

# Separate SCF<sup>CDC4</sup> recognition elements target Cdc6 for proteolysis in S phase and mitosis

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**The Cdc6 DNA replication initiation factor is targeted for ubiquitin-mediated proteolysis by the E3 ubiquitin ligase SCF<sup>CDC4</sup> from the end of G<sub>1</sub> phase until mitosis in the budding yeast *Saccharomyces cerevisiae*. Here we describe a dominant-negative *CDC6* mutant that, when overexpressed, arrests the cell cycle by inhibiting cyclin-dependent kinases (CDKs) and, thus, prevents passage through mitosis. This mutant protein inhibits CDKs more efficiently than wild-type Cdc6, in part because it is completely refractory to SCF<sup>CDC4</sup>-mediated proteolysis late in the cell cycle and consequently accumulates to high levels. The mutation responsible for this phenotype destroys a putative CDK phosphorylation site near the middle of the Cdc6 primary amino acid sequence. We show that this site lies within a novel Cdc4-interacting domain distinct from a Cdc4-interacting site identified previously near the N-terminus of the protein. We show that both sites can target Cdc6 for proteolysis in late G<sub>1</sub>/early S phase whilst only the newly identified site can target Cdc6 for proteolysis during mitosis.**

**Keywords:** Cdc4/Cdc6/cell cycle/proteolysis/replication

## Introduction

Precise and tightly controlled DNA replication is an essential feature of the eukaryotic cell cycle. The regulated assembly of pre-replicative complexes (pre-RCs) at origins of DNA replication during the G<sub>1</sub> phase is a critical step in this process. In budding yeast the main cyclin (Cln)-dependent kinase (CDK), Cdc28, triggers the initiation of DNA replication from origins containing pre-RCs and, at the same time, prevents the assembly of new pre-RCs (Diffley, 1996, 2001; Dutta and Bell, 1997; Donaldson and Blow, 1999; Pasero and Schwob, 2000). Thus, pre-RCs can assemble only early in G<sub>1</sub> phase when Cdc28 kinase activity is low.

The Cdc6 protein is required for the assembly of pre-RCs (Cocker *et al.*, 1996; Detweiler and Li, 1997; Tanaka *et al.*, 1997) and Cdc6 levels are tightly regulated during the cell cycle by CDK activity (Piatti *et al.*, 1995; Drury *et al.*, 1997, 2000; Elsasser *et al.*, 1999; Calzada *et al.*, 2000). The *CDC6* gene is transcribed periodically in early

G<sub>1</sub> phase (Zhou and Jong, 1990; Zwerschke *et al.*, 1994; Piatti *et al.*, 1995; McInerny *et al.*, 1997). In addition to this transcriptional regulation, evidence has emerged of an intricate system for ensuring the effective elimination of Cdc6 throughout the cell cycle. We have previously defined three distinct modes of Cdc6 proteolysis based on *cis*- and *trans*-acting genetic requirements (Drury *et al.*, 2000). Cdc6 proteolysis during pre-Start G<sub>1</sub> (mode 1) is moderately rapid ( $t_{1/2}$  ~15 min), is independent of the two best-characterized cell cycle-regulated pathways for ubiquitin-mediated proteolysis, the SCF and the APC/cyclosome, and does not require any of the eight consensus Cdc28 phosphorylation sites in Cdc6.

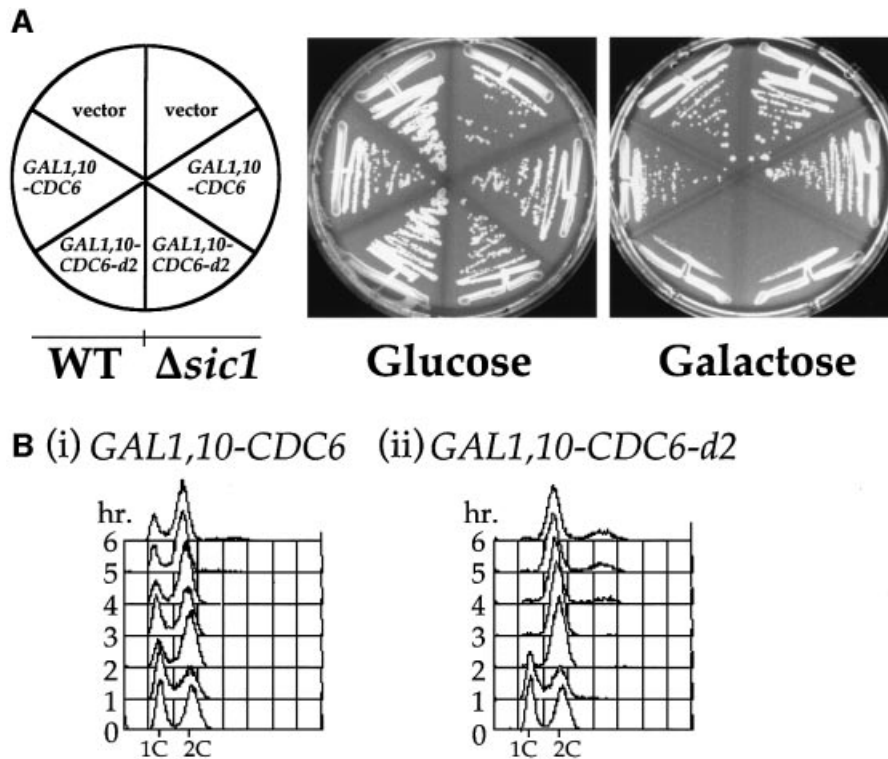
Near the end of G<sub>1</sub> phase, Cdc6 proteolysis becomes extremely rapid ( $t_{1/2}$  < 2 min). This rapid 'mode 2' proteolysis requires SCF<sup>CDC4</sup>. Mode 2 degradation occurs only after the activation of the G<sub>1</sub> cyclin-dependent kinase. Moreover, it requires at least some subset of CDK consensus phosphorylation sites in Cdc6, since a mutant in which all eight consensus sites have been mutated is resistant to mode 2 proteolysis. Whilst it requires G<sub>1</sub> cyclins, mode 2 proteolysis does not require the S-phase-specific cyclins Clb5 or Clb6, suggesting that it is direct phosphorylation of Cdc6 by Cln-Cdc28 that targets it for proteolysis.

