26. Knoblich, J. A. Mechanisms of asymmetric cell division during animal development. *Curr. Opin. Cell Biol.* **9,** 833–841 (1997).
27. Lu, B., Jan, L. Y. & Jan, Y. N. Asymmetric cell division: lessons from flies and worms. *Curr. Opin. Genet. Dev.* **8,** 392–399 (1998).
28. Yamashita, M., Yoshikuni, M., Hirai, T., Fukada, S. & Nagahama, Y. A monoclonal antibody against the PSTAIR sequence of $p34^{cdc2}$ catalytic subunit of maturation-promoting factor and key regulator of the cell cycle. *Dev. Growth Differ.* **33,** 617–624 (1991).

Acknowledgements

We thank V. Amin and K. Adams for technical assistance; F. Uhlmann and K. Nasmyth for communicating results before publication; and T. Matsumoto, S. Subramani and M. Yanagida for strains and reagents. We also thank V. Buck, L. Johnston, S. Jensen and D. Mulhivill for comments and critical reading of the manuscript, and members of the Hyams and Millar laboratories for their encouragement and support. This work was supported by a Wellcome Trust grant to J.S.H. and by a MRC grant to J.B.A.M.

Correspondence and requests for materials should be addressed to J.B.A.M. (e-mail: jmillar@nimr.mrc.ac.uk).

Cdc6 cooperates with Sic1 and Hct1 to inactivate mitotic cyclin-dependent kinases

Arturo Calzada*, Maria Sacristán*, Elisa Sánchez & Avelino Bueno

Instituto de Microbiología-Bioquímica e Instituto de Biología Molecular y Celular del Cáncer, Departamento de Microbiología y Genética, Campus Miguel de Unamuno, Universidad de Salamanca, CSIC 37007 Salamanca, Spain
** These authors contributed equally to this work*

Exit from mitosis requires the inactivation of mitotic cyclin-dependent kinases (CDKs). In the budding yeast, *Saccharomyces cerevisiae*, inactivation of CDKs during late mitosis involves degradation of B-type cyclins as well as direct inhibition of cyclin–CDK complexes by the CDK-inhibitor protein Sic1 (refs 1–3). Several striking similarities exist between Sic1 and Cdc6, a DNA replication factor essential for the formation of pre-replicative complexes at origins of DNA replication[4–9]. Transcription of both genes is activated during late mitosis by a process dependent on Swi5 (ref. 10). Like Sic1, Cdc6 binds CDK complexes *in vivo*[11,12] and downregulates them *in vitro*[11]. Here we show that Cdc6, like Sic1, also contributes to inactivation of CDKs during late mitosis in *S. cerevisiae*. Deletion of the CDK-interacting domain of Cdc6 does not inhibit the function of origins of DNA replication during S phase, but instead causes a delay in mitotic exit; this delay is accentuated in the absence of Sic1 or of cyclin degradation. By contributing to mitotic exit and inactivation of CDKs, Cdc6 helps to create the conditions that are required for its subsequent role in the formation of pre-replicative complexes at origins of DNA replication.

We first examined whether Cdc6 acts as a CDK inhibitor when overexpressed *in vivo*. We constructed *S. cerevisiae* strains in which expression of *CDC6*, or a mutated version lacking the amino-terminal 47 amino acids (*Δ47cdc6*)[13], were regulated by the galactose-inducible *GAL1-10* promoter. Exponential cultures were grown in the absence of galactose, and cells were then arrested in G2/M with the microtubule-depolymerizing drug nocodazole. Expression of *CDC6* or *Δ47cdc6* was then induced by adding galactose to the medium. Samples were taken at regular intervals and processed for CDK kinase assays. Overexpression of wild-type Cdc6 inhibited the activity of the cyclin B–CDK complex Clb2–Cdc28, whereas the induction of *Δ47cdc6* did not affect Clb2–Cdc28 activity, as expected (Fig. 1a). Cdc6-mediated inactivation was independent of Sic1-mediated Clb2–Cdc28 inhibition (Fig. 1b). Similarly, inhibition of Cdc28 activity by Cdc6 did not require Hct1 (ref. 2), an activator of cyclin B degradation by means of the anaphase promoting complex (APC; Fig. 1c). Together with the *in vitro* ability of Cdc6 to inhibit Cdc28 (ref. 11), these results indicate that Cdc6 can function as a CDK inhibitor, and that this function is exerted through the Cdc28-interacting domain present in the first 47 amino acids of the Cdc6 protein.

