promoter. We were interested in determining whether the degradation of Cdc6p expressed from its own promoter was also independent of Clb5 and Clb6 activation. After cells are released from α factor arrest, there is a burst of *CDC6* transcription [23,33–35] and synthesis [23,29] before entry into S phase. Therefore, we examined Cdc6p levels after release from an α factor arrest in either wild-type cells or a *clb5,6Δ* mutant containing just the endogenous *CDC6* gene. Figure 5b shows that both the appearance and disappearance of Cdc6p soon after release from α factor occurred at the same time in the wild type and in *clb5,6Δ* mutants. There was a delay in Cdc6p appearance in the next cell cycle in the *clb5,6Δ* mutant. This was presumably due to a delay in entering mitosis, in response to the delay in S-phase completion. This shows that the degradation of Cdc6p expressed from

Figure 4

The SP/TP motifs are required for rapid degradation of Cdc6p in late G1. Asynchronous cultures of YLD16 (*GAL-CDC6*) and YLD35 (*GAL-CDC6-S₇P(1-8)A*) cells were synchronised with α factor in YP-Raf. Galactose was added to induce Cdc6p or Cdc6-S₇P(1-8)Ap (upper arrows); 30 min later, the cells were released into the cell cycle in YP-Raf, Gal. Samples were taken at the indicated times to determine the budding index (lower left panel), for FACS analysis (lower right panels) and to be processed for immunoblotting to detect Cdc6p, Cdc6-S₇P(1-8)Ap and Sic1p (upper panels). Lanes 1–9 correspond to the times at which the samples were taken, in 10 min intervals from 0 to 80 min.

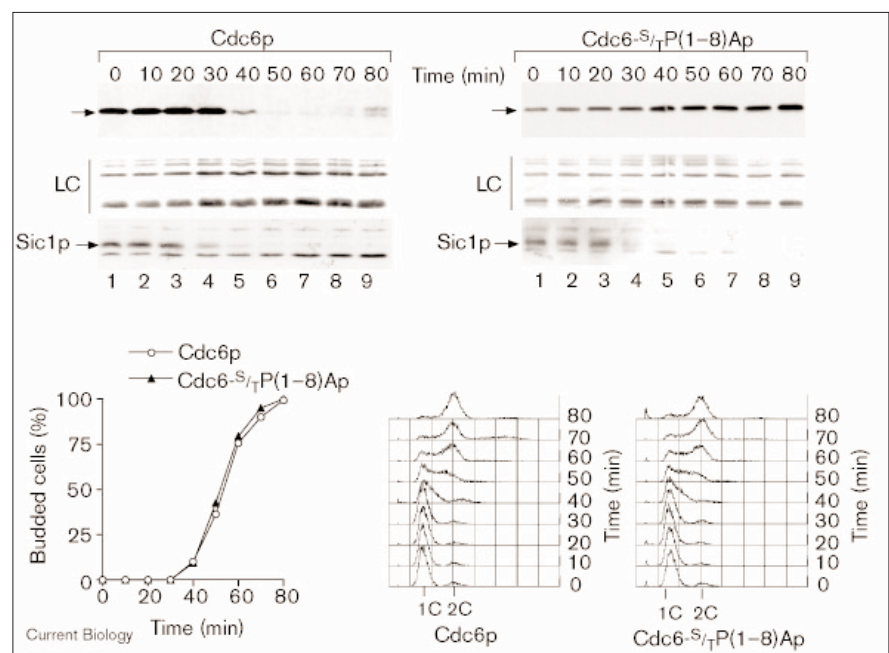
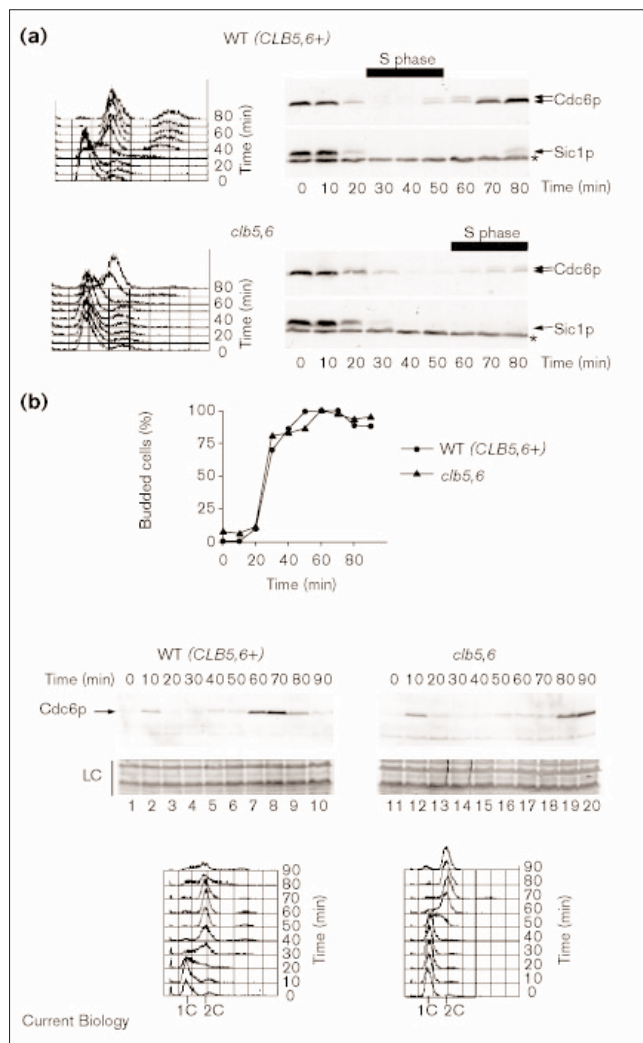