Later in the cell cycle, after activation of the B type cyclins, Cdc6 proteolysis is significantly slower than it is during S phase ( $t_{1/2}$  ~15 min). This 'mode 3' proteolysis, however, is still entirely dependent upon SCF<sup>CDC4</sup> and also requires some subset of CDK consensus sites. In our previous work, modes 2 and 3 were distinguished primarily on the basis of proteolysis rate. Here we show that Cdc6 possesses two distinct SCF<sup>CDC4</sup>-interaction domains, one, previously identified, near the N-terminus of the protein and a second novel domain located near the middle of the protein. Interestingly, this second domain contains two CDK consensus sites and mutation of either site stabilizes the protein during G<sub>2</sub>/M. Mode 2 proteolysis works through both SCF<sup>CDC4</sup> sites but primarily through the first, whereas mode 3 is entirely dependent upon the second. This is a novel mechanism for modulating the rate of SCF<sup>CDC4</sup>-dependent proteolysis during the cell cycle.

## Results

### *CDC6-d2 overexpression prevents mitosis*

To better understand the function and regulation of Cdc6 in DNA replication we isolated and characterized dominant-negative mutant alleles of *CDC6* (Perkins and Diffley, 1998). In this screen, a plasmid containing the *CDC6* gene under the control of the *GALI,10* promoter was randomly mutagenized by passage through a mutator *Escherichia coli* strain. This library of mutants was transformed into yeast and plated on glucose-containing plates.



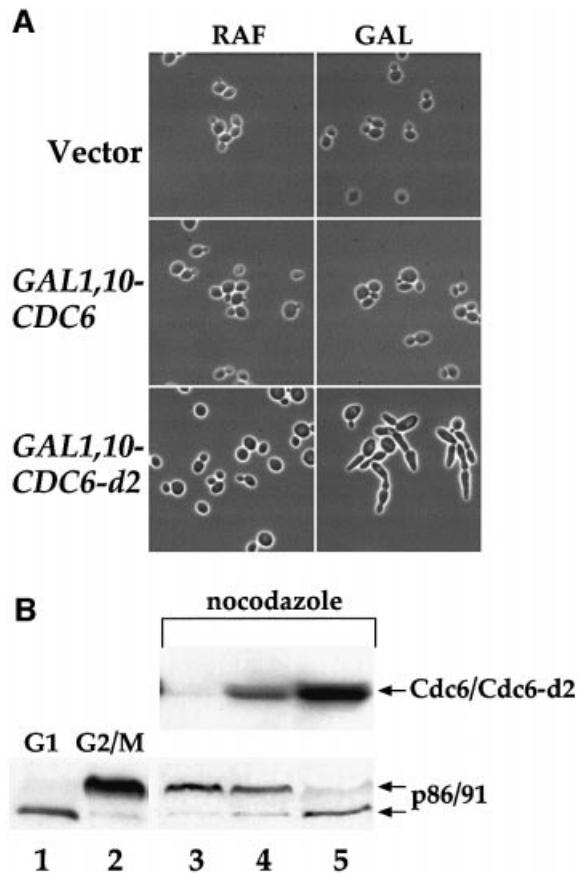
**Fig. 1.** (A) W303-1a or W303-1a $\Delta$ *sic1* were transformed with pRS426 (Christianson *et al.*, 1992), pRS426 *GAL1,10-CDC6* or pRS426 *GAL1,10-CDC6-d2* and grown on glucose (no expression from the *GAL1,10* promoter) or galactose to induce expression of the *CDC6* gene. Expression of *CDC6-d2* but not *CDC6* resulted in an inhibition to growth. As can be seen on the right-hand half of each plate this effect was not dependent on *SIC1*. (B) W303-1a with an integrated copy of either (i) *GAL1,10-CDC6* or (ii) *GAL1,10-CDC6-d2* was grown in YPRaf until mid-log phase. At this point the cells were transferred into YPGal and allowed to continue cycling. Samples were taken every hour and processed for flow cytometry.

Transformants were then replica plated on to galactose-containing plates to allow expression of the mutant proteins. From 20 000 transformants, two candidates were identified that grew on glucose- but not galactose-containing medium. One of these, *CDC6-d1*, which has previously been described in detail, prevented normal S phase progression by interfering with pre-RC assembly (Perkins and Diffley, 1998). The second, *CDC6-d2*, is described in this paper. Figure 1A shows that yeast carrying either the wild-type *GAL1,10-CDC6* plasmid or the vector alone formed colonies on both glucose- and galactose-containing plates. However, yeast carrying the *GAL1,10-CDC6-d2* plasmid grew well on glucose but showed severely retarded growth on galactose-containing plates.

To begin to determine the mechanism of growth suppression, we asked whether Cdc6-d2 overexpression arrested cells at a specific stage of the cell cycle. Cells containing either wild-type *CDC6* or *CDC6-d2* under the control of the *GAL1,10* promoter were grown to mid-log phase in raffinose-containing medium and then transferred to fresh medium containing galactose. At the time intervals indicated in Figure 1B, samples were taken and the DNA content was measured by flow cytometry. Figure 1A shows that overexpression of wild-type Cdc6 does not prevent cell proliferation. As shown in Figure 1B, this overexpression does have an effect on cell cycle kinetics as can be seen by the increase in the fraction of cells with a 2C DNA content, consistent with previous

work (Bueno and Russell, 1992). The expression of Cdc6-d2, however, prevents proliferation (Figure 1A) and cells accumulate with an apparent 2C DNA content (Figure 1B). In other experiments, when Cdc6-d2 was expressed after release from a nocodazole arrest, cells were unable to complete mitosis, indicating that Cdc6-d2 can block cell cycle progression after S phase is complete (data not shown). Thus, unlike Cdc6-d1, which inhibited S phase but not mitosis, Cdc6-d2 prevents passage through mitosis but does not prevent DNA replication.

Microscopic examination revealed that cells expressing *GAL1,10-CDC6-d2* developed abnormal cell morphology characterized by the presence of highly elongated buds (Figure 2A). This morphology is similar to that of *cdc4* temperature-sensitive mutant cells at the restrictive temperature (Hartwell *et al.*, 1973, 1974; Hereford and Hartwell, 1974; Hartwell, 1976). It is also similar to the phenotype of cells overexpressing the Cdc28-Clb kinase inhibitor (CKI) Sic1 (Schwob *et al.*, 1994; Dahmann *et al.*, 1995; Noton and Diffley, 2000) and of cells bearing mutations that compromise Cdc28-Clb activity (Schwob *et al.*, 1994), presumably because Cdc28-Clb kinase activity inhibits polarized bud growth induced by Cln-Cdc28 kinase (Lew and Reed, 1993, 1995). This phenotype suggested that Cdc6-d2 was causing a G<sub>2</sub>/M arrest by inhibiting Cdc28-Clb kinase. Cdc6 can interact with and inhibit Cdc28-Clb kinase *in vitro* (Elsasser *et al.*, 1996; Calzada *et al.*, 2000) and *in vivo* (Desdouets *et al.*, 1998; Calzada *et al.*, 2000); thus, it was possible that the