Null mutants of *HCT1* (*Δhct1*) are viable and their exit from mitosis is only moderately delayed compared with wild-type cells[2]. This is probably because defects in cyclin proteolysis are compensated by direct CDK inhibition[2]. If Cdc6, together with Sic1, inhibits CDKs as cells exit mitosis it would be expected that *cdc6* mutant cells would show defects in exiting this stage of the cell cycle. We first confirmed that the *Δ47cdc6* strain did not exhibit any defects in DNA replication. Rates of plasmid loss are very similar between *Δ47cdc6* and *Δ47cdc6 Δhct1* strains and appropriate wild-type or *Δhct1* controls (per cent of loss rates, mean ± s.d: wild type, 1.81 ± 0.16; *Δ47cdc6*, 1.51 ± 0.23; *Δhct1*, 2.72 ± 0.31; *Δ47cdc6 Δhct1*, 2.28 ± 0.18. Second, we used two-dimensional gel analyses to show that *Δ47cdc6* cells fire replication origins as efficiently as wild-type cells (as shown for *ARS1* in Supplementary Information Fig. 1).

To study whether *Δ47cdc6* mutant cells were defective in their exit from mitosis, we first used nocodazole block-and-release experiments, because this allowed us to analyse fully all the events from mitosis until entry into G1 phase. In keeping with defects in the exit from mitosis, the decline of cells with mitotic spindles was delayed in *Δ47cdc6* and *Δhct1* cells compared with *CDC6* wild-type cells. Also, Clb2-associated kinase activity and Clb2 protein levels declined slightly more rapidly in the wild-type control than in mutant cells (see Supplementary Information Fig. 2). We then analysed exit from mitosis in cells synchronized in telophase, by arresting a *cdc15* mutant at the restrictive temperature of 37 °C (ref. 14). Thus, exponential cultures of *cdc15-2* and isogenic *cdc15-2 Δ47cdc6* and *cdc15-2 Δhct1* strains were blocked at 37 °C for 4 h. Cells were then released from the block and further incubated at 20 °C; samples were taken at regular intervals and processed for immunofluorescence, analysed by western blot and assayed for kinase activity (Fig. 2). In both *cdc15-2 Δ47cdc6* and *cdc15-2 Δhct1* mutants, the rate of disappearance of elongated spindles was delayed compared with the control (Fig. 2b). Consistent with this delay, on release from the telophase arrest, activity of Clb2-associated H1 kinase declined faster in the *cdc15-2* control than in *cdc15-2 Δ47cdc6* or *cdc15-2 Δhct1* (Fig. 2b). Levels of Clb2 protein declined both in the *cdc15-2* control and in *cdc15-2 Δ47cdc6* mutant cells. In keeping with previous observations[2], Clb2 was stable in the strain with *HCT1* deleted.

We also found that the *Δ47cdc6* mutant is synthetically lethal when combined with mutations in the *CDC23* gene, which encodes a component of the APC[15–17], just as observed previously for *Δsic1* (ref. 2). Several *Δ47cdc6 cdc23-1* haploid strains were selected at 25 °C and found to be inviable above 28 °C, a temperature at which *Δ47cdc6* and *cdc23-1* single mutants grow at a similar rate as the wild type (see Supplementary Information Fig. 3). Synthetic lethality of *Δ47cdc6* with an APC mutant suggests either that the APC is involved in the regulation of Cdc6 protein stability, or that Cdc6 cooperates with the APC in promoting progression through mitosis, in which APC also participates[2,15–17]. As proteolysis of Cdc6 is not regulated by the APC[18], our data, together with the synthetic lethality described for *cdc6-1 cdc14-1* mutants[19], suggest a scheme in which Cdc6 cooperates with Sic1 in CDK inhibition. We also found that *Δ47cdc6* is synthetically lethal at intermediate temperatures when combined with other mutants defective in mitotic exit, such as *cdc14-1*. Several haploid *Δ47cdc6 cdc14-1* strains were selected and found to be inviable at 31 °C (see Supplementary Information Fig. 3). This synthetic lethality was comparable to the genetic interactions observed in *cdc14-1 Δsic1* and *cdc14-1 Δhct1* double mutants[19,20]. *Δ47cdc6 cdc14-1* double mutants arrested at 31 °C as