Figure 5



Clb5 and *Clb6* are not required for degradation of Cdc6p in late G1. (a) Wild-type (WT; *CLB5,6+*, *GAL-CDC6*) and YKL125 (*clb5,6Δ*, *GAL-CDC6*) cells were grown in YP medium containing galactose (YP-Gal) and arrested in G1 with α factor. They were then released into the cell cycle in YP-Gal. Samples were taken at the indicated times for FACS analysis (left) and to be processed for immunoblotting (right). (b) A similar experiment as in (a) was performed with wild-type (*CLB5,6+*) and YKL124 (*clb5,6Δ*) cells. After release from an α factor block in YPD, samples were taken at the times indicated for FACS (bottom panels), to determine the budding index (upper panel) and to be processed for immunoblotting (middle panels). Lanes 1–10 and 11–20 correspond to the times at which the samples were taken, in 10 min intervals from 0 to 90 min.

its own promoter occurs downstream of Start but does not require activation by *Clb5* and *Clb6*. Taken together, these experiments show that mode 2 activation requires *Cln-Cdc28* but does not require *Clb5* and *Clb6*.

Pre-Start degradation of Cdc6p is inhibited by Cdc28p

In nocodazole-arrested cells, Cdc6p degradation was entirely dependent on SCF^{CDC4}. Thus, mode 1 appears to

be unable to degrade Cdc6p later in the cell cycle. We were, therefore, interested in testing the possibility that mode 1 is inhibited by Cdc28p. To begin to address this, we exploited the fact that mode 1 was capable of rapidly degrading Cdc6-S/T-P(1–8)Ap while modes 2 and 3 were not. Figure 6a shows that Cdc6-S/T-P(1–8)Ap was rapidly degraded in *cdc4* cells arrested at the restrictive temperature. As *cdc4* mutants arrest with high levels of *Cln-Cdc28p* kinase, low levels of *Clb-Cdc28p* kinase and inactive SCF^{CDC4}, we conclude that mode 1 is still active after *Cln-Cdc28p* kinase activation but before *Clb-Cdc28p* kinase activation.