**Fig. 2.** (A) YGP28 (vector), YGP24 (*GAL1,10-CDC6*) or YGP25 (*GAL1,10-CDC6-d2*) cells were grown in YPRaf to mid-log phase and then transferred into either fresh YPRaf or YPGal and allowed to grow for 6 h. Three microlitres from each culture were examined by phase contrast microscopy. (B) YGP28 (lane 3), YGP24 (lane 4) and YGP25 (lane 5) cells were grown in YPRaf to mid-log phase and then blocked in  $G_2/M$  with nocodazole. Once 90% of the cells had a large budded phenotype they were transferred into YPGal also containing nocodazole. After a further 2.5 h, samples were taken and processed for immunoblotting to examine the phosphorylation status of the DNA polymerase  $\alpha$  subunit p86/91. Control samples to visualize the usual pattern of p86/91 phosphorylation in  $G_1$  (lane 1) and  $G_2/M$  (lane 2) cells are also shown.

dominant-negative effect of overexpression of Cdc6-d2 was the result of Cdc28-Clb kinase inhibition. Consistent with this, removal of the N-terminal 47 amino acids of Cdc6-d2, which contain the Cdc28-Clb-interacting domain, eliminates the growth arrest phenotype (data not shown).

The second largest subunit (B subunit) of DNA polymerase  $\alpha$  primase is phosphorylated late in the cell cycle by Cdc28-Clb kinase (Foiani *et al.*, 1995). This phosphorylation causes a shift in mobility in SDS-polyacrylamide gels, and inhibition of Cdc28-Clb kinase in nocodazole-arrested cells is sufficient to cause dephosphorylation of the B subunit (Desdouets *et al.*, 1998). Overexpression of wild-type Cdc6 in nocodazole-arrested cells can drive dephosphorylation of B subunit when SCF<sup>CDC4</sup> is inactive (Desdouets *et al.*, 1998). However, Figure 2B, lane 4, shows that overexpression of wild-type Cdc6 in nocodazole-arrested cells does not lead to dephosphorylation of B subunit when SCF<sup>CDC4</sup> is active. By contrast, overexpression of the mutant Cdc6-d2 leads

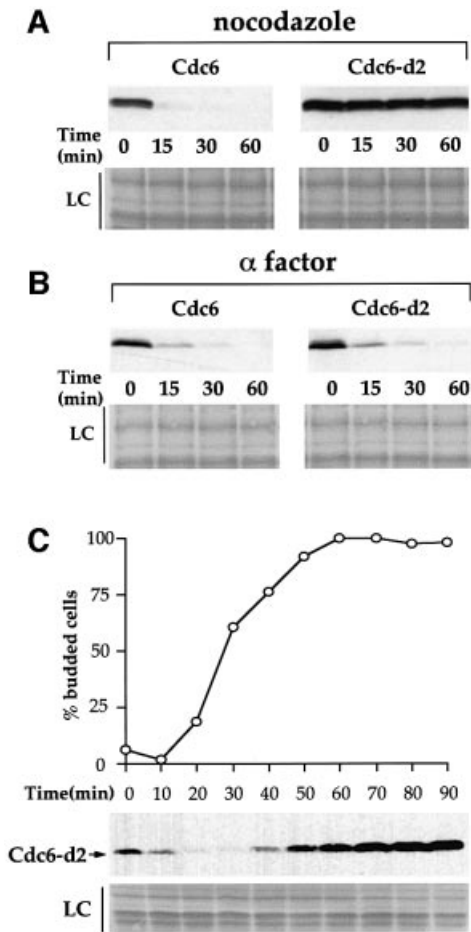
to efficient dephosphorylation of B subunit even when SCF<sup>CDC4</sup> is active (Figure 2B, lane 5).

These results are consistent with the idea that overexpression of Cdc6-d2 blocks cells in  $G_2/M$  by inactivating Cdc28-Clb kinase. CDK inhibition could be due to direct action of Cdc6 on Cdc28. Alternatively, since both Cdc6 (Piatti *et al.*, 1995; Elsasser *et al.*, 1996; Drury *et al.*, 1997) and the CKI Sic1 (Schwob *et al.*, 1994; Knapp *et al.*, 1996; Verma *et al.*, 1997b) are targeted for degradation by SCF<sup>CDC4</sup>, the effects might be indirect. That is, overexpression of the mutant Cdc6 might, in some way, interfere with SCF<sup>CDC4</sup>, causing inappropriate accumulation of Sic1 and consequent cell cycle arrest. Such a mechanism has been shown to explain how overexpression of budding yeast Cdc6 drives re-replication in fission yeast (Wolf *et al.*, 1999a). However, as shown in Figure 1A, overexpression of Cdc6-d2 is still lethal in cells lacking Sic1, thus, the dominant-negative phenotype is not due to stabilization of Sic1.

### **Cdc6-d2 proteolysis does not occur in $G_2/M$ but is normal in $G_1$ and S phases**

The results described above suggest that Cdc6-d2 overexpression prevents entry into mitosis by inactivating Cdc28-Clb kinase. The wild-type Cdc6 protein appears to be less efficient at inhibiting Cdc28-Clb kinase since its overproduction does not block cell proliferation. One possible explanation for this difference might be that the mutant protein is more stable than wild-type Cdc6. This would allow the protein to accumulate to higher levels and, thus, inhibit Cdc28-Clb kinase more effectively. This possibility is consistent with the fact that Cdc6-d2 overexpression in a *CDC4*<sup>+</sup> background results in a phenotype very similar to the overexpression of wild-type Cdc6 in a *cdc4* mutant background (Drury *et al.*, 1997; Sanchez *et al.*, 1999). It is also consistent with the fact that Cdc6-d2 accumulates to higher levels than wild-type Cdc6 when expressed in nocodazole-arrested cells (Figure 2B). To test this hypothesis directly, we examined the rate at which wild-type and mutant proteins disappeared in cells arrested in mitosis. In Figure 3A, cells were first arrested with nocodazole in raffinose-containing medium and Cdc6 or Cdc6-d2 was expressed by switching cells to galactose-containing medium for 30 min. Transcription was then repressed by transferring cells to glucose-containing medium and further translation was blocked with cycloheximide. The disappearance of Cdc6 was examined by immunoblot. This experiment shows that, as described previously, the wild-type protein disappears rapidly in nocodazole-arrested cells; however, the mutant Cdc6-d2 protein is almost completely stable in these cells. This experiment indicates that Cdc6-d2 is resistant to mode 3 proteolysis.