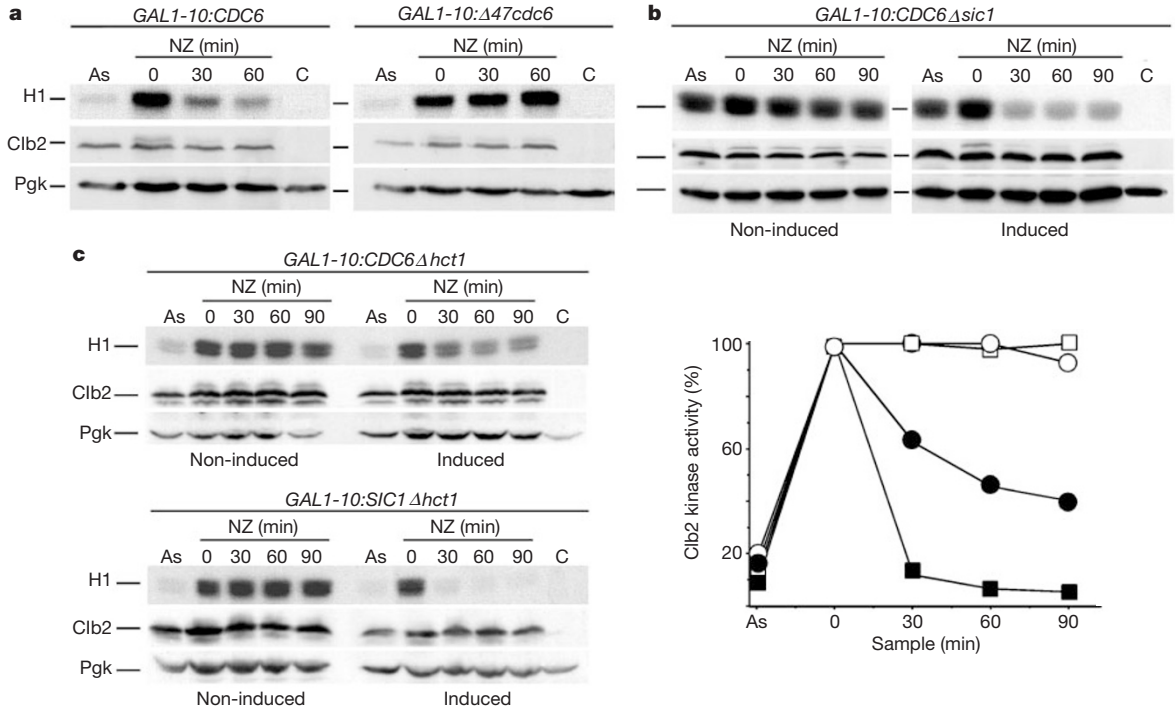

Figure 1 Cdc6 promotes Clb2-associated kinase inhibition by a mechanism independent of Clb2 degradation. Cultures of congenic wild type (**a**), *Δsic1* (**b**) and *Δhct1* (**c**) cells expressing *CDC6*, *Δ47cdc6* or *SIC1* under the control of the galactose-inducible promoter *GAL1-10* were arrested in mitosis with nocodazole (NZ) before the addition of galactose. Samples were taken at regular intervals and processed for Clb2-associated H1 kinase activity assays and western analysis (Pgk as a loading control). Asynchronous (As) and untagged Clb2 (C) controls are shown for reference. In **c**, circles indicate *GAL1-10:CDC6*, squares *GAL1-10:SIC1*, open symbols non-induced and closed symbols induced samples.

