

# Binding of cyclin-dependent kinases to ORC and Cdc6p regulates the chromosome replication cycle

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**Cdc6p and the origin recognition complex (ORC) are essential for assembly of a pre-replicative complex (preRC) at origins of replication, before the initiation of DNA synthesis. In the absence of Cdc6p, cells fail to initiate DNA replication and undergo a "reductional" mitosis, in which the unreplicated chromosomes are randomly segregated to the spindle poles. We show here that the cells harboring a mutation in the essential Cdc6p Walker A-box arrest in late mitosis, probably at anaphase. This cell cycle block requires either the three Cdc28p phosphorylation sites within the N terminus of Cdc6p or a short region (aa 8–17) that contains a Cy (Cyclin) interaction sequence. These same two Cdc6p mutants that allow a reductional mitosis are defective in binding Cdc28p kinase. In addition to Cdc6p, ORC also binds to cyclin-dependent kinases (CDKs). Interestingly, Sic1p, a CDK inhibitor protein, blocked the S phase-specific Cdc28p-Clb5p kinase from interacting with ORC, but did not prevent the G<sub>1</sub>-specific Cdc28p-Cln2p kinase-ORC interaction. We suggest that ORC, Cdc6p, and Sic1p bind to different CDKs in a cell cycle-dependent manner to temporally regulate events that (i) allow preRC formation after mitosis, (ii) prevent mitosis before DNA replication can occur, and (iii) promote initiation of DNA replication.**

The initiation of DNA replication in *Saccharomyces cerevisiae* and other eukaryotes is a cell cycle-regulated process requiring the assembly of a pre-replicative complex (preRC) at origins of DNA replication during G<sub>1</sub> phase (1–4). The preRC contains several conserved initiation proteins and assembles before DNA synthesis (4). PreRC proteins include the origin recognition complex (ORC) that binds to and marks replication origins throughout the entire cell cycle, the Cdc6 protein (Cdc18 in *Schizosaccharomyces pombe*; ref. 5), the minichromosome maintenance (MCM) helicase, and Cdt1 (6). Cdc6p is an unstable protein that interacts with ORC and promotes loading of the six-subunit MCM complex to origins in an ATP-dependent manner (7–15). Although not yet identified in budding yeast, Cdt1 is also required for MCM binding to chromatin (6). The preRC renders chromosomes competent for initiation of DNA replication, but initiation does not occur because cells have not yet committed to S phase. Both cyclin-dependent protein kinases (CDKs; Cdc28p-Clb5p and -Clb6p) and Cdc7p-Dbf4p are required for S phase commitment by activating origin firing and promoting loading of Cdc45p (16–29).

Yeast Cdc28p interacts with two types of cyclins, the Cln and Clb cyclins, to form an active CDK (30). The G<sub>1</sub> cyclins, Cln1p and Cln2p, control bud formation, spindle pole body duplication, and one step in the initiation of DNA replication. The Cln-CDKs promote DNA replication by allowing expression and activation of the other type of CDK-cyclin, the B-type CDKs containing Clb5p or Clb6p cyclins (31, 32). In late G<sub>1</sub>, the Clns promote degradation of the B-type CDK inhibitor, Sic1p, and the initiation protein Cdc6p (33–35). There are six *CLB* genes (*CLB1-6*) in budding yeast. Clb5p- and Clb6p-dependent kinases promote origin activation in a temporally controlled manner (36) and activation of origins by loading Cdc45p (23), whereas Clb1–4p kinases act later in the cell cycle, during G<sub>2</sub> and M phase.

DNA replication is controlled by CDKs in at least two ways. First, preRC assembly can only occur when CDKs are inactive, thereby restricting preRC assembly to the period from mitotic exit to late G<sub>1</sub> (37–42). During this period, CDKs are kept inactive by a functioning anaphase-promoting complex (APC/cyclosome) and the presence of the CDK inhibitor Sic1p. The APC/cyclosome containing the adaptor Cdc20p promotes exit from mitosis by ubiquitinating the anaphase inhibitor Pds1 and Clb cyclins, such as Clb5p (43). The Cdc28p inhibitor Sic1p (p40) inhibits the kinase activity of any Cdc28p-Clb cyclin until late G<sub>1</sub> (44). Later in the cell cycle, when CDK activity is high during S, G<sub>2</sub>, and early mitosis, assembly of the preRC cannot occur. However, CDKs provide a second function because they are required for origin activation in late G<sub>1</sub> (37–39, 45). CDK regulation of preRC assembly includes control of cell localization of MCM proteins and stability or availability of initiation regulatory proteins such as Sic1p and Cdc6p (34, 40–42). After inhibition of APC and destruction of Sic1p, CDKs are activated and promote loading of Cdc45p and other proteins at each origin in a temporally regulated manner (23, 24, 46).

In addition to regulating the assembly of the preRC, cells also have checkpoint mechanisms to coordinate progression from one phase of the cell cycle to the next (47–50). Checkpoints act to inhibit subsequent cell cycle events if there is a delay or error in a preceding stage. For instance, the S phase checkpoint acts to inhibit the metaphase to anaphase transition in budding yeast when DNA damage or incomplete DNA replication occurs during S phase. DNA damage or incomplete DNA replication is sensed and results in the activation of the Mec1p and Rad53p kinases that mediate repair and cell cycle arrest. Johnston and colleagues (51) have also described a G<sub>1</sub>–M phase checkpoint that operates in budding yeast to prevent mitosis in the absence of the initiation of DNA replication. This less well characterized pathway is independent of the Mec1p checkpoint kinase that mediates the inhibition of mitosis in response to incomplete DNA replication or damage. Furthermore, not all yeast strains have retained this checkpoint mechanism. The G<sub>1</sub>–M checkpoint is dominant in heterozygous diploids, suggesting that some standard yeast strains have mutations in this pathway.

Depletion of Cdc6p prevents replication initiation and results in a reductional mitosis in which cells randomly segregate their unreplicated chromosomes (8). Under these circumstances, the G<sub>1</sub>–M checkpoint is not operating. Paradoxically, we described a nonfunctional *cdc6* mutant (in the same strain background)

Abbreviations: ORC, origin recognition complex; preRC, pre-replicative complex; MCM, minichromosome maintenance; FACS, fluorescence-activated cell sorter; CDK, cyclin-dependent kinase; APC, anaphase-promoting complex; Cy, cyclin; IP, immunoprecipitate.

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that prevented mitosis in the absence of DNA replication (12). Cdc6p is predicted to be an ATP binding protein and a mutant that changed a conserved lysine to glutamate (K114E) in the Walker A box was not able to initiate DNA replication or load the MCM proteins at the origin. So, in contrast to depletion of Cdc6p, cells expressing *cdc6(K114E)* (hereafter referred to as *cdc6<sup>KE</sup>*) did not pass through mitosis, suggesting that the inhibition of mitosis required Cdc6p itself. The inhibitory activity was mapped to the N-terminal 50 aa of Cdc6p, a region that is dispensable for its essential role in initiation (12).