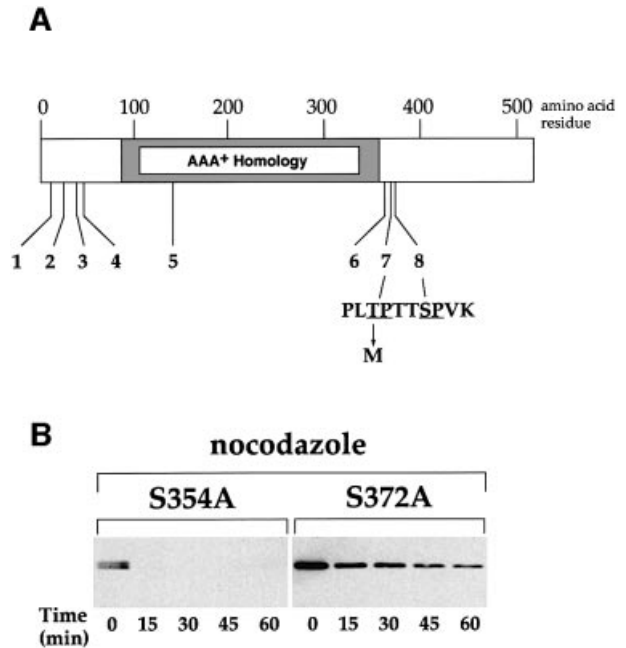
To determine whether this effect on Cdc6 proteolysis is a specific cell cycle effect or some more general defect, we examined the proteolysis of wild-type and mutant Cdc6 proteins at other points in the cell cycle. In  $\alpha$ -factor-arrested cells Cdc6 proteolysis occurs by a pathway (mode 1) that is distinct from mode 3; it is independent of SCF<sup>CDC4</sup> and does not require any of the CDK consensus sites in Cdc6. Figure 3B shows that Cdc6 and Cdc6-d2 are both degraded at very similar rates in  $\alpha$ -factor-arrested cells. Thus, Cdc6-d2 is not defective as a substrate for



**Fig. 3.** YGP24 (*GAL1,10-CDC6*) and YGP25 (*GAL1,10-CDC6-d2*) cells were grown in YPrAf until mid-log phase, the cultures were then split into two and blocked with either nocodazole (A) or  $\alpha$  factor (B). After blocking *GAL1,10-CDC6* or *GAL1,10-CDC6-d2* was induced by the addition of galactose. Thirty minutes later, transcription from the *GAL1,10* promoter and translation were repressed ( $T = 0$ ). Further samples were taken at the times indicated and processed for immunoblotting. LC is the loading control showing a region of Ponceau-S-stained membrane corresponding to 55–66 kDa. (C) YP25 cells were grown in YPrAf and blocked with  $\alpha$  factor to synchronize them. Once the cells were blocked galactose was added to induce expression of *GAL1,10-CDC6-d2*. After 30 min the cells were washed and released from the block into YPrAf/Gal. Samples were taken at the time of release and every subsequent 10 min to be processed for immunoblotting and to assess the budding index.

mode 1 proteolysis while it is completely defective as a substrate for mode 3 proteolysis.

Mode 2 proteolysis during late  $G_1$  phase and S phase is more rapid than mode 3 proteolysis but, like mode 3, requires SCF<sup>CDC4</sup>. We were, therefore, interested in determining whether Cdc6-d2 was generally defective in all SCF<sup>CDC4</sup> proteolysis or if it is specifically defective in mode 3 proteolysis. To test this, cells were arrested in  $G_1$  with  $\alpha$  factor, transferred to galactose-containing medium in  $\alpha$  factor and released from the  $\alpha$ -factor arrest into fresh galactose-containing medium. In this experiment, therefore, Cdc6 is constitutively expressed during and after release from the  $G_1$  arrest. We have shown previously that the very rapid mode 2 proteolysis drastically reduces the steady-state levels of Cdc6 from approximately the time of bud emergence until the end

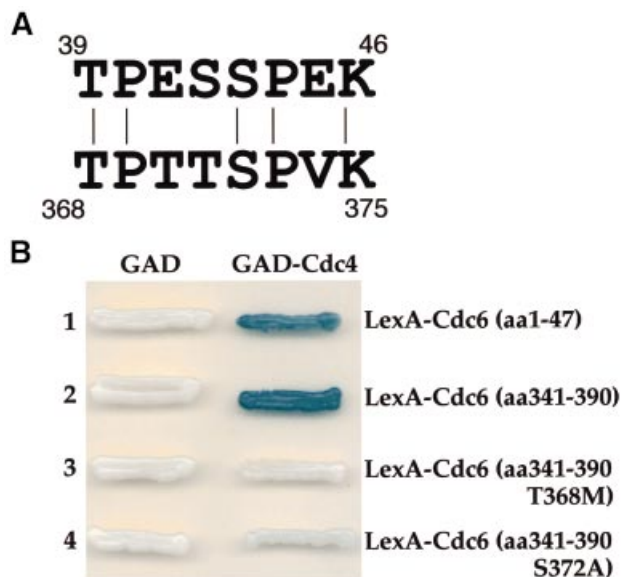


**Fig. 4.** (A) A schematic diagram to show the relative positions of the eight <sup>9</sup>T<sub>P</sub> motifs in Cdc6. The amino acid sequence around sites 7 and 8 is shown and the arrow denotes a T to M substitution at amino acid position 368 caused by the mutation in *CDC6-d2*. Amino acid residue numbers are at the top of the diagram; the region of similarity in various AAA<sup>+</sup> family members is indicated as a grey box (Perkins and Diffley, 1998; Neuwald *et al.*, 1999). (B) *GAL1,10* promoter shut-off experiments with cells blocked in  $G_2/M$  by nocodazole and expressing either *GAL1,10-CDC6-S354A* (phosphorylation site 6) or *GAL1,10-CDC6-S372A* (phosphorylation site 8). Transcription and translation were repressed and a sample was taken ( $T = 0$ ) to be processed for immunoblotting. Subsequent samples were taken at the times indicated.

of S phase in such an experiment (Drury *et al.*, 1997, 2000). Figure 3C shows that Cdc6-d2 mutant protein is also unable to accumulate during this time interval. The reduction in Cdc6-d2 levels during S phase is very similar to that seen with the wild-type protein (Drury *et al.*, 2000; and data not shown) indicating that Cdc6-d2 can still be targeted for proteolysis during S phase. At later time points in this experiment, Cdc6-d2 accumulates to quite high levels, consistent with a defect in degradation later in the cell cycle. Thus, Cdc6-d2 is specifically defective as a substrate for mode 3 proteolysis and is not affected for mode 1 or 2 proteolysis.