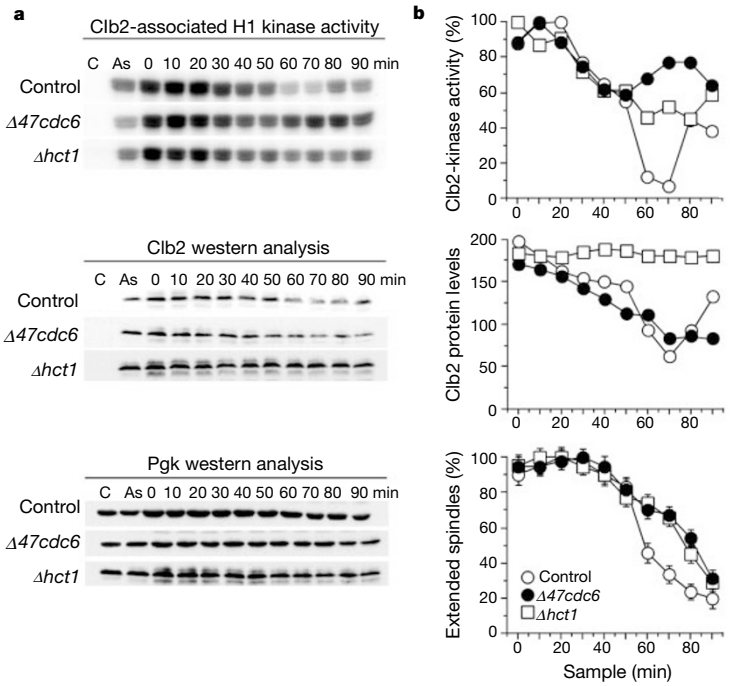

Figure 2 Exit from mitosis is delayed in *Δ47cdc6* mutant cells compared with *CDC6* wild-type cells. Cultures of *cdc15-2* (control), *cdc15-2 Δ47cdc6* (*Δ47cdc6*) and *cdc15-2 Δhct1* (*Δhct1*) strains were synchronized in telophase by incubating them at 37 °C for 4 h. Cultures were then released at 20 °C and samples taken at indicated intervals and processed for Clb2-associated H1 kinase activity assays (**a**, top panels), Clb2 and Pgk western analysis (**a**, middle and lower panels) and tubulin staining (**b**, lower plot) to measure the exit from mitosis. Plots of the relative H1 kinase activity and Clb2 protein abundance (normalized to Pgk levels) are shown in **b**.

single, elongated budded cells with two distinct nuclei (not shown), similar to *cdc14-1* mutant cells at 37 °C (ref. 21) and consistent with a block late in mitosis. These genetic interactions suggest that the Cdc28-interaction domain at *CDC6* participates in mitotic exit.

If Cdc6 cooperates with Hct1 and Sic1 to promote mitotic exit, the role of Cdc6 in this process might be easier to observe in cells lacking Hct1 or Sic1. We also characterized, therefore, the phenotype of the double mutant *Δ47cdc6 Δhct1*. These mutant cells, although viable, were larger (Fig. 3b) and grew slower than wild-type or single-mutant controls, showing a significant lag in late mitosis (Fig. 3). Consistent with this lag, asynchronously growing cells showed a marked increase in 2C DNA cell contents as compared with wild-type cells (Fig. 3a) and had approximately 50% of the levels of Clb2–Cdc28 kinase activity to nocodazole-arrested cells (Fig. 3d). Furthermore, tubulin staining revealed that 80.1 ± 2.71% (mean ± s.d.) of these cells had an extended mitotic spindle reminiscent of defects late in mitosis (Fig. 3c), a percentage significantly higher than those observed in wild-type (14.1 ± 0.76%), *Δ47cdc6* (16.3 ± 0.97%) and *Δhct1* (26.5 ± 1.32%) controls (this defect is rescued by *SIC1*; see Supplementary Information Fig. 4). A telophase block-and-release experiment was then performed to compare the Clb2-associated kinase activity, and also to measure the extended spindle index in *cdc15-2 Δhct1* and *cdc15-2 Δ47cdc6 Δhct1* strains (Fig. 3e). After a 4-h block at 37 °C, cells were released and further incubated at 25 °C, taking samples at regular intervals. Consistent with a role for Cdc6 in the exit from mitosis, in *cdc15-2 Δ47cdc6 Δhct1* cells both the rate of disappearance of telophase spindles and the drop in Clb2–Cdc28 kinase activity was significantly delayed compared with the *cdc15-2 Δhct1* control (Fig. 3e).

In a similar manner, we examined the effects of combining *Δ47cdc6* with deletion of the *SIC1* gene. We found that the double mutant *Δ47cdc6 Δsic1* is inviable at all temperatures. To study the double-mutant phenotype, a conditional *Δ47cdc6 Δsic1 GAL1-10:cdc6-1* haploid strain was constructed. This strain grew in galactose-based medium at 25 °C. Nevertheless, repression of the *GAL1-10* promoter at 37 °C induced the appearance of large budded cells with replicated DNA, two nuclei and extended mitotic spindles (Fig. 4), consistent with a defect in late anaphase. This result provides further evidence supporting a role for Cdc6 in the exit from mitosis, in combination with Sic1.