We next tested whether inactivation of Cdc28p later in the cell cycle could activate mode 1. We arrested cells in nocodazole then inactivated all forms of Cdc28p using the temperature-sensitive *cdc28* degtron mutant [36,37]. This treatment is sufficient to promote re-entry of Mcm4 into the nucleus [36] and to promote the assembly of pre-RCs at origins [13]. Figure 6b shows that, after Cdc28p inactivation in nocodazole-arrested cells, both wild type and Cdc6-S/T-P(1–8)Ap were degraded at the same rate. Therefore, inactivation of Cdc28p promotes reactivation of mode 1 degradation in mitosis.

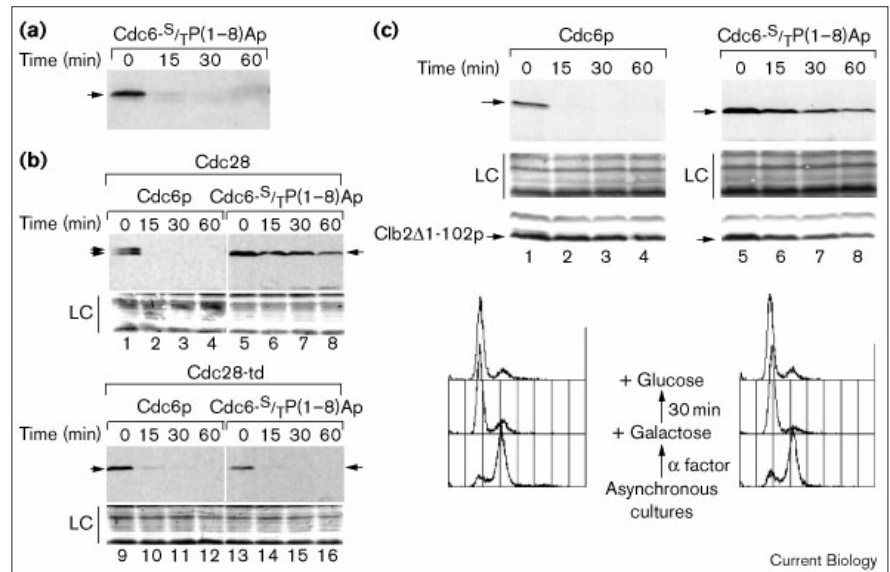
Finally, we determined whether activation of *Clb* kinase is sufficient to inactivate mode 1 degradation during G1. Cells were arrested in α factor and *Clb2Δ1-102* was expressed. *Clb2Δ1-102* is a form of *Clb2* in which the destruction box, required for APC/C-mediated degradation, has been removed. Expression of *Clb2Δ1-102* can induce S phase in α -factor-arrested cells but does not induce budding, a *Cln*-dependent phenomenon. Figure 6c shows that, after *Clb2Δ1-102* expression, Cdc6-S/T-P(1–8)Ap became stabilised relative to wild-type Cdc6p. Therefore, *Clb* expression is sufficient to inactivate mode 1 during G1.

Mode 1 cannot degrade Cdc6p made in the previous cell cycle

The finding that Cdc6p degradation before Start does not require SCF does not appear consistent with the results of an experiment we previously published showing that *cdc4* mutant cells are defective in degrading Cdc6p if released from a nocodazole block into an α -factor block at the restrictive temperature [29]. In this experiment, Cdc6p, expressed from its own promoter, first appears in both mutant and wild-type cells late in mitosis. Most of the Cdc6p in wild-type cells is degraded soon afterwards. In *cdc4* mutant cells however, Cdc6p levels do not significantly decline. One possible explanation for this is that Cdc6p, which is synthesised before exit from mitosis, is normally degraded by SCF^{CDC4} and cannot be degraded by the mode 1 pathway. To test this, wild-type and *cdc4* mutant cells containing *GAL-CDC6* were first arrested in mitosis with nocodazole in raffinose-containing medium, transferred to 37°C for 15 minutes and galactose was then added for a further 15 minutes to induce Cdc6p. The