#### The mutation in Cdc6-d2 identifies a CDK consensus site required for mode 3 proteolysis

DNA sequence analysis of the *CDC6-d2* allele identified a single point mutation causing a change from C to T at bp 1103, which resulted in a replacement of threonine at amino acid 368 with methionine (Figure 4A). Cdc6 contains eight SP or TP motifs that could potentially serve as CDK phosphorylation sites; the mutation in Cdc6-d2 destroys the seventh of these sites. This site is not a 'complete' consensus site since it does not contain a basic residue at the +3 position (Langan *et al.*, 1989; Shenoy *et al.*, 1989). Cdc6 is a member of a superfamily of ATPases (the AAA<sup>+</sup> family) characterized by an extensive region of homology (Perkins and Diffley, 1998; Neuwald *et al.*, 1999). The mutation in Cdc6-d2 occurs ~30 amino acids C-terminal to this region of similarity. In a 50 amino



**Fig. 5.** (A) Similarity of a Cdc4-interaction domain in the N-terminus of Cdc6 with sequence around the mutation in Cdc6-d2. The numbers denote the amino acid in Cdc6 at the start and finish of the two sequences. (B) Two-hybrid analysis with these two regions of Cdc6.

acid residue region surrounding this mutation, there are six proline and three glycine residues, suggesting that this region may be unstructured. There are two other SP motifs in this region and we were interested in determining whether mutation of these sites also affected the stability of Cdc6. To examine this we used site-directed mutagenesis to construct two alleles of *CDC6*, changing serine 354 to alanine in one case (S354A) and serine 372 to alanine in the other (S372A). Figure 4C shows that Cdc6-S354A was highly unstable in nocodazole-arrested cells, similar to wild-type Cdc6. However, Cdc6-S372A was significantly more stable under these conditions, though less stable than Cdc6-d2 (compare Figure 3A with 4B). Thus CDK sites seven and eight both contribute to proteolysis during mitosis.

#### **Cdc6 contains two separate Cdc4p-interaction domains**

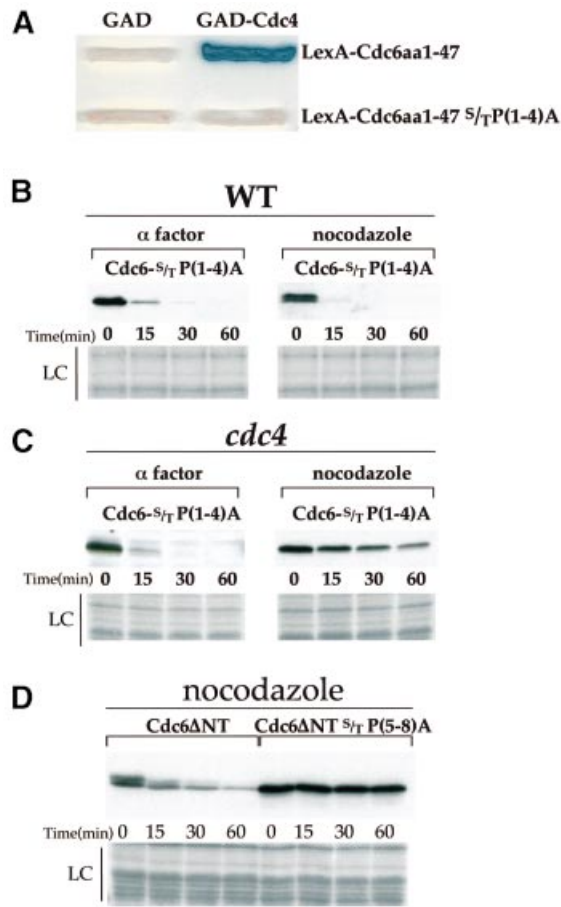
Our previous analysis had suggested that the N-terminal 47 amino acids of Cdc6 played an important role in SCF<sup>CDC4</sup>-dependent proteolysis, thus it was somewhat surprising to find that sequences in the middle of the protein were also involved in proteolysis. We showed previously that the N-terminal 47 amino acids of Cdc6 interacted with Cdc4 in a two-hybrid assay and that the strongest interaction required sequences between amino acids 17 and 47 (Drury *et al.*, 1997). We noticed a short region of sequence similarity between this region and the region encompassing the mutation in Cdc6-d2 (Figure 5A). This sequence contains an incomplete CDK consensus site (without the basic residue at +3) followed by a complete consensus site. We were interested in whether this sequence comprises a hitherto unidentified Cdc4-interaction domain. To address this, we fused a 50 amino acid polypeptide encompassing this sequence to the LexA DNA binding domain and examined its ability to interact with Cdc4 in a two-hybrid experiment. Figure 5B shows

that, like the N-terminal 47 amino acids of Cdc6, amino acids 341–390 interacted strongly with Cdc4, indicating that this region can act as a Cdc4-interaction domain *in vivo*.

Interaction of SCF<sup>CDC4</sup> with substrates such as Sic1 requires phosphorylation at CDK sites (Feldman *et al.*, 1997; Skowrya *et al.*, 1997; Verma *et al.*, 1997a). We considered the possibility that mutation of CDK sites 7 and 8, which causes stabilization of Cdc6 in nocodazole-arrested cells, might affect interaction with Cdc4. To examine this, polypeptides corresponding to amino acid residues 341–390 but containing single amino acid substitutions in the CDK consensus sites were tested for their ability to interact with Cdc4 in the two-hybrid system. Figure 5B shows that mutation of either phosphorylation site dramatically reduced Cdc4 interaction. The T368M mutant (from Cdc6-d2), which in the context of the full-length protein is completely stable in nocodazole-arrested cells (Figure 3A), shows no detectable interaction with Cdc4, while the S372A mutant, which is more stable but not completely stable (Figure 4B), still shows weak interaction. Thus, there is a strong correlation between stability in G<sub>2</sub>/M and ability to interact with Cdc4.

The results described above strongly suggest that Cdc6 contains an additional Cdc4-interaction domain between amino acids 341 and 390, which is critical for mode 3 proteolysis. The existence of a functionally important Cdc4-interaction domain in addition to the N-terminal site is supported by several other experiments. First, Figure 6A shows that a polypeptide containing the N-terminal 47 amino acids of Cdc6 interacts with Cdc4 in the two-hybrid system and, consistent with the importance of CDK phosphorylation, mutation of the four SP/TP motifs in this polypeptide abrogates this interaction. However, as shown in Figure 6B, when these four mutations are engineered into the full-length Cdc6 protein, the resulting protein is as rapidly degraded in nocodazole-arrested cells as it is in  $\alpha$ -factor-arrested cells. As shown in Figure 6C, degradation of this protein in nocodazole-arrested cells is still completely dependent upon Cdc4, arguing strongly that there must be an additional Cdc4-interaction site in the protein. Secondly, we had previously shown that deletion of the N-terminal 47 amino acids of Cdc6 significantly stabilized the protein in nocodazole-arrested cells. We noted, however, that this truncated protein was still degraded, although the degradation was significantly slower (Drury *et al.*, 1997). Furthermore, the degradation of this truncated protein was faster in nocodazole-arrested cells than it was in  $\alpha$ -factor-arrested cells. In these original experiments, we were not adding cycloheximide at the time of transcriptional repression to prevent further translation of stable messages. Using cycloheximide, in Figure 6D we show that this truncated protein is, indeed, degraded in nocodazole-arrested cells although the rate of degradation is considerably slower than the rate for full-length protein (compare Figures 3A and 6D). Moreover, mutation of the remaining four CDK consensus sites in this truncated protein renders it completely stable in nocodazole-arrested cells. Although we do not know why the truncated protein is degraded more slowly than the full-length protein, we note that this truncation also removes a putative nuclear localization signal that resides in the N-terminal 47 amino acids (Jong *et al.*, 1996). Regardless,