CDC6 is essential in the formation of pre-replicative complexes at origins of DNA replication[5,22]. It is known that the periodic transcription of the *CDC6* gene in rapidly proliferating cells starts late in mitosis[23,24] and this is correlated with the appearance of the Cdc6 initiation protein[7]. We have discovered a major reason explaining why Cdc6 is synthesized at this stage of the cell cycle: namely, that it contributes to the inactivation of CDK at the end of mitosis, cooperating with Sic1 in the direct inhibition of cyclin B–CDK complexes (Fig. 4c).

Our findings show that in budding yeast Cdc6 has a dual role, in both chromosome replication and in mitosis, and it will be of

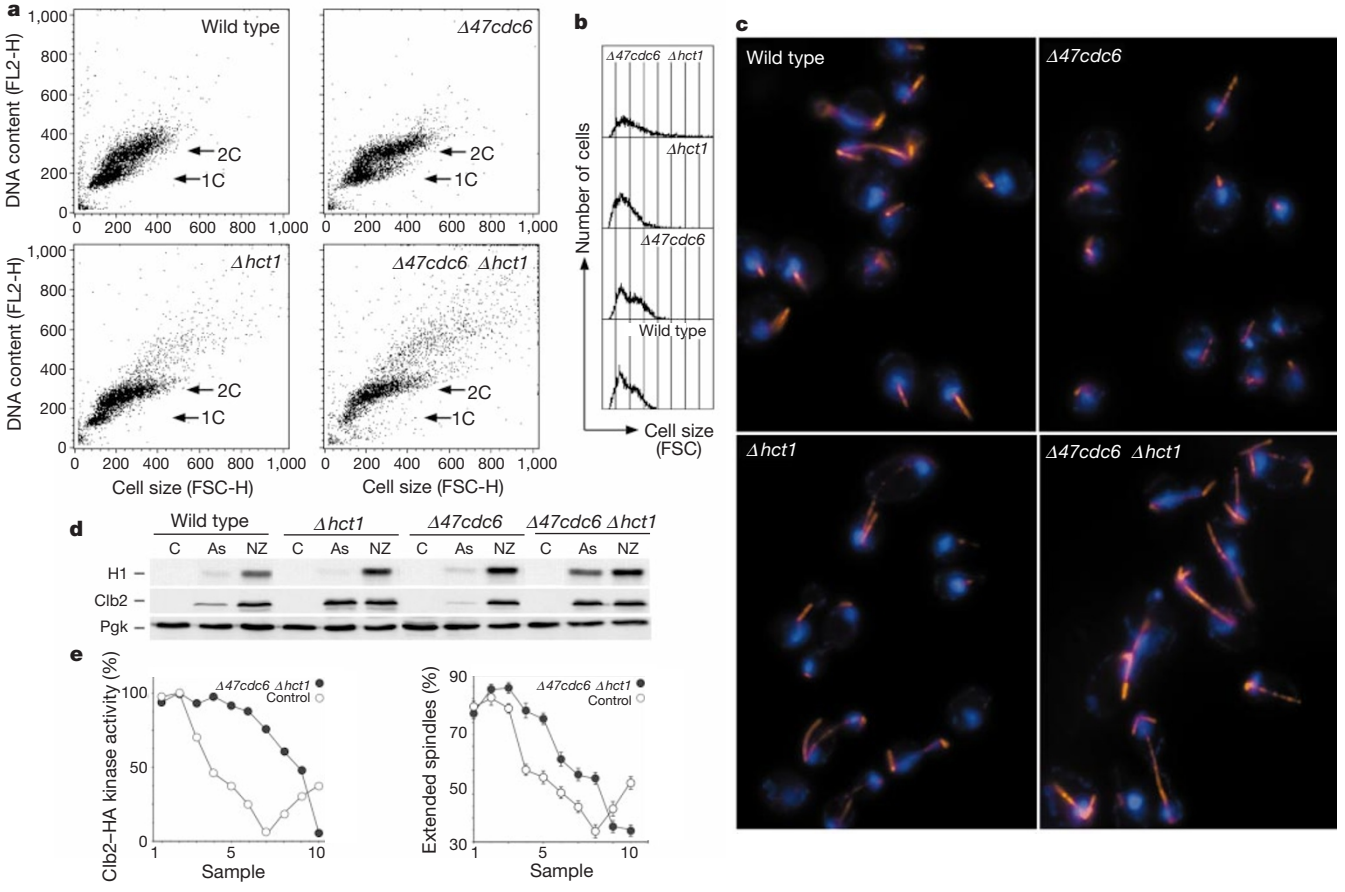

Figure 3 The 47 amino-terminal truncation in *CDC6* confers a strong mitotic delay to *Δhct1* cells. Dot plots (**a**) of DNA content versus cell size and histograms (**b**) of cell size from samples of asynchronous cultures (28 °C) of wild-type, *Δ47cdc6*, *Δhct1* and *Δhct1* Δ47cdc6 isogenic strains. **c**, Micrographs of these cells co-stained with anti-tubulin and DAPI. **d**, Immunoprecipitated Clb2-associated H1 kinase activity of the same cultures (As) as compared to nocodazole-arrested cells (NZ) and an untagged Clb2 control (C). Clb2 and Pgk western blots are shown. **e**, A telophase block-and-release experiment of *cdc15-2 Δhct1* (control) and *cdc15-2 Δhct1 Δ47cdc6* strains. Plots of Clb2–HA kinase activity and extended spindles are shown. Samples are telophase-arrested cells (1), or taken every 10 min after the release (2–10).

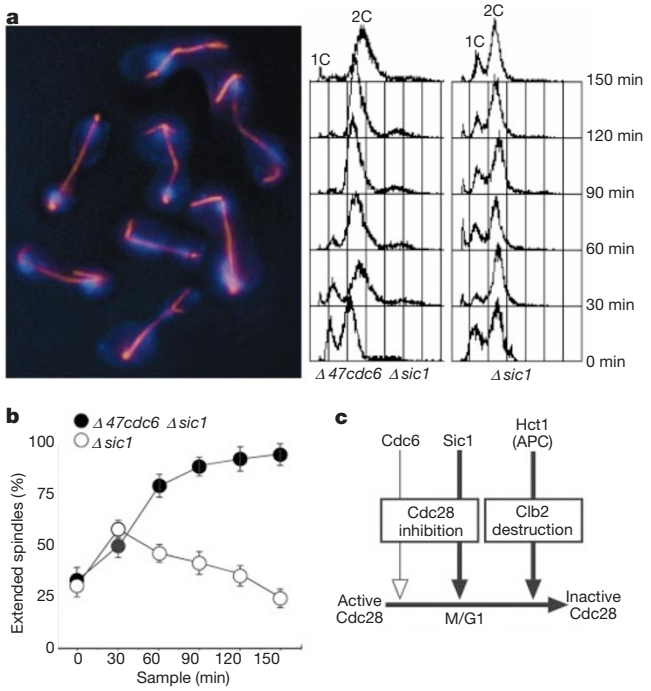