Figure 6

Clb-Cdc28 kinase inactivates mode 1 of Cdc6p degradation. (a) *cdc4-1* cells were grown in YP-Raf at 24°C, blocked in G1 with α factor and then transferred to 37°C in YP-Raf, Gal for 30 min. The cells were subsequently released into the cell cycle in YP-Raf, Gal at 37°C, causing them to block at the *cdc4-1* restriction point; 2 h after release from α factor, Cdc6 expression was repressed. Samples were taken and processed for immunoblotting. The arrow indicates Cdc6p. (b) Cells containing *GAL-Ubr1/Cdc28-td* (YLD44 and YLD46) or *GAL-Ubr1* alone (YLD47 and YLD49) as a control were grown in YP-Raf at 24°C and subsequently blocked in G2 with nocodazole. Both Ubr1 and either Cdc6p or Cdc6-S/T-P(1-8)Ap were induced by the addition of galactose. After 30 min, the cultures were shifted to 37°C to inactivate Cdc28p. After a further 30 min, Cdc6p or Cdc6-S/T-P(1-8)Ap expression was repressed. Samples were taken at the time of transcriptional repression and at the subsequent times indicated and processed for immunoblotting. Lane numbers correspond to the times at the top of the immunoblot; arrows indicate Cdc6p. (c) Strains containing *GAL-CLB2 Δ 1-102* and either *GAL-CDC6* (YLD50) or *GAL-CDC6-S/T-P(1-8)A* (YLD51)



were grown in YP-Raf and blocked with α factor. Both Clb2 Δ 1-102p (lower arrows) and Cdc6p or Cdc6-S/T-P(1-8)Ap (upper arrows) were induced by the addition of galactose. After 30 min, expression of Cdc6p and

Cdc6-S/T-P(1-8)Ap was repressed. Samples were taken and processed for immunoblotting (upper panels) and FACS analysis (lower panels). Lane numbers correspond to the times at the top of the immunoblot.

cells were then transferred to 37°C in medium lacking nocodazole but containing glucose and α factor. Thus, a pulse of Cdc6p was made in mitosis and its fate followed as cells exited mitosis and entered G1. Figure 7a shows that, while Cdc6p rapidly disappeared from wild-type cells, it persisted in the *cdc4* mutant cells for a long period of time.

Although this suggests that mode 1 degradation cannot operate on Cdc6p made in the previous cell cycle, it is also possible that Cdc4p is required at some point between the nocodazole- and α factor arrest to activate mode 1. To test this, wild-type and *cdc4* mutant cells were released from the nocodazole block into α factor at 37°C as in Figure 7a except that *CDC6* transcription was transiently induced after cells had passed through mitosis. Figure 7b shows that, in contrast to Figure 7a, Cdc6p synthesised after cells had passed through mitosis was rapidly degraded in the *cdc4* mutant. This result is most consistent with the idea that Cdc6p made before exit from mitosis must be degraded by SCF^{CDC4} and cannot be degraded by the SCF^{CDC4}-independent pre-Start mode of degradation.