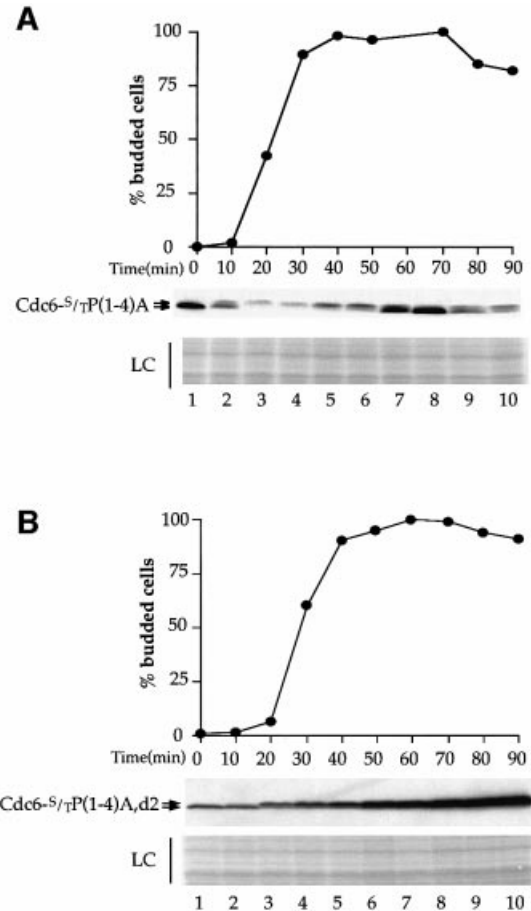




**Fig. 6.** (A) The requirement for the <sup>S/T</sup>P motifs in the N-terminal fragment of Cdc6 was tested in a two-hybrid assay with Cdc4p. The top row shows the two-hybrid interaction between the N-terminus of Cdc6 and Cdc4p described previously. In the second row the N-terminus but with the <sup>S/T</sup>P motifs mutated to A[<sup>S/T</sup>P(1-4)A] does not interact with GAD-Cdc4. (B) Promoter shut-off experiments with Cdc6-<sup>S/T</sup>P(1-4)A in cells blocked in G<sub>1</sub> or G<sub>2</sub>/M show that these motifs are not required for instability in G<sub>1</sub> or G<sub>2</sub>/M. However, this instability is dependent on *CDC4* (C) in G<sub>2</sub>/M since Cdc6-<sup>S/T</sup>P(1-4)A is stable in *cdc4ts* cells blocked in nocodazole. (D) Cdc6ΔNT is more stable than the full-length protein. It is, however, degraded at a slower rate as can be seen above in a promoter shut-off in G<sub>2</sub>/M blocked cells. Mutation of the remaining <sup>S/T</sup>P(5-8)A in this truncated protein stabilizes it.

these experiments, taken together, demonstrate the existence of a second, functionally important Cdc4-interaction domain between amino acids 341 and 390, which contains two critical CDK phosphorylation sites.

Our results thus far show that Cdc6 contains two Cdc4-interaction domains; one is located in the N-terminal 47 amino acids (Cdc4 site 1) and one is located between amino acids 341 and 390 (Cdc4 site 2). We have shown that mutation which abrogates Cdc4 site 2 interaction (i.e. Cdc6-d2) prevents SCF<sup>CDC4</sup>-dependent mode 3 degradation in nocodazole-arrested cells but not SCF<sup>CDC4</sup>-dependent mode 2 degradation during S phase. Conversely, mutations that abrogate Cdc4 site 1 interaction [i.e. Cdc6<sup>S/T</sup>P(1-4)A] do not affect SCF<sup>CDC4</sup>-dependent mode 3 degradation in nocodazole-arrested cells. Thus, we were interested in determining whether the degradation of this mutant during S phase by mode 2 was affected. Figure 7A shows that the levels of this mutant



**Fig. 7.** (A) YLD66 cells grown in YPRaf were synchronized with α factor. Once the cells were blocked, galactose was added to induce the Cdc6-<sup>S/T</sup>P(1-4)A protein. After a further 30 min the cells were released from the block into YPRaf/Gal. Samples were taken at the time of release (*T* = 0) and every 10 min after for a duration of 90 min to assess the budding index and for immunoblotting to detect levels of Cdc6-<sup>S/T</sup>P(1-4)A. (B) A similar experiment was performed with strain YLD69 [*GAL1,10-CDC6-S/T P(1-4)A,d2*].

protein, when constitutively expressed, are significantly reduced during S phase although these levels are not reduced to the same extent as the wild-type protein, suggesting some role for site 1 in mode 2 proteolysis. However, this experiment, together with the experiment in Figure 3C, shows that neither of the two Cdc4-interaction sites is sufficient to account fully for mode 2. Thus, we considered the possibility that either site can target Cdc6 for mode 2 proteolysis. To test this, we examined the proteolysis of a Cdc6 mutant in which both Cdc4-interaction sites are inactivated by mutation of the important CDK consensus sites. To do this, all four CDK sites in the N-terminus, together with CDK site 7, were mutated. Figure 7B shows that, unlike mutants in either site 1 or site 2 alone, this protein is stable during S phase. Thus, these two Cdc4-interaction sites act redundantly during S phase.

## Discussion

The proteolysis of the budding yeast Cdc6 protein is under surprisingly complex regulation. Previously, we distin-

guished two distinct modes of SCF<sup>CDC4</sup>-dependent Cdc6 proteolysis during late G<sub>1</sub>/early S phase and during G<sub>2</sub>/M primarily on the basis of degradation rate. Here, we have extended this distinction by showing that Cdc6 is targeted for SCF<sup>CDC4</sup>-dependent degradation by two different SCF<sup>CDC4</sup>-interaction domains, which are regulated differently during the cell cycle. One of these interaction domains, in the N-terminal 47 amino acids, is only utilized during late G<sub>1</sub> and S phases while the other, located in the middle of the primary amino acid sequence is utilized from late G<sub>1</sub> through mitosis (see Figure 7A). As a consequence, Cdc6 proteolysis during late G<sub>1</sub>/S phase is not dependent upon either domain individually but requires one or the other domain while proteolysis in G<sub>2</sub>/M is entirely dependent upon the second domain.