Figure 4 *Δsic1 Δ47cdc6* double-mutant cells are defective in the exit from mitosis. Asynchronously growing cultures of *Δ47cdc6 Δsic1 GAL1-10:cdc6-1* and *Δsic1 GAL1-10:cdc6-1* strains were repressed and shifted to 37 °C to analyse DNA content (**a**) and extended spindle morphology (**b**) during a time-course experiment. *Δ47cdc6 Δsic1* cells from the last time point (**a**) were co-stained with anti-tubulin antibodies and DAPI to show spindle and nuclear morphology, respectively. **c**, A model for the relative importance of Cdc6, Sic1 and Hct1 in CDK inactivation at the exit from mitosis.

considerable interest to see whether the same is true for homologues of Cdc6 in other species of eukaryote. □

Methods

Strains and cell cycle control

All strains used in this work were backcrossed three times with a 15Dau strain[12]. All of them carried a *CLB2HA* allele[2]. They were constructed by tetrad analysis, checking appropriate segregation of markers after each cross genetically, by Southern blot and/or polymerase chain reaction (PCR) and segregation of *CLB2HA* by western blot. To construct the *Δ47cdc6* strain that carries this allele as the only copy of the *CDC6* allele, two PCRs were performed. We generated a fragment with 596 base pairs (bp) (flanked by *Eco*RI–*Nde*I sites) containing the 5′-flanking sequence upstream from the *CDC6* ATG (oligonucleotides 5′-TAGAATTCGAGGCTTTCTCGAGG-3′ and 3′-CATGGTATATGC AAGGTATACAT-5′) and a 633-bp fragment (flanked by *Nde*I and *Xba*I sites) from the 145–778 bp of the *CDC6* open reading frame (oligonucleotides 5′-TACATATGTTTGGC TCACAGTCT-3′ and 3′-CCATACCGATTATCAGATCTATAC-5′). The two fragments were ligated using the *Nde*I sites and cloned into a pGEM-T vector. The internal *Mlu*I–*Xba*I fragment was fully sequenced and used to replace the wild-type *Mlu*I–*Xba*I fragment contained in the genomic *Bam*HI–*Hin*dIII 5.5-kilobase (kb) fragment. A *URA3* gene was introduced as marker into the *CDC6* downstream from the *Eco*RI site and the resulting *Bam*HI–*Hin*dIII fragment was used to transform 15Dau cells. The resulting genomic constructions were tested by PCR and sequencing.