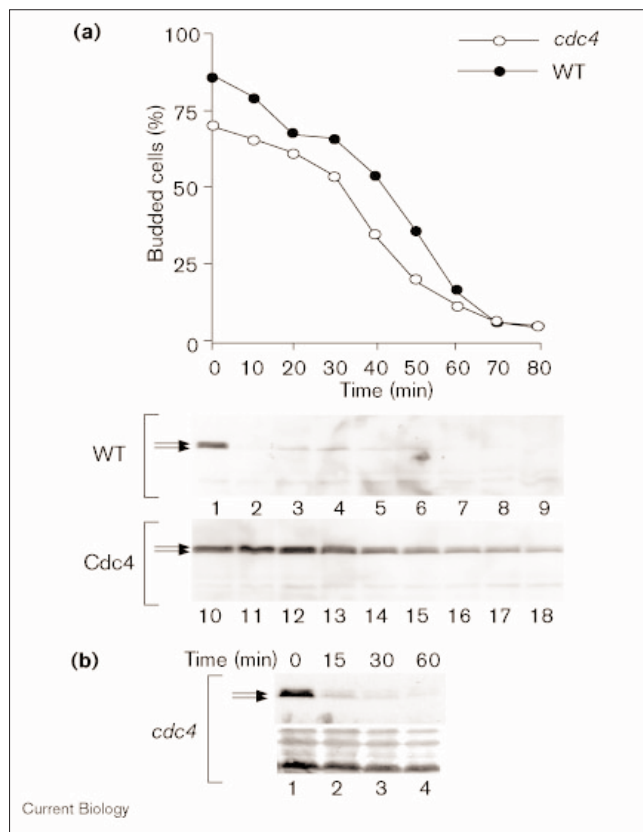
Discussion

In this paper, we have defined three modes of Cdc6p proteolysis that can be distinguished on the basis of rates and *cis*- and *trans*-acting mutations. These are summarised in Figure 8. Before Start, we found that Cdc6p was degraded through mode 1. Mode 1 degradation was moderately rapid

and characterised by two major features. First, it was independent of SCF and APC/C and, second, it did not require any of the potential Cdc28p phosphorylation sites in Cdc6p. We do not know how mode 1 degradation works but, Cdc6 Δ NTP, a mutant in which the amino-terminal 47 amino acids of Cdc6p are deleted, was stable in G1-arrested cells, arguing that signals required for mode 1 degradation reside in the amino terminus. Although the experiments used to define mode 1 degradation presented here involved expression of Cdc6p from the *GAL 1,10* promoter, our previous experiments showed that Cdc6p expressed from its own promoter is synthesised and then degraded during pre-Start G1 in wild-type cells ([29]; Figure 3a), showing that Cdc6p proteolysis is active during pre-Start G1. Mode 2 degradation was extremely rapid and characterised by the fact that it required SCF^{CDC4} and at least some of the potential Cdc28p phosphorylation sites in Cdc6p. It is likely that this mode of degradation requires multiple phosphorylation sites as neither Cdc6-S/T-P(1-4)Ap nor Cdc6-S/T-P(5-8)Ap was completely stable during S phase (data not shown). Finally, later in the cell cycle, Cdc6p degradation slowed down. This degradation remained dependent on SCF^{CDC4} and Cdc28p phosphorylation sites. Modes 2 and 3 differed not only in rate but also in the form of Cdc28p kinase required for their activation (see below).

Each mode of Cdc6p degradation was regulated by Cdc28p. Several lines of evidence suggest that SCF-independent

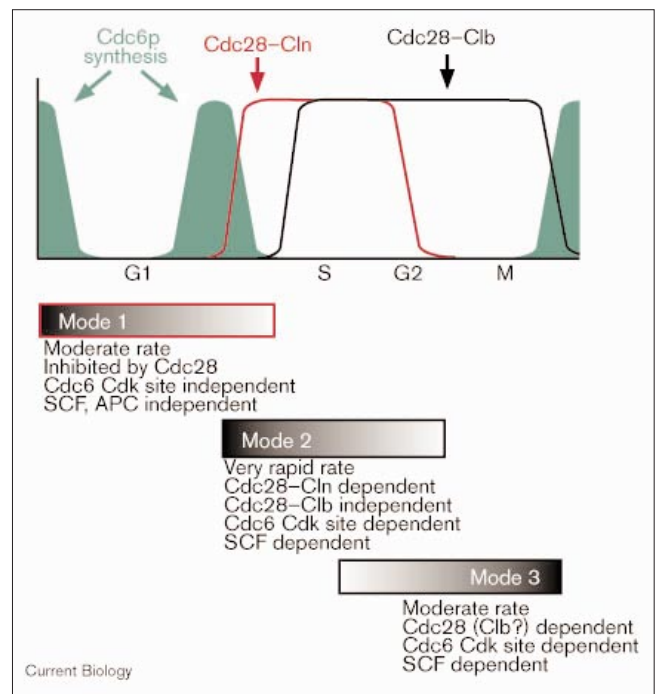
Figure 7



CDC4 is required for the degradation of Cdc6p made in G2 but not for the Cdc6p made in G1. (a) A pulse of Cdc6p was given in mitosis to wild-type (WT, YLD15) and *cdc4* (YLD16) cells (see text). The cells were subsequently released into G1 at 37°C without further Cdc6p expression. Samples were taken at the indicated times to measure the budding index (upper panel) and to be processed for immunoblotting (middle and lower panel). Lanes 1–9 and 10–18 correspond to the times at which the samples were taken, in 10 min intervals from 0 to 80 min. (b) This experiment was performed in a manner similar to that in (a) except that Cdc6 was induced for 30 min in G1 (α -factor block) at 37°C and then repressed rather than in mitosis. Samples were taken at the time of repression and at the indicated times to be processed for immunoblotting.