Cdc18, the fission yeast homologue of Cdc6p, is required for the initiation of DNA replication (5) and also inhibits passage through mitosis when the protein is overproduced (52, 53). Like Cdc6p, Cdc18 is an unstable protein. Cdc18 contains six consensus CDK phosphorylation sites (five within the N terminus) that mediate its turnover, because mutation of these sites increases Cdc18p stability (54). Inhibition of B-type CDK activity also increased the stability of Cdc18. Like Cdc6p, which binds Cdc28 cyclin (55), Cdc18 interacts through its N terminus with the CDK subunit Cdc2 associated with the B-type cyclins (Cdc13 and Cig2; ref. 56). Increasing Cdc18 levels through overproduction leads to repeated rounds by DNA synthesis by promoting replication re-initiation. This may reflect an inhibition of mitotic CDK activity through direct binding and by promotion of new initiation events when Cdc18 is greatly overproduced.

Here we show that the ability to inhibit mitosis is abrogated by one of two separate mutations within the N terminus of Cdc6p. The first changes the three Cdc28 consensus phosphorylation sites at T7, T23, and S43 to alanine (TTS). The second, by deleting a 10-aa region ( $\Delta 8-17$ ) containing a match to the Cy (Cyclin) motif. The Cy motif is found in other eukaryotic proteins and mediates binding to CDK-cyclins (57–60). The ability of Cdc6p to inhibit mitosis is also correlated with binding to Cdc28p. A genetic analysis indicates that increasing B-type CDK activity can overcome the mitotic arrest of the *cdc6<sup>KE</sup>* mutant. The inhibition of mitosis is independent of *RAD53*, indicating that it is independent of the G<sub>1</sub>-S or S phase checkpoint pathways. These results suggest that the N terminus of Cdc6p has a function during mitosis and might be involved in the G<sub>1</sub>-M checkpoint pathway. We also suggest that Cdc6p, together with Sic1p and the APC/cyclosome, antagonizes CDK activity in late mitosis to early G<sub>1</sub>, perhaps directly at origins of replication. Finally, we demonstrate that Sic1p, in addition to regulating CDK activity, controls substrate accessibility.

## Materials and Methods

**Strains and Plasmids.** Genetic manipulation and transformation of yeast was done by standard techniques (61, 62). All *S. cerevisiae* strains were derivatives of W303 (63). Mutations in *CDC6* were introduced by using site-directed mutagenesis of single-stranded uracil-containing phagemid DNA. After sequencing a restriction fragment containing the mutation, the mutations were transferred to pMW71 (pRS415-*CDC6*) for testing complementation and into pMW333 (pRS405-*cdc6<sup>KE</sup>*) for integration into the *LEU2* locus as described (12). The plasmids pWS945 (pGAL-*CLB5*) and yCpG2 (pGAL-*CLN1*) were obtained from B. Futcher (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). pJA92 (*RAD53 URA3*) was from S. Elledge (Baylor College of Medicine, Houston). pMW479 (pGAL-*CDC4*) was constructed by cloning a *Bam*HI-*Sac*I PCR fragment using genomic DNA as template into the pGAL expression plasmid pVF1. *CDC4* was entirely sequenced to verify that it was wild type. Galactose-dependent induction of Cdc4p was determined by Western blot using rabbit polyclonal antibodies against Cdc4p (from M. Goebel, Indiana University School of Medicine, Bloomington, IN).

**Immunoprecipitation of HA-CDC6 and Associated Proteins.** Baculovirus transfer vectors were constructed in pAcSG2 (PharMin-

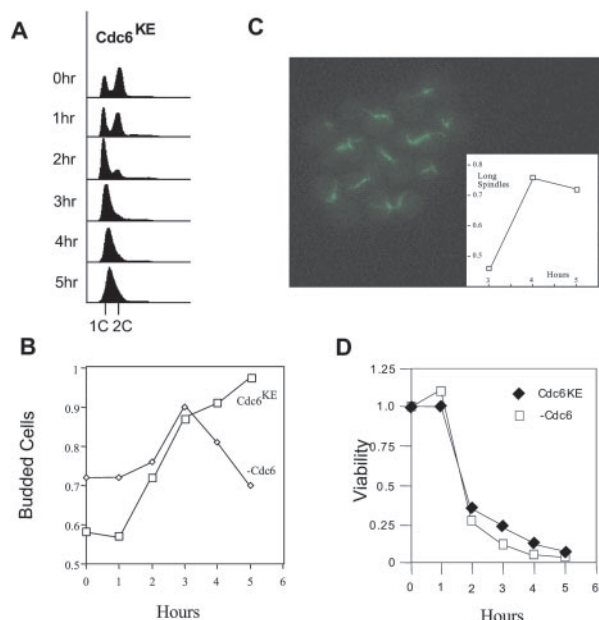
gen) to encode HA-HIS6-*CDC6* wild type (pMW44) and the TTS (pMW475) and  $\Delta 8-17$  (pMW476) derivative mutants. An *Nco*I site was introduced at the N terminus of *CDC6* and the epitope tag was inserted into this site. Recombinant viruses were constructed by using the BaculoGold system as described (7). The viruses were separately infected into  $2 \times 10^7$  Sf9 cells on 10-cm plates and harvested after 40 h at 27°C. The multiplicity of infection was adjusted so that the HA-HIS6-Cdc6 proteins were produced equally. Sf9 cells were lysed in 1 ml Sf9 lysis buffer (10 mM Tris-HCl (pH 7.5)/0.2% Nonidet P-40/1 mM EDTA/1 mM EGTA) containing 150 mM NaCl and a mixture of protease and phosphatase inhibitors. After centrifugation at 14,000 rpm, the soluble fraction was taken and the NaCl concentration was adjusted to 0.5 M. Monoclonal antibody 12CA5 coupled to protein A Sepharose beads (10  $\mu$ l bed volume) was added to the lysate and mixed at 4°C for 2 h. Antibody beads were collected and washed four times in the same buffer containing 500 mM NaCl and then once in same buffer containing 150 mM NaCl. W303 was grown to an OD<sub>600</sub> = 0.5, harvested, washed once with water, and resuspended in 2 ml of yeast Nonidet P-40 lysis buffer (20 mM Tris-HCl (pH 7.5)/150 mM NaCl/1 mM EGTA/0.5% Nonidet P-40) containing a mixture of protease and phosphatase inhibitors. Bead-beated yeast cell extracts were made as described (25) and equal volumes of yeast extract (500  $\mu$ l) were added to each of the Sf9 immunoprecipitates. After a further 2-h incubation at 4°C, the beads were washed four times in the same buffer, resuspended in protein sample buffer, and separated on SDS/12.5% PAGE gels. These gels were silver-stained to visualize Cdc6p or blotted and probed with rabbit polyclonal antibodies directed against Cdc28p (1:2000 from B. Futcher) to detect associated proteins. Control beads from wild-type Sf9 cell lysates (i.e., not charged with HA-HIS6-Cdc6p) did not immunoprecipitate Cdc28p (data not shown).