The mechanism by which these two SCF<sup>CDC4</sup> elements are differentially regulated is unclear. It is possible that SCF<sup>CDC4</sup> itself changes during the cell cycle, either in its subunit composition or by some post-translational modification, so that it is unable to recognize site 1 in G<sub>2</sub>/M. Although we cannot rule out this possibility, there is currently no evidence to support this idea. Alternatively, some additional regulation may act through Cdc6 itself to determine the availability of these interaction sites. The rapid mode 2 proteolysis normally begins near the onset of S phase and our previous work showed that, although it required CDK activity, it did not require the activity of the S-phase-promoting cyclins, Clb5 and Clb6. Moreover, mode 2 Cdc6 proteolysis was not delayed in strains lacking Clb5 and Clb6. This suggested that the G<sub>1</sub> cyclins may play a direct role in mode 2 degradation. In nocodazole-arrested cells, when Cdc6 is degraded via mode 3, the Clns are present at very low levels because mitotic cyclins like Clb2 repress their expression (Amon *et al.*, 1993). Thus, the inactivity of site 1 in nocodazole-arrested cells may indicate that this site is a specific substrate of the Cln kinase and is not phosphorylated by Clb-associated kinases. We think this is unlikely for two reasons. First, the sequences of the two sets of CDK sites are very similar and second, overexpression of Cln2 in nocodazole-arrested cells does not destabilize Cdc6-d2 (L.S.Drury and J.F.X.Diffley, data not shown).

We suggest another possibility. In addition to several CDK consensus sites and a Cdc4-interacting domain, the N-terminal 47 amino acids also contain a region that interacts very tightly with Clb, but not with Cln-associated Cdc28 (Elsasser *et al.*, 1996). The tight association of Cdc28-Clb with this N-terminal region may act to prevent either phosphorylation of the N-terminus or association of the phosphorylated N-terminus with Cdc4. Further work will be required to separate the Clb-Cdc28 binding domain from the Cdc4-interacting domain and test this hypothesis.

The complexity of Cdc6 proteolysis suggests that elimination of the protein from cells is likely to be important. Mode 2 degradation acts to eliminate Cdc6 rapidly from cells before DNA replication begins and, thus, appears to play a role in preventing the relicensing of origins that have already fired. The experiments in this paper, together with the results of Bueno and Russell (1992) suggest an additional reason for removing Cdc6 rapidly from cells: when not degraded, Cdc6 can interfere with progression into mitosis. In the experiments described here, the high Cdc6 levels generated in the

*CDC6-d2* mutant appear to prevent entry into mitosis. It is possible that even when expressed at lower levels, stable Cdc6 might cause some mitotic defect. Cdc4 is required to degrade the CKI Sic1 in G<sub>1</sub> phase to allow cells to enter S phase. Interestingly, Cdc4 is still essential in cells lacking Sic1 (Schwob *et al.*, 1994) and such double mutants (*cdc4<sup>ts</sup>, sic1Δ*) are defective in progressing through mitosis (Goh and Surana, 1999). Perhaps the inability to degrade Cdc6 contributes to this defect.

The ability of stable Cdc6 to prevent mitosis correlates with an apparent reduction in CDK activity *in vivo* as shown by dephosphorylation of DNA polymerase  $\alpha$ . Given that this loss of CDK activity *in vivo* appears to be independent of Sic1 and that Cdc6 can interact with and inhibit Cdc28-Clb kinases *in vivo* and *in vitro*, it is most likely that stable Cdc6 inhibits mitosis by directly inhibiting Cdc28 kinase. The reason for this CKI activity is unclear. The domain responsible for kinase inhibition can be removed from Cdc6 without affecting its ability to assemble pre-RCs and support normal DNA replication (Drury *et al.*, 1997). It is, therefore, likely that CDK inhibition by Cdc6 is, in some way, a redundant activity. It is possible that this activity of Cdc6 acts with other mechanisms to ensure full inhibition of Cdc28 activity at the end of mitosis. It is also possible that the tight interaction of Cdc28 with Cdc6 may inhibit Cdc6 function; however, elimination of this interaction does not lead to over-replication because several redundant mechanisms prevent reassembly of pre-RCs later in the cell cycle (Diffley, 2001).

In both budding and fission yeasts, Cdc6 (*cdc18*) is targeted for degradation by CDK-dependent, SCF<sup>CDC4</sup>-mediated polyubiquitylation. Whether the fission yeast protein has a similar organization of SCF<sup>CDC4</sup>-interaction sites remains to be determined. However, like budding yeast Cdc6, fission yeast *cdc18* normally disappears during S phase and remains unstable in cells arrested in mitosis (Kelly *et al.*, 1993; Kominami and Toda, 1997; Baum *et al.*, 1998; Jallepalli *et al.*, 1998; Lopez-Girona *et al.*, 1998; Wolf *et al.*, 1999b). In vertebrates, Cdc6 proteolysis may be more complex. Chromatin-bound Cdc6 appears to remain stable during S phase (Williams *et al.*, 1997; Coverley *et al.*, 2000) though soluble Cdc6 is degraded at this time (Coverley *et al.*, 2000). Additionally, human Cdc6 appears to be targeted for degradation at the end of mitosis by the APC/C (Mendez and Stillman, 2000; Petersen *et al.*, 2000). Thus, although details may differ, proteolysis at some point during the cell cycle may be a common feature of Cdc6 regulation.

In conclusion, we have shown that Cdc6 proteolysis via SCF<sup>CDC4</sup> occurs through two different Cdc4-interaction sites, which are regulated differently during the cell cycle. This represents a novel way in which the rate of a protein's degradation can be modulated by a single ubiquitylation pathway.

## Materials and methods

### Strains and media

Strains (listed in Table I) were all derived from W303-1a. Cells were routinely grown in YP containing glucose (YPD), raffinose (YPRaf) or galactose (YPGal) at 2% (Rose *et al.*, 1989). Cell cycle blocks with nocodazole or  $\alpha$  factor were as described previously (Diffley *et al.*, 1994).