mode 1 degradation is inhibited by Cdc28p. First, wild-type Cdc6p was almost completely stable in nocodazole-arrested cells in which SCF had been inactivated. Second, Cdc6-^S/_TP(1–8)Ap, which could be degraded by mode 1, was considerably more stable in G2 than in G1. Third, expression of Clb2Δ1–102 in α -factor-arrested cells caused the stabilisation of Cdc6-^S/_TP(1–8)Ap. Finally, inactivation of Cdc28p in mitosis-arrested cells caused Cdc6-^S/_TP(1–8)Ap to become unstable. The inhibition of mode 1 by Cdc28p appeared to be Clb specific as it was still active in *cdc4*-arrested cells. We suggest that mode 1 becomes activated when Clb–Cdc28p kinase is inactivated at the end of mitosis. Mode 1 is then inactivated by one or more of the Clb–Cdc28p kinases later in the cell cycle. At present we

Figure 8



Three modes of Cdc6 degradation during the cell cycle. The model is described in the text. The different forms of degradation are distinguished on the basis of different *cis*- and *trans*-acting factors. The fading of the boxes indicates ambiguities about which factors inactivate modes 1 and 2 and activate mode 3.

do not know how Clb–Cdc28p inhibits mode 1 degradation. The fact that Cdc6-^S/_TP(1–8)Ap, which presumably cannot be phosphorylated by Cdc28p, is stable in mitosis suggests that Cdc28p may be acting by inhibiting the degradation machinery itself rather than through Cdc6p. Nevertheless, the fact that wild-type Cdc6p made in the previous cell cycle cannot be degraded during pre-Start G1 suggests that Cdc28p acts directly on Cdc6p. It is possible that Cdc28p inhibits mode 1 both by inhibiting the degradation machinery and by modifying the substrate at a non-consensus phosphorylation site. It is interesting to note that, although human Cdc6 levels do not fluctuate dramatically during the cell cycle, Cdc6p is degraded upon entry into quiescence [24–26]. Perhaps this degradation occurs by a pathway related to mode 1.

The rapid SCF-dependent mode 2 degradation of Cdc6p was activated by Cln–Cdc28p kinase in late G1. As activation of mode 2 degradation did not require Clb5 and Clb6 and was not delayed in *clb5,6* mutants, and as Cdc6-^S/_TP(1–8)Ap was resistant to mode 2 degradation, we suggest that Cln–Cdc28p kinase acts directly on Cdc6p. Recent biochemical analysis has suggested that Clb5–Cdc28p kinase is more efficient than Cln2–Cdc28p kinase in promoting SCF^{CDC4}-dependent polyubiquitination *in vitro* [27].

Our results *in vivo* clearly demonstrate that Clb5 and Clb6 are completely dispensable for timely degradation of Cdc6p before S-phase entry. The reason for the discrepancy is not clear but it is possible that some modification of Cdc6p in addition to Cln–Cdc28p phosphorylation, or some additional factor missing from the *in vitro* reactions, is required for full activity of mode 2.

CDC6 is transcribed at the end of mitosis in rapidly growing cells; there is, however, also a burst of *CDC6* transcription and synthesis after cells are released from a G1 arrest [23,33–35] (see Figure 8). Interestingly, although *CDC6* transcription occurs downstream of Start in such conditions, it occurs well before the transcription of *CLN1* and *CLN2*. Our results suggest that this may be important to allow time for Cdc6p to act in Mcm loading before full Cln activation and rapid Cdc6p degradation.