**Other Methods.** Tubulin staining (64), fluorescence-activated cell sorter (FACS) analysis and chromatin binding experiments to look at Orc6p phosphorylation were carried out as described (65). Baculoviruses expressing ORC (66) and Cdc28p-Clb5p (67) have been described. The Sic1p baculovirus was obtained from Mike Tyers (University of Toronto), and the Cln2p baculovirus from Ray Deshaies (California Institute of Technology, Pasadena). Baculovirus transfer vectors to express Clb1p and Clb6p were constructed by cloning the *Hind*III-*Nhe*I *CLB1* and *CLB6* restriction fragments into pAcPK31 (7) to give pMW10 and pMW11, respectively. Baculoviruses were constructed by using the BaculoGold system (PharMingen). For the kinase assays in Fig. 6C, the immunoprecipitates (IPs) were washed in 50 mM Tris-HCl/10 mM MgCl<sub>2</sub> and then resuspended in 10  $\mu$ l 50 mM Tris-HCl/10 mM MgCl<sub>2</sub>/1 mM ATP containing 1  $\mu$ g histone H1 and 10  $\mu$ Ci (1 Ci = 37 GBq) [ $\gamma$ -<sup>32</sup>P]ATP. The reactions were incubated for 10 min at 30°C and then separated on SDS/12.5% PAGE gels.

## Results

**Cells Expressing *cdc6<sup>KE</sup>* Arrest in G<sub>2</sub>/M in a *RAD53*-Independent Manner.** Depletion of Cdc6p resulted in a failure to initiate DNA replication and after a period, a “reductional” mitosis occurred in which unreplicated chromosomes formed spindle attachments and randomly segregated to the two spindle poles (8). Subsequent cytokinesis resulted in cell death because cells accumulated with fractional (0.5C) DNA contents. The *cdc6<sup>KE</sup>* Walker A mutant also failed to initiate DNA replication because it did not load the MCMs onto chromatin; however, these cells did not undergo a reductional mitosis, but arrested with a 1C DNA content (12).

To determine whether the *cdc6<sup>KE</sup>* mutation caused cells to arrest in G<sub>1</sub> or G<sub>2</sub>/M, we compared the morphology of cells expressing this mutant form of Cdc6p with cells lacking any Cdc6p. Cells expressing *cdc6<sup>KE</sup>* from its natural promoter and wild-type *CDC6* under control of the methionine repressible



**Fig. 1.** Cells expressing *cdc6<sup>KE</sup>* arrest after entry into mitosis. (A) FACS profile of YB504 (*MET3-CDC6 cdc6<sup>KE</sup>*) showing accumulation of cells with 1C DNA content after repression of wild-type *MET3-CDC6*. (B) Budding index of K4055 (*MET3-CDC6 Δcdc6*) and YB504 (*MET3-CDC6 cdc6<sup>KE</sup>*) after repression of wild-type *MET3-CDC6*. (C) Tubulin immunofluorescence of YB504 at 5-h time point. The *Inset* shows that these cells accumulate with long spindles. (D) Viability curve of YB504 and K4055 following shut-off of *CDC6*.

*MET3* promoter were grown to early exponential phase. The wild-type Cdc6p had a half-life of  $\approx 5$  min and disappeared rapidly after the addition of methionine to the medium (8). Cells expressing only *cdc6<sup>KE</sup>* accumulated as large budded cells with a 1C DNA content and with long spindles (Fig. 1A–C), indicating that cells accumulated in mitosis, probably late anaphase, and not in  $G_1$ .

A trivial explanation for the mitotic arrest phenotype seen with the *cdc6<sup>KE</sup>* mutant could be that this allele allowed initiation of replication at a very small subset of origins. Such limited initiation events could activate the *RAD53*-dependent  $G_1/S$  or S phase checkpoint response pathway that prevents mitosis when DNA replication is blocked (47–49). We thought that *RAD53* was not likely to be involved in the *CDC6*-dependent mitotic arrest because in the absence of DNA replication, sister chromatid cohesion is not possible. It is thought that *RAD53* inhibits the metaphase to anaphase transition by inhibiting cleavage of Pds1p and Scc1p, thereby preventing sister chromatid separation (68, 69). However, to test whether this arrest was *RAD53*-dependent, the *rad53-1* mutation was introduced into the *cdc6<sup>KE</sup>* strain and DNA content was measured by FACS following shut off of the wild-type *MET3-CDC6*. Two *rad53-1* segregants from the cross were analyzed. One of the *cdc6<sup>KE</sup> rad53-1* strains (YB791) still arrested with a 1C DNA content, but unexpectedly, a second *cdc6<sup>KE</sup> rad53-1* strain (YB792) underwent reductional mitosis (data not shown). Both *cdc6<sup>KE</sup> rad53-1* strains were transformed with a wild-type copy of *RAD53* on an *ARS-CEN* plasmid. *RAD53* reversed the hydroxyurea (HU) sensitivity of both strains as expected; however, it had no effect on the reductional mitosis of the YB792 strain. Therefore, the mitotic block in the first *cdc6<sup>KE</sup>* strain was *RAD53*-independent. The reductional mitosis seen in the second strain was also *RAD53*-independent and must have been caused by an alteration at another locus, consistent with previous findings that not all *S. cerevisiae* strains have an intact  $G_1$ -M checkpoint pathway (51).

One of the functions of a checkpoint response is to maintain cell viability by delaying the cell cycle until the error in cell division



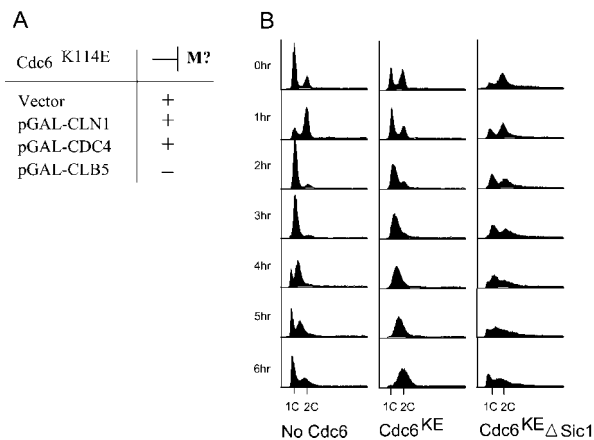
**Fig. 2.** Summary of *CDC6* mutational analysis. The sequence of the N-terminal 50 aa is shown at the top. Cells expressing *cdc6<sup>KE</sup>* containing the indicated N-terminal mutation were grown to early exponential phase. Then wild-type *MET3-CDC6* was repressed by the addition of methionine, samples were taken every hour for 6 h and scored by FACS for the accumulation of fractional (0.5C) DNA content. All of the mutants were tested (except  $\Delta N43$ ) and found to efficiently complement a *CDC6* deletion.

process is corrected. We tested whether the *cdc6<sup>KE</sup>*-dependent arrest maintained cell viability by comparing the number of viable cells over time following shut off of wild type *MET3-CDC6* in the *cdc6<sup>KE</sup>* mutant strain to the strain containing no additional *CDC6*. Both strains lost viability at a similar rate to 5–10% after 5 h (Fig. 1D). This observation indicated that this arrest was not a true checkpoint, because the entry into mitosis with unreplicated chromosomes was a lethal event. The arrest more likely reflects some function (besides MCM loading) that wild-type Cdc6p promotes during mitosis, and this function is also required for the completion of mitosis under these circumstances.