**Table I.** Yeast strains

Strain	Genotype
YGP24	<i>MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3 112 can1-100, GAL1,10-CDC6, (HIS3)</i>
YGP25	<i>MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3 112 can1-100, GAL1,10-CDC6-d2, (HIS3)</i>
YGP28	<i>MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3 112 can1-100, pRS303 (HIS3)</i>
YGP54	<i>MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3 112 can1-100, GAL1,10-CDC6S372A, (HIS3)</i>
YLD65	<i>MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3 112 can1-100, GAL1,10-CDC6-<sup>S</sup>/<sub>7</sub>P (1-4)A, (HIS3)</i>
YLD66	<i>MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3 112 can1-100, cdc4-1, GAL1,10-CDC6-<sup>S</sup>/<sub>7</sub>P (1-4)A, (HIS3)</i>
YLD67	<i>MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3 112 can1-100, GAL1,10-CDC6 ΔNT (LEU2)</i>
YLD68	<i>MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3 112 can1-100, GAL1,10-CDC6ΔNT<sup>S</sup>/<sub>7</sub>P (5-8)A, (LEU2)</i>
YLD69	<i>MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3 112 can1-100, GAL1,10-CDC6-<sup>S</sup>/<sub>7</sub>P (1-4)A,d2, (HIS3)</i>
L40	<i>MATa his3Δ200 trp1-901 leu2-3 112 ade2 lys2-801am URA3:::(lexAop)<sub>8</sub>-lacZ LYS2:::(lexAop)<sub>4</sub>-HIS3</i>

**Table II.** Oligonucleotide primers

Primer	Sequence 5'-3'
49362	CGCGGATCCGTTGCGTATATCATTCGCATC
67395	AAAAAGGATCCATGCAGTTTGGCTCACAGTCT
49628	AAAAAGGATCCGATGTCAGCTATACCAAT
67393	GTTGCAGTTCACAGATGTTGCACCAGAATCCGCGCCAGAA
124310	AAAAAGAATTCAGTATCGAAATCTATGAGTT
124311	TTTTTGTGCGACCTATATGTAGTTCAAGCCTATTT
95854	CCTTTGACGCCAACTACTGCCCGGTAAGAAATCG
95856	GAAAAGCGGTTTCTGCTGGCGCCAACAACAAGAGGATCATTG
T7A	GTCAGCTATACCAATAGCCCCAACTAAGCGTATC
T23A	CTATTTGACGATGCTCCAGCGGCGCCTCCACGACCTTG
T39A	GTTGCAGTTCACAGATGTTGCACCAGAATCCGCGCCAGAA
S43A	GATGTTACACCAGAATCCGCGCCAGAAAACCTGCAGTTTGG

### Plasmid constructs

The plasmids containing *GAL1,10-CDC6* have been described previously (Drury *et al.*, 1997). To make the plasmid containing the *GAL1,10-CDC6-<sup>S</sup>/<sub>7</sub>P(1-4)A* allele (pLD25), site-directed mutagenesis was performed using a QuikChange™ site-directed mutagenesis kit (Stratagene). The starting template was *GAL1,10-CDC6* in pRS303 and sites were mutated sequentially with the oligonucleotide primers T7A, T23A, T39A and S43A (see Table II). The final product was sequenced to ensure that only the directed mutations were present. The same strategy was used to make the S354A and the S372A alleles using oligonucleotide primers 95856 and 95854, respectively (Table II). To make the *GAL1,10-CDC6-<sup>S</sup>/<sub>7</sub>P(1-4)A,d2* allele the smaller *PstI* fragment was subcloned from pGP26 (pRS303-*GAL1,10-CDC6-d2*) into the larger *PstI* fragment from pLD25 and the orientation was confirmed by sequencing.

The plasmid pGP19 is pRS305 containing the *GAL1,10-CDC6ΔNT* subcloned from pLD4 (Drury *et al.*, 1997). pT54 was made by using the oligonucleotide primers 67395 and 49362 to PCR the *CDC6ΔNT* gene from pLD14 [*GAL1,10-CDC6<sup>S</sup>/<sub>7</sub>P(1-8)A*] so that it contains *<sup>S</sup>/<sub>7</sub>P(5-8)A* (T.Seki, personal communication).

To make the two-hybrid constructs, the oligonucleotide primers 124310 and 124311 were used to PCR a 150 bp fragment from *CDC6*, *CDC6-d2* or *CDC6S372A* and cloned into pBTM116 using the *EcoRI* and *SaI* restriction enzyme recognition sites. Similarly, to analyse the N-terminal Cdc4-interaction domain, the oligonucleotide primers 49628 and 67393 were used to PCR a fragment encoding the first 47 amino acids from *CDC6* from pLD25 and these were subcloned into pBTM116. The pACT vector encoding GAD-Cdc4 was described previously (Drury *et al.*, 1997).

### Cell cycle experiments

Cell cycle synchronization was performed as described previously (Diffley *et al.*, 1994).  $\alpha$ -factor block and release experiments entailed washing the blocked cells twice with 2 vol of fresh medium and returning them to fresh medium at the same cell density.

### *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. Galactose was added to a final

concentration of 2%. The time of induction was generally 30 min. Transcriptional repression was achieved by the addition to the cell suspension of glucose to a final concentration of 2%. At the same time cycloheximide was added to a final concentration of 1 mg/ml to prevent further translation of any mRNA.

### Cell extracts, immunoblotting and loading controls

Protein extracts from trichloroacetic acid-fixed yeast cells were made as described (Desdouets *et al.*, 1998). The samples were boiled in sample buffer for 3 min and 5–10  $\mu$ l was loaded on 10% a SDS gel and submitted to PAGE. For immunoblot analysis the proteins were transferred on to Hybond ECL membrane (Amersham) and blocked with 5% dried milk in Tris-buffered saline containing 0.1% Tween-20. Detection of wild-type Cdc6 and the various mutant Cdc6 proteins was performed using the monoclonal 9H/85 at 5  $\mu$ g/ml and detection of the DNA polymerase  $\alpha$  subunit p86/91 was with the 6D2 antibody (Desdouets *et al.*, 1998). The proteins were detected using ECL reagents (Amersham) as per the manufacturer's instructions. To ensure equal sample loading and even transfer from the polyacrylamide gel to the membrane the blots were stained with Ponceau S (Sigma) and scanned before antibody incubation.

### Two-hybrid analysis

Various pBTM116 and pACT constructs were transformed into the yeast strain L40 (Hollenberg *et al.*, 1995). The transformants were tested for protein-protein interaction by streaking on selective agar plates buffered with phosphate buffer to pH 7.0 and containing 5-bromo-chloroindol-3-yl  $\beta$ -D-galactopyranoside (X-Gal) at a concentration of 40  $\mu$ g/ml.

### FACS analysis

Samples for flow cytometric analysis were collected and processed as described previously (Santocanale and Diffley, 1996). Analysis was performed using a Becton Dickinson FACScan.

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