As Cln activation must occur before Clb activation, our results suggest that a cell's capacity to form pre-RCs is diminished — in this case by Cdc6p degradation — before, not concomitant with, S-phase entry. The nuclear localisation of Mcm proteins is regulated in a similar manner [36]. Mcm4 becomes excluded from the nucleus as it is displaced from chromatin during S phase. Mcm4 which is not chromatin bound is, however, exported much earlier, at the end of G1 phase. This exclusion of the free Mcm proteins requires active Cln–Cdc28p kinase but, like mode 2 degradation, is not delayed in the *clb5,6* mutant. Together, these results suggest that Cln–Cdc28p acts through several independent pathways to downregulate pre-RC assembly, independent of its ability to promote S phase via Clb activation.

Many details of Cdc6p proteolysis are unresolved. What factors are involved in mode 1 degradation? Do the different modes of degradation target different pools of Cdc6p (for example, chromatin-bound versus free Cdc6p, nuclear versus cytoplasmic, etc.)? What is the functional significance of degrading different pools of Cdc6p? The results presented here provide a framework for understanding the proteolysis of this key cell-cycle regulator.

Materials and methods

Strains and media

The strains (see Supplementary material) were all derived from W303-1a. Cell cycle blocks with nocodazole or α factor were as previously described [3]. FACS analysis was as previously described [38]. The strains YLD52 and YLD53 contain a single copy of *CDC6* or *CDC6-S/P(1–8)A* (for a description of this allele, see below) under the control of the *GAL1,10* promoter and were made by plasmid shuffle [39] in a strain in which the endogenous *CDC6* gene was deleted.

Plasmid constructs

The plasmids containing *GAL1,10–CDC6* have been described previously [29]. To make the *GAL1,10–CDC6-S/P(1–8)A* allele, site-directed mutagenesis was performed using a QuikChange™ site-directed mutagenesis kit (Stratagene). The starting template was *GAL1,10–CDC6* in pRS303 [40] and sites were mutated sequentially with the oligonucleotide primers listed in the Supplementary material. The

final product was sequenced to ensure that only the directed mutations were present. The fragment containing *GAL1,10–CDC6-S/P(1–8)A* was subsequently subcloned using flanking *NotI* and *SalI* restriction sites in the polylinker into the same acceptor sites in pRS303 (pLD14) and pRS306 (pLD15). The construct expressing *GAL1,10–CLB2–Myc* will be described elsewhere (K. Bousset, personal communication). The construct expressing *GAL1,10–CLB2Δ1–102* (pKB19) was made by subcloning a fragment containing *GAL1,10–CLB2Δ1–102* from pKL145 [36] into pRS304.

GAL1,10 promoter induction, repression and protein stability experiments

For induction of the *GAL1,10* promoter, cells were grown in YP medium containing raffinose as a carbon source, or synthetic medium containing sucrose. Galactose was added to a final concentration of 2%. The time of induction was generally 30 minutes but, in the case of cells grown in sucrose, the induction period was 45 minutes. Transcriptional repression was achieved by the addition of glucose to a final concentration of 2% to the cell suspension. At the same time, cycloheximide was added to a final concentration of 1 mg/ml to prevent further translation of any mRNA.

Cell extracts and immunoblotting

Protein extracts from harvested yeast cells were made by TCA precipitation after glass bead lysis [41]. Immunoblots were performed as described [29]. Detection of Sic1p was with a polyclonal antibody (a gift from L. Johnston), and detection of Myc-tagged Clb2 and Clb2Δ1–102p was with 9E10. After processing for immunoblotting, the membrane was stained with amido black [32,42] to detect total proteins and ensure equal sample loading and even transfer from the polyacrylamide gel to the membrane.

Supplementary material

Two tables listing the genotypes of the strains used in this study and oligonucleotide primers used for site-directed mutagenesis are available at <http://current-biology.com/supmat/supmatin.htm>.

Acknowledgements

We gratefully acknowledge discussions with Karim Labib and we thank Karim and Takashi Toda for reading this manuscript. We thank Lee Johnston for the gift of anti-Sic1p antibody.

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