**Inhibition of Mitosis Requires One of Two Sequences in Cdc6p.** A structure-function analysis was used to determine which of the amino acids within the N terminus of Cdc6<sup>KE</sup>p were responsible for the mitotic arrest in the absence of DNA replication. A series of nested deletions beginning at the N terminus was created and after introduction into cells, DNA content was determined by sampling every hour for 5 h following shut-off of the wild-type *CDC6* gene. Deletion of the first eight amino acids had no effect on the mitotic arrest, however, all subsequent deletions were unable to maintain an arrest and accumulated with 0.5C DNA content (summarized in Fig. 2). Further deletions within this N-terminal region defined aa 8–17 as critical for this function. Deletion of 8–17 residues removed the sequence RRNL that matched the Cy box (zRxL) defined in higher eukaryotes as a sequence that mediates binding to the cyclin subunit of CDKs (57–60). However, a smaller deletion comprising this sequence ( $\Delta 13$ –17, RRNLF) had no effect, indicating that the upstream sequence is required. All of the *CDC6* N-terminal mutants (containing wild-type K114), including the point mutants (below), were able to efficiently complement a deletion of *CDC6* when using a plasmid-based assay and had no discernable growth defects. This was expected, because the N-terminal 50 aa of Cdc6p are nonessential.

Cdc6p contains three consensus Cdc28p kinase phosphorylation sites within the N terminus at positions 7, 23, and 43. All three serine and threonine residues were changed to alanine. This triple mutant (TTS) was unable to arrest in mitosis and accumulated with fractional DNA content (Fig. 2). However, no single phosphorylation site was essential for this activity, because the individual point mutants still maintained the 1C DNA arrest. (The first deletion of residues 2–7 removes the first Cdc28p phosphorylation site, T7.) From an analysis of all possible double site mutants, the first residue was the most critical because the T7A T23A double mutant accumulated with fractional DNA content, the T7A S43A mutant did so partially, and the T23A



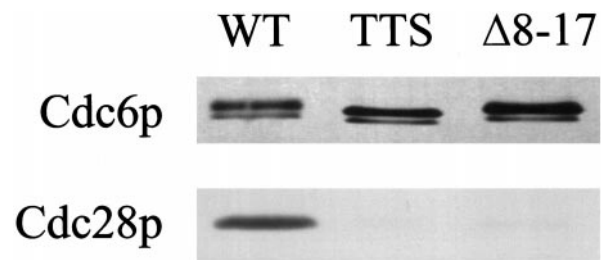


**Fig. 3.** Increasing Cdc28p-Clb kinase activity abrogates the mitotic block. (A) YB504 (*MET3-CDC6 cdc6<sup>KE</sup>*) was transformed with vector (yCP50) or GAL expression plasmids for *CLN1*, *CDC4*, or *CLB5* and grown to early exponential phase in minimal raffinose (2%) medium lacking uracil and methionine. Galactose (2%) and methionine (2 mM) were added at time 0 and samples were taken to analyze DNA content every 2 h for 8 h. (B) FACS profiles showing that deletion of *SIC1* also allows *cdc6<sup>KE</sup>* cells to go through a reductional mitosis. These cells were grown in SCM medium lacking methionine. Methionine (2 mM) was added at time 0 and cells were processed for FACS analysis at the indicated times. K4055 (*MET3-CDC6 Δcdc6*), YB504 (*MET3-CDC6 cdc6<sup>KE</sup>*), YB777 (*MET3-CDC6 cdc6<sup>KE</sup> Δsic1*).

S43A mutant still arrested with a 1C DNA content. Furthermore, the double mutant T7A Δ13–17 deletion still arrested in mitosis, again indicating that aa 8–12 were also critical for the arrest. These data suggest that mutation of two separate regions of Cdc6p can abrogate the mitotic arrest: the Cdc28p consensus phosphorylation sites and a short 10-aa sequence from residues 8–17. The sequences may function together to recruit a factor that mediates the arrest.

**Increasing Cdc28p/Clb Kinase Levels Can Overcome the Mitotic Arrest Caused by *cdc6<sup>KE</sup>*.** Because the mutational analysis suggested that a CDK interaction at the N terminus might be important for the mitotic arrest, we next examined the effect of overproducing various factors that affect Cdc28p activity (Fig. 3A). Cells containing *MET3-CDC6*, *cdc6<sup>KE</sup>*, and various plasmids capable of overproducing *CLN1*, *CDC4*, or *CLB5* and a vector control were grown in raffinose medium lacking methionine. At an early exponential phase of growth, methionine and galactose were added to the medium to both repress the wild-type copy of *CDC6* and to induce expression of the respective genes. Time points were taken every 2 h for 8 h. Neither the vector control nor expression of *CLN1* or *CDC4* could overcome the mitotic arrest. We also tried Gal expression of *CLN2*; however, this in itself was lethal to the cells and greatly broadened the 1C and 2C DNA peaks by FACS. In contrast, Gal-induced expression of *CLB5* overcame the mitotic arrest, indicating that increasing Cdc28p-Clb5p kinase levels reversed the inhibitory effect of Cdc6<sup>KE</sup>p on mitosis.

Because Sic1p is an inhibitor of Cdc28p-Clb kinase but not Cdc28p-Cln kinase (33, 44), we constructed a strain containing *sic1Δ MET3-CDC6 cdc6<sup>KE</sup>* (YB777). When the wild-type *CDC6* was shut off in these cells lacking *SIC1*, they also underwent a reductional mitosis (Fig. 3B). Transformation of an *ARS CEN* plasmid containing wild type *SIC1* into the *sic1Δ* strain (YB777) restored the ability to block entry into mitosis and also the normal growth properties of the strain (data not shown). This observation further indicates that increasing Cdc28p-Clb kinase activity can overcome the mitotic arrest induced by *cdc6<sup>KE</sup>* cells that have failed to initiate DNA replication.



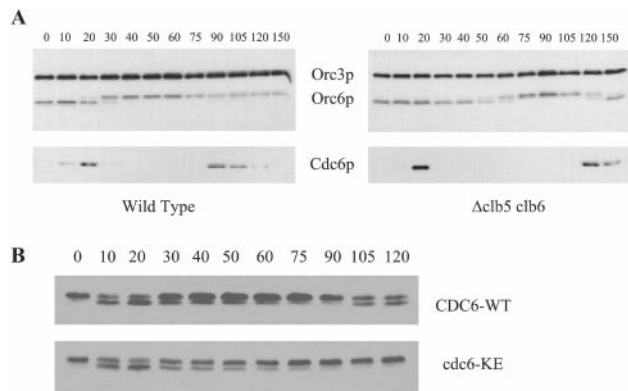
**Fig. 4.** Cdc28p is coimmunoprecipitated with wild-type Cdc6p, but not the two mutants (TTS and Δ8–17) that go through a reductional mitosis. (Upper) Cdc6p following immunoprecipitation by silver staining. (Lower) Western blot for Cdc28p. Experimental details are described in *Materials and Methods*.