Yeast strains were grown in rich YEP medium (1% yeast extract, 2% peptone) containing 2% glucose. For block-and-release experiments, cells were grown in YEP with 2% glucose at 28 °C and synchronized by adding 15 μg ml^{-1} nocodazole for 150 min, except where indicated. Cells were collected by centrifugation and released in nocodazole-free medium for 90 min at 28 °C. Overexpression experiments were done with cells growing in YEP medium with 2% raffinose at 32 °C; after 120 min of nocodazole blocking, 2.5% galactose was added to induce (or 2% glucose to repress) expression from the *GAL1* promoter. The cell cycle position was monitored by standard FACS analysis. Analysis of plasmid loss rate was performed as described[25].

Kinase analysis and western blot

Cell extracts were prepared as described[12] and protein concentrations were determined using BCA Protein Assay Kit (Pierce). Immunoprecipitation and immunoblotting were carried out with anti-haemagglutinin (HA) (12CA5, Roche) and anti-Pgk (mouse monoclonal, Molecular Probes) antibodies. Kinase assays were performed with H1 histone (Calbiochem) as substrate on immunoprecipitates at 25 °C as described[12], and quantified with a phosphorimager. H1 histone signals were normalized to the nocodazole-arrest level.

Microscopy

To evaluate exit from mitosis, elongated spindles were counted and plotted. Staining of microtubules was performed as described[26] using anti-tubulin antibody TAT-1, and DNA was visualized after staining with 4,6-diamidino-2-phenylindole (DAPI). Fluorescence images were collected using a Leica Q550CW microscope with a ×63 objective and a Sensys CCD (charge-coupled device) camera (Photometrics). More that 300 cells were examined for each time point, and the experiment was repeated three times, to gain an estimate of error.

Received 7 February; accepted 18 June 2001.