**Mitotic Inhibition by Cdc6<sup>KE</sup>p Is Correlated with Binding to Cdc28p.** It has been reported that Cdc6p interacts with Cdc28p and inhibits its kinase activity (55). Furthermore, it was reported that this Cdc28p interaction is mediated by the N-terminal ≈50 aa of Cdc6p (55). Because the mutational analysis and genetic data suggested that Cdc6p might inhibit mitosis through an interaction with Cdc28p-Clb kinase, we tested whether wild-type Cdc6p and the two mutants that failed to arrest in mitosis could coimmunoprecipitate Cdc28p. It was difficult to detect binding to Cdc28p after immunoprecipitation of HA3-Cdc6p from yeast cells, but another experimental approach was informative. HA epitope-tagged versions of wild-type Cdc6p or the two mutants were produced in Sf9 insect cells by using recombinant baculoviruses. The HA-Cdc6p was immunoprecipitated under stringent salt conditions and the immunoprecipitates washed extensively. This efficient purification yielded very few contaminating insect cell proteins. The immunoprecipitates were then mixed with wild-type yeast whole cell extracts and, after a further 2-h incubation, the presence of Cdc28p was examined by immunoblotting (Fig. 4).

Similar amounts of wild-type Cdc6p and the two mutants were recovered in the immunoprecipitates (Fig. 4 Upper). Fig. 4 Lower shows a Western blot for Cdc28p. Although the wild-type Cdc6p protein coimmunoprecipitated Cdc28p, as reported (55), both of the mutants that fail to arrest in mitosis (TTS and Δ8–17) produced proteins that were defective in coimmunoprecipitating Cdc28p (Fig. 4 Lower). Together with the genetic data presented above, these results suggest that a Cdc6p–Cdc28p interaction is important in restraining mitosis in the absence of DNA replication initiation. This could be through Cdc6p phosphorylation, binding of the Cdc28p–cyclin, inhibition of Cdc28p–cyclin activity, or all three.

**Cells Expressing *cdc6<sup>KE</sup>* Activate S Phase Cdc28p-Clbs on Schedule During Passage Through G<sub>1</sub>.** Cells in G<sub>1</sub> that do not have active Cdc28p-Clb kinase, but have activated Cdc28p-Cln kinase, have a multibudded phenotype. In contrast, cells that have not activated Cdc28p-Cln kinase arrest as unbudded cells. Because cells expressing only *cdc6<sup>KE</sup>* arrested with a dumb-bell morphology, it is likely that they had activated Cdc28p-Clb5p during passage through G<sub>1</sub>, even though replication initiation had not occurred. We sought an independent confirmation that Cdc28p-Clb5p was activated normally and sought a marker for Cdc28p-Clb5p activation during G<sub>1</sub>. Orc6p was a good candidate because it is phosphorylated as cells initiate DNA synthesis, and dephosphorylated as cells exit mitosis and enter G<sub>1</sub> (65). Furthermore, Orc6p phosphorylation required the *ORC6* Cdc28p consensus phosphorylation sites (65), but it was not known whether Cdc28p-Cln or -Clb kinases were responsible.

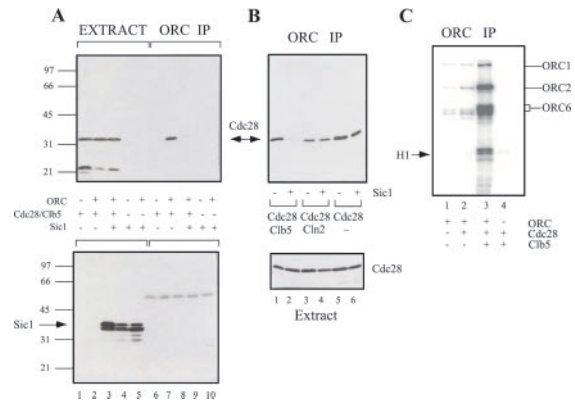
To determine whether *CLB5* or *CLB6* was responsible for the Orc6p phosphorylation as cells entered S phase, the timing of Orc6p phosphorylation in wild-type and *Δclb5 clb6* cells was compared after a synchronous release from an alpha factor-mediated G<sub>1</sub> arrest. Cdc28p-Cln kinase is known to be activated



**Fig. 5.** Orc6p phosphorylation requires *CLB5 CLB6* and Orc6p phosphorylation occurs on schedule in the *cdc6<sup>KE</sup>* mutant during passage through G<sub>1</sub>. (A) Cells (W303 *MATa* and W303 *MATa*  $\Delta$ *clb5 clb6*) arrested in G<sub>1</sub> by using alpha factor were released into the cell cycle. Samples were taken at the indicated minutes and processed into soluble and chromatin containing fractions as described (64). Western blots of the chromatin fractions are shown. (B) MET3-CDC6 cells also expressing wild-type CDC6 or *cdc6<sup>KE</sup>* were arrested in mitosis by using the *dbf2-1* temperature-sensitive mutation (37°C) for 3 h. Methionine was added 30 min before release at 25°C and samples were taken at the indicated minutes following shift to 25°C and blotted for Orc6p.

on schedule in the  $\Delta$ *clb5 clb6* cells; however, there is an  $\approx$ 30-min delay in the initiation of DNA replication because of a failure to activate Cdc28p-Clb5p and -Clb6p kinases (31, 32). Under these circumstances, initiation is thought to be dependent on the later Cdc28p-Clb3p and -Clb4p kinases. At the alpha factor arrest, Orc6p was in the dephosphorylated form in the two strains (Fig. 5A). Orc3p was shown as a loading control. Orc6p was phosphorylated at 30 min after release in the wild-type strain, but was significantly delayed to 60 min in the  $\Delta$ *clb5 clb6* mutant. FACS analysis indicated that the  $\Delta$ *clb5 clb6* strain also delayed entry into S phase relative to the wild type by 30 min (data not shown), as reported (32). These results suggest that Clb5p and Clb6p were required for the phosphorylation of Orc6p before S phase. Cdc6p bound to chromatin 20 min after release from the alpha factor arrest and was removed by 30 min in both the wild-type and  $\Delta$ *clb5 clb6* strains, indicating that Cdc28p-Clb5p and -Clb6p kinases were not required for either Cdc6p binding or removal from the chromatin.

Using Orc6p phosphorylation as a marker, we then tested whether Clb5p and Clb6p were activated on schedule in cells that were transiting G<sub>1</sub>-expressing *cdc6<sup>KE</sup>* as compared with cells expressing wild-type Cdc6p. Cells were synchronized in late mitosis by using the *dbf2-1* mutation. While arrested, the wild-type *MET3-CDC6* gene was repressed by the addition of methionine for 30 min and the cells were allowed to enter G<sub>1</sub> at the permissive temperature for *dbf2-1*, expressing either wild-type *CDC6* or *cdc6<sup>KE</sup>* from the endogenous *CDC6* promoter. Orc6p was dephosphorylated within 10 min of release and was rephosphorylated beginning at 30 min in both strains (Fig. 5B), thus the Cdc28p-Clb5p and -Clb6p were activated in both strains at the same time. The wild-type cells passed through mitosis and entered G<sub>1</sub> phase again during the 105–120-min interval as evidenced by the dephosphorylation of Orc6p (Fig. 5B). However, the *cdc6<sup>KE</sup>* cells arrested with a 1C DNA content by FACS (data not shown) and Orc6p remained phosphorylated. These results demonstrated that Cdc28p-Clb5p and -Clb6p kinases were activated in the *cdc6<sup>KE</sup>* mutant during passage through G<sub>1</sub>, consistent with the large-budded arrest phenotype of this strain. Furthermore, cells actually exited mitosis from the *dbf2-1* arrest point after shutting off wild-type *CDC6* and expressing only *cdc6<sup>KE</sup>*, suggesting that the Cdc6<sup>KE</sup>p mitotic block occurred earlier than the *DBF2* execution point.



**Fig. 6.** Cdc28p kinase coimmunoprecipitates with ORC. (A) Sf9 insect cells were infected with baculoviruses expressing ORC, Cdc28p, Clb5p, and Sic1p in various combinations. The same amount of ORC was immunoprecipitated in each case by using monoclonal anti-ORC antibodies when ORC was expressed. The IPs were blotted for associated proteins: Cdc28p (Upper) and Sic1p (Lower). (B) Sic1p prevents the Cdc28p-Clb5p interaction with ORC. Upper and Lower show Cdc28p Western blots following immunoprecipitation of ORC from insect cells expressing the indicated proteins, as above. (C) ORC, Cdc28p, and Clb5p were coexpressed in insect cells in various combinations. ORC was immunoprecipitated as above and the IPs were tested for H1 kinase activity following addition of 1  $\mu$ g H1 and [ $\gamma$ -<sup>32</sup>P]ATP.

**Sic1p Regulates Cdc28–Cyclin Interaction with ORC.** Because Orc1p, Orc2p, and Orc6p contain Cdc28p phosphorylation sites and are phosphorylated *in vitro* by purified Cdc28p-Clb5p kinase (data not shown and Fig. 6C), we tested whether ORC could also physically interact with Cdc28p kinase. Baculovirus expressing ORC and either Cdc28p alone or Cdc28p together with Cln2p, Clb1p, Clb5p, or Clb6p were co-infected into Sf9 cells. Soluble Sf9 extracts were made as described (7) and ORC protein was immunoprecipitated by using a mixture of monoclonal antibodies against the ORC subunits. The IP was separated on SDS/PAGE gels and then tested for coimmunoprecipitation of Cdc28p by Western blotting. Cdc28p was coimmunoprecipitated with ORC no matter what cyclin subunit was coexpressed and even in the absence of any cyclin subunit (Fig. 6B, lanes 1, 3, and 5, and data not shown). Cdc28–cyclin was not immunoprecipitated in the absence of ORC protein (Fig. 6A, lane 6). We additionally verified that each cyclin subunit was being coimmunoprecipitated together with Cdc28p by making S35-labeled Cdc28p–cyclin Sf9 extracts as described (7). S35-labeled extracts were mixed with (nonlabeled) Sf9 extract expressing ORC, ORC was immunoprecipitated, and after separation on SDS/PAGE gels, Cdc28–cyclin coimmunoprecipitated only when ORC protein was also present in the extract (data not shown).

We then tested whether the CDK inhibitor Sic1p could either interact with ORC or prevent interaction of various Cdc28–cyclin pairs with ORC. Sic1p could not be coimmunoprecipitated with ORC (Fig. 6A, lanes 9 and 10). However, coexpression of Sic1p with ORC and Cdc28–cyclin prevented the interaction of Cdc28p-Clb5p with ORC (Fig. 6A, lanes 7 and 8, and B, lanes 1 and 2), but not the interaction of Cdc28p-Cln2p (Fig. 6B, lanes 3 and 4) or Cdc28 (Fig. 6B, lanes 5 and 6) with ORC. This observation is consistent with the observation that Sic1p interacts with and inhibits Cdc28p-Clb, but not Cdc28p-Cln kinases.

We tested whether the Cdc28p kinase in the ORC immunoprecipitates was active. ORC was immunoprecipitated from baculovirus-infected Sf9 cells expressing ORC alone, ORC plus Cdc28p, or ORC plus Cdc28p-Clb5p. After extensive washing, histone H1 kinase activity was measured. Fig. 6C shows that although no H1 kinase activity was present in the ORC alone IP or when Cdc28p was also expressed, a very active H1 kinase is present in the ORC IP when Cdc28p-Clb5p were coexpressed. In

addition, the Orc1p, Orc2p, and Orc6p subunits were phosphorylated by Cdc28-Clb5p in the IP. There was also weak phosphorylation of these ORC subunits in the ORC alone and ORC-Cdc28p IP (Fig. 6C, lanes 1 and 2), probably reflecting the association of an insect cell kinase. Only these three ORC subunits contain consensus Cdc28p phosphorylation sites. Orc1p has one site, Orc2p has six sites, and Orc6p has four sites. Finally, an ORC-Cdc28p interaction likely also occurs in yeast. Following immunoprecipitation of ORC from exponentially growing yeast, we detected a CDC28-dependent ORC and H1 kinase activity, because the ORC-associated kinase activity was seen in wild-type cells but not in *cdc28-4* cells (data not shown).

## Discussion

We have described mutations within the N-terminal nonessential domain of Cdc6p that define regions required for the inhibition of mitosis in the absence of DNA replication initiation. One mutation deleted a motif that matches the Cy box consensus present in higher eukaryotic organisms shown to mediate binding to cdk2-cyclinA (57–60). This Cdc6p deletion mutant also failed to coimmunoprecipitate Cdc28p, consistent with it acting as a Cdc28-cyclin interaction domain. We have noticed that the CDK inhibitor Sic1p also contained a match to the Cy motif near the C terminus at positions 260 to 263 (KRRL). A C-terminal deletion analysis of *SIC1* found that the 26 C-terminal aa were required for growth arrest caused by overproduction of a nondegradable form of Sic1p (M.W. and B.S., unpublished data). One essential sequence within these 26 aa was the Cy motif and similar findings to ours have been published (70). This data supports the hypothesis that the Cy motif is also present in budding yeast and mediates interaction with the B-type Cdc28p complexes, the inhibitory target of Sic1p. Our data, shown in Fig. 6, also demonstrate that Sic1p has an additional function to block Clb-CDKs from interacting with ORC. Interestingly, the Cdc28p-Cln2p kinase could still bind ORC in the presence of Sic1p. We suggest that Sic1p not only ensures a temporal pattern of CDK activation, but also regulates access of specific CDKs to their key substrates.