1. Donovan, J. D., Toyn, J. H., Johnson, A. L. & Johnston, L. H. p40SDB25, a putative CDK inhibitor, has a role in the M/G1 transition in *Saccharomyces cerevisiae*. *Genes Dev.* **8,** 1640–1653 (1994).
2. Schwab, M., Schulze-Lutum, A. & Seufert, W. Yeast Hct1 is a regulator of Clb2 cyclin proteolysis. *Cell* **90,** 683–693 (1997).
3. Visintin, R., Prinz, S. & Amon, A. CDC20 and CDH1: a family of substrate-specific activators of APC-dependent proteolysis. *Science* **17,** 460–463 (1997).
4. Liang, C., Weinreich, M. & Stillman, B. ORC and Cdc6p interact and determine the frequency of initiation of DNA replication in the genome. *Cell* **81,** 667–676 (1995).
5. Cocker, J. H., Piatti, S., Santocanale, C., Nasmyth, K. & Diffley, J. F. X. An essential role for the Cdc6p protein in budding yeast pre-replicative complexes. *Nature* **379,** 180–182 (1996).
6. Aparicio, O. M., Weinstein, D. M. & Bell, S. P. Components and dynamics of DNA replication complexes in *S. cerevisiae*: redistribution of MCM proteins and Cdc45p during S phase. *Cell* **91,** 59–69 (1997).
7. Detweiler, C. S. & Li, J. Cdc6p establishes and maintains a state of replication competence during G1 phase. *J. Cell Sci.* **110,** 753–763 (1997).
8. Donovan, S., Harwood, J., Drury, L. S. & Diffley, J. F. Cdc6p-dependent loading of Mcm proteins onto pre-replicative chromatin in budding yeast. *Proc. Natl Acad. Sci. USA* **94,** 5611–5616 (1997).
9. Tanaka, T., Knapp, D. & Nasmyth, K. Loading of an Mcm protein onto DNA replication origins is regulated by Cdc6p and CDKs. *Cell* **90,** 649–660 (1997).
10. Spellman, P. T. *et al.* Comprehensive identification of cell cycle-regulated genes of the yeast *Saccharomyces cerevisiae* by microarray hybridization. *Mol. Biol. Cell* **9,** 3273–3297 (1998).
11. Elsasser, S., Lou, F., Wang, B., Campbell, J. L. & Jong, A. Interaction between yeast Cdc6 protein and B-type cyclin/Cdc28 kinases. *Mol. Biol. Cell* **7,** 1723–1735 (1996).
12. Calzada, A., Sánchez, M., Sánchez, E. & Bueno, A. The stability of the Cdc6 protein is regulated by CDK/cyclin B complexes in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **275,** 9734–9741 (2000).
13. Drury, L. S., Perkins, G. & Diffley, J. F. X. The Cdc4/34/53 pathway targets Cdc6p for proteolysis in budding yeast. *EMBO J.* **16,** 5966–5976 (1997).
14. Yeong, F. M., Lim, H. H., Padmashree, C. G. & Surana, U. Exit from mitosis in budding yeast: biphasic inactivation of the Cdc28-Clb2 mitotic kinase and the role of Cdc20. *Mol. Cell* **5,** 501–511 (2000).
15. Irniger, S., Piatti, S., Michaelis, C. & Nasmyth, K. Genes involved in sister chromatid separation are needed for B-type cyclin proteolysis in budding yeast. *Cell* **81,** 269–278 (1995).
16. King, R. W. *et al.* 20S complex containing *CDC27* and *CDC16* catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. *Cell* **81,** 279–288 (1995).
17. Peters, J. M. Subunits and substrates of the anaphase-promoting complex. *Exp. Cell Res.* **248,** 339–349 (1999).
18. Pichler, S., Piatti, S. & Nasmyth, K. Is the yeast Anaphase Promoting Complex needed to prevent re-replication during G2 and M phases? *EMBO J.* **16,** 5988–5997 (1997).
19. Yuste-Rojas, M. & Cross, F. R. Mutations in *CDC14* result in high sensitivity to cyclin gene dosage in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **263,** 60–72 (2000).
20. Visintin, R. *et al.* The phosphatase Cdc14 triggers mitotic exit by reversal of Cdk-dependent phosphorylation. *Mol. Cell* **2,** 709–718 (1998).
21. Culotti, J. & Hartwell, L. H. Genetic control of the cell division cycle in yeast III. Seven genes controlling nuclear division. *Exp. Cell Res.* **67,** 389–401 (1971).
22. Mizushima, T., Takahashi, N. & Stillman, B. Cdc6p modulates the structure and DNA binding activity of the origin recognition complex *in vitro*. *Genes Dev.* **14,** 1631–1641 (2000).
23. Zwerschke, W., Rottjakob, H.-W. & Kuntzel, H. The *Saccharomyces cerevisiae CDC6* gene is transcribed at late mitosis and encodes an ATP/GTPase controlling S phase initiation. *J. Biol. Chem.* **269,** 23352–23356 (1994).
24. Piatti, S., Lengauer, C. & Nasmyth, K. Cdc6 is an unstable protein whose de novo synthesis in G1 is important for the onset of S phase and for preventing a 'reductional' anaphase in the budding yeast *Saccharomyces cerevisiae*. *EMBO J.* **14,** 3788–3799 (1995).
25. Hogan, E. & Koshland, D. Addition of extra origins of replication to a minichromosome suppressed its mitotic loss in *cdc6* and *cdc14* mutants of *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA* **89,** 3098–3102 (1992).
26. Bähler, J. & Pringle, J. R. Pom1p, a fission yeast protein kinase that provides positional information for both polarized growth and cytokinesis. *Genes Dev.* **12,** 1356–1370 (1998).

Supplementary information is available from *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

Acknowledgements

We thank J. Correa, K. Labib, S. Moreno, L. Pelloquin and the ASP laboratory for many discussions, and K. Labib for critically reading this manuscript. We are grateful to R. Basco, J. Correa, J. Diffley, S. Ufano and C. Vázquez for yeast strains and anti-Pgk antibody. We also thank N. Skinner for editing the text. This research was supported by CICYT and PGC grants to A.B.

Correspondence and requests for materials should be addressed to A.B. (e-mail: abn@gugu.usal.es).