The second mutation that abrogated the mitotic block was a triple alanine substitution of the three N-terminal Cdc28p phosphorylation sites in Cdc6p. The corresponding protein was also defective in coimmunoprecipitating Cdc28p. Because we were able to overcome the mitotic arrest phenotype by increasing the amount of Cdc28p-Clb activity, either by overproducing Clb5p or deleting the Cdc28p-Clb inhibitor *SIC1*, together this data suggests that, like Sic1p, Cdc6p plays a role in passage through mitosis by interaction with Cdc28-Clb kinases.

The *CDC6* homologues share a conserved core region shown by the recently solved crystal structure of an archaeal Cdc6 orthologue to encode a two-domain, ATP-binding module of the AAA+ family, and a C-terminal winged-helix that may interact with DNA or other proteins (71). An alignment of human, *Xenopus*, *S. pombe*, and *S. cerevisiae* *CDC6* homologues indicates very little sequence conservation in their N-terminal regions, and in fact the N termini have very different lengths. So it was surprising to find that the alignment showed sequence conservation just covering the ten amino acids from positions 8–17 in the budding yeast protein. Furthermore, all of the Cdc6 proteins had CDK consensus phosphorylation sites in their N termini.

Perhaps the mitotic function of this N-terminal domain is conserved in the various Cdc6 proteins.

Cdc6p may be one component of the previously described G<sub>1</sub>-M phase checkpoint that blocks mitosis in the absence of replication initiation; however, the *CDC6*-dependent arrest could be a separate phenomenon. The mitotic arrest seen in the *cdc6<sup>KE</sup>* mutant was *RAD53*-independent and our data indicated that another locus in addition to *CDC6* was also involved.

An apparent discrepancy with our finding of a mitotic inhibitory role for the Cdc6p N terminus is the report that depletion of Cdc45p also resulted in a reduction of mitosis in the absence of initiation of DNA replication (28). There are several explanations for this finding. One is that not all *S. cerevisiae* strains retain the ability to block mitosis in the absence of initiation and this pathway may be defective in the particular strain used. Alternatively, the nonfunctional K114E mutation in Cdc6p might be required to reveal the mitotic arrest, because this mutant is unable to load the Mcm proteins during late mitosis. The absence of the preRC may be required for the mitotic block.

Recent studies in *Xenopus* have suggested that binding of cyclinE-cdk2 to preRCs is necessary for the initiation of DNA replication (72). Also, many tumor cells overexpress cyclin E, and it is known that this circumstance can lead to genetic instability and inappropriate regulation of S phase (73). It is therefore likely that inappropriate temporal regulation of CDKs will disrupt the events that lead to accurate inheritance of the genome. In this paper we have uncovered additional mechanisms to ensure that cyclin-dependent kinases are precisely coordinated with the events necessary to lead to initiation of DNA replication. As cells exit mitosis and proceed through G<sub>1</sub> phase, Clb-cyclin kinases are kept inactive by the combination of an active APC and the presence of CDK inhibitor Sic1p. We suggest that the Cdc6p on chromatin during the G<sub>1</sub> phase of the cell cycle (12) may also act to inhibit Clb-cyclin kinase activity as cells exit mitosis, and therefore provide an opportunity for preRCs to be formed. Paradoxically, Clb-like CDKs are required in late G<sub>1</sub> phase to activate S phase and initiation of DNA replication at each origin. Cdc6 is located at each origin because it binds directly to ORC (14). Thus, Cdc6 could potentially create a local environment in which CDKs would be prevented from prematurely activating preRCs. We have also demonstrated that Cln-CDKs can bind to ORC in the presence of Sic1p, but that Clb-CDKs cannot. Cln-CDKs trigger the destruction of Cdc6p and Sic1p, thereby removing Cdc6p from origins. In the absence of Sic1p, the Clb-CDKs then act on preRCs to initiate DNA replication. Thus, Sic1p acts as a molecular chaperone to orchestrate temporal-specific CDK function at replication origins. The presence of Cdc6 at origins during G<sub>1</sub> phase also ensures that cells do not enter into mitosis without having replicated DNA, but it is clear that additional mechanisms must be operating to ensure the dependence of mitosis on DNA replication.

**Note Added in Proof.** Similar conclusions have recently been published (74). “Cdc6 cooperates with Sic1 and Hct1 to inactivate mitotic cyclin-dependent kinases.”

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1. Diffley, J. F. X. (1996) *Genes Dev.* **10**, 2819–2830.
2. Stillman, B. (1996) *Science* **274**, 1659–1663.
3. Dutta, A. & Bell, S. P. (1997) *Annu. Rev. Cell. Dev. Biol.* **13**, 293–332.
4. Lei, M. & Tye, B. K. (2001) *J. Cell Sci.* **114**, 1447–1454.
5. Kelly, T. J., Martin, G. S., Forsburg, S. L., Stephen, R. J., Russo, A. & Nurse, P. (1993) *Cell* **74**, 371–382.
6. Lygerou, Z. & Nurse, P. (2000) *Science* **290**, 2271–2273.
7. Liang, C., Weinreich, M. & Stillman, B. (1995) *Cell* **81**, 667–676.
8. Piatti, S., Lengauer, C. & Nasmyth, K. (1995) *EMBO J.* **14**, 3788–3799.

9. Coleman, T. R., Carpenter, P. B. & Dunphy, W. G. (1996) *Cell* **87**, 53–63.
10. Donovan, S., Harwood, J., Drury, L. S. & Diffley, J. F. X. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 5611–5616.
11. Perkins, G. & Diffley, J. F. X. (1998) *Mol. Cell* **2**, 23–32.
12. Weinreich, M., Liang, C. & Stillman, B. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 441–446.
13. Neuwald, A. F., Aravind, L., Spouge, J. L. & Koonin, E. V. (1999) *Genome Res.* **9**, 27–43.
14. Mizushima, T., Takahashi, N. & Stillman, B. (2000) *Genes Dev.* **14**, 1631–1641.



15. Seki, T. & Diffley, J. F. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 14115–14120.
16. Aparicio, O. M., Weinstein, D. M. & Bell, S. P. (1997) *Cell* **91**, 59–69.
17. Hardy, C. F., Dryga, O., Seematter, S., Pahl, P. M. & Sclafani, R. A. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 3151–3155.
18. Lei, M., Kawasaki, Y., Young, M. R., Kihara, M., Sugino, A. & Tye, B. K. (1997) *Genes Dev.* **11**, 3365–3374.
19. Owens, J. C., Detweiler, C. S. & Li, J. J. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 12521–12526.
20. Bousset, K. & Diffley, J. F. X. (1998) *Genes Dev.* **12**, 480–490.
21. Donaldson, A. D., Fangman, W. L. & Brewer, B. J. (1998) *Genes Dev.* **12**, 491–501.
22. Mimura, S. & Takisawa, H. (1998) *EMBO J.* **17**, 5699–5707.
23. Zou, L. & Stillman, B. (1998) *Science* **280**, 593–596.
24. Aparicio, O. M., Stout, A. M. & Bell, S. P. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 9130–9135.
25. Weinreich, M. & Stillman, B. (1999) *EMBO J.* **18**, 5334–5346.
26. Jares, P. & Blow, J. J. (2000) *Genes Dev.* **14**, 1528–1540.
27. Nougarede, R., Della Seta, F., Zarzov, P. & Schwob, E. (2000) *Mol. Cell. Biol.* **20**, 3795–3806.
28. Tercero, J. A., Labib, K. & Diffley, J. F. (2000) *EMBO J.* **19**, 2082–2093.
29. Zou, L. & Stillman, B. (2000) *Mol. Cell. Biol.* **20**, 3086–3096.
30. Futcher, B. (1996) *Yeast* **12**, 1635–1646.
31. Kuhne, C. & Linder, P. (1993) *EMBO J.* **12**, 3437–3447.
32. Schwob, E. & Nasmyth, K. (1993) *Genes Dev.* **7**, 1160–1175.
33. Schwob, E., Bohm, T., Mendenhall, M. D. & Nasmyth, K. (1994) *Cell* **79**, 233–244.
34. Verma, R., Annan, R. S., Huddleston, M. J., Carr, S. A., Reynard, G. & Deshaies, R. J. (1997) *Science* **278**, 455–460.
35. Drury, L. S., Perkins, G. & Diffley, J. F. (2000) *Curr. Biol.* **10**, 231–240.
36. Donaldson, A. D., Raghuraman, M. K., Friedman, K. L., Cross, F. R., Brewer, B. J. & Fangman, W. L. (1998) *Mol. Cell* **2**, 173–182.
37. Correa-Bordes, J. & Nurse, P. (1995) *Cell* **83**, 1001–1009.
38. Dahmann, C., Diffley, J. F. X. & Nasmyth, K. A. (1995) *Curr. Biol.* **5**, 1257–1269.
39. Piatti, S., Bohm, T., Cocker, J. H., Diffley, J. F. X. & Nasmyth, K. (1996) *Genes Dev.* **10**, 1516–1531.
40. Labib, K., Diffley, J. F. & Kearsley, S. E. (1999) *Nat. Cell Biol.* **1**, 415–422.
41. Nguyen, V. Q., Co, C., Irie, K. & Li, J. J. (2000) *Curr. Biol.* **10**, 195–205.
42. Noton, E. & Diffley, J. F. (2000) *Mol. Cell* **5**, 85–95.
43. Shirayama, M., Toth, A., Galova, M. & Nasmyth, K. (1999) *Nature (London)* **402**, 203–207.
44. Mendenhall, M. D., al-Jumaily, W. & Nugroho, T. T. (1995) *Prog. Cell Cycle Res.* **1**, 173–185.
45. Wuarin, J. & Nurse, P. (1996) *Cell* **85**, 785–787.
46. Tanaka, T. & Nasmyth, K. (1998) *EMBO J.* **17**, 5182–5191.
47. Elledge, S. J. (1996) *Science* **274**, 1664–1672.
48. Longhese, M. P., Foiani, M., Muzi-Falconi, M., Lucchini, G. & Plevani, P. (1998) *EMBO J.* **17**, 5525–5528.
49. Weinert, T. (1998) *Cell* **94**, 555–558.
50. Rhind, N. & Russell, P. (2000) *J. Cell Sci.* **113**, 3889–3896.
51. Toyn, J. H., Johnson, A. L. & Johnston, L. H. (1995) *Mol. Cell. Biol.* **15**, 5312–5321.
52. Nishitani, H. & Nurse, P. (1995) *Cell* **83**, 397–405.
53. Jallepalli, P. V. & Kelly, T. J. (1996) *Genes Dev.* **10**, 541–552.
54. Jallepalli, P. V., Brown, G. W., Muzi-Falconi, M., Tien, D. & Kelly, T. J. (1997) *Genes Dev.* **11**, 2767–2779.
55. Elsassner, S., Lou, F., Wang, B., Campbell, J. L. & Jong, A. (1996) *Mol. Biol. Cell* **7**, 1723–1735.
56. Brown, G. W., Jallepalli, P. V., Huneycutt, B. J. & Kelly, T. J. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 6142–6147.
57. Zhu, L., Harlow, E. & Dynlacht, B. D. (1995) *Genes Dev.* **9**, 1740–1752.
58. Adams, P. D., Sellers, W. R., Sharma, S. K., Wu, A. D., Nalin, C. M. & Kaelin, W. G. (1996) *Mol. Cell. Biol.* **16**, 6623–6633.
59. Chen, J., Saha, P., Kornbluth, S., Dynlacht, B. D. & Dutta, A. (1996) *Mol. Cell. Biol.* **16**, 4673–4682.
60. Russo, A. A., Jeffrey, P. D., Patten, A. K., Massague, J. & Pavletich, N. P. (1996) *Nature (London)* **382**, 325–331.
61. Rose, M., Winston, F. & Hieter, P. (1990) *Methods in Yeast Genetics: A Laboratory Course Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
62. Guthrie, C. & Fink, G. R. (1991) *Methods Enzymol.* **194**.
63. Thomas, B. J. & Rothstein, R. (1989) *Cell* **56**, 619–630.
64. Lee, L., Klee, S. K., Evangelista, M., Boone, C. & Pellman, D. (1999) *J. Cell Biol.* **144**, 947–961.
65. Liang, C. & Stillman, B. (1997) *Genes Dev.* **11**, 3375–3386.
66. Bell, S. P., Mitchell, J., Leber, J., Kobayashi, R. & Stillman, B. (1995) *Cell* **83**, 563–568.
67. Duncker, B. P., Pasero, P., Braguglia, D., Heun, P., Weinreich, M. & Gasser, S. M. (1999) *Mol. Cell. Biol.* **19**, 1226–1241.
68. Sanchez, Y., Bachant, J., Wang, H., Hu, F., Liu, D., Tetzlaff, M. & Elledge, S. J. (1999) *Science* **286**, 1166–1171.
69. Uhlmann, F., Wernic, D., Poupard, M. A., Koonin, E. V. & Nasmyth, K. (2000) *Cell* **103**, 375–386.
70. Hodge, A. & Mendenhall, M. (1999) *Mol. Gen. Genet.* **262**, 55–64.
71. Liu, J., Smith, C. L., DeRyckere, D., DeAngelis, K., Martin, G. S. & Berger, J. M. (2000) *Mol. Cell* **6**, 637–648.
72. Furstenthal, L., Kaiser, B. K., Swanson, C. & Jackson, P. K. (2001) *J. Cell Biol.* **152**, 1267–1278.
73. Spruck, C. H., Won, K. A. & Reed, S. I. (1999) *Nature (London)* **401**, 297–300.
74. Calzada, A., Sacristan, M., Sanchez, E. & Bueno, A. (2001) *Nature (London)* **412**, 355–358.