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Stable Association of Mitotic Cyclin B/Cdc2 to Replication Origins Prevents Endoreduplication

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Summary

We show that in fission yeast the mitotic B type cyclin Cdc13/Cdc2 kinase associates with replication origins in vivo. This association is dependent on the origin recognition complex (ORC), is established as chromosomes are replicated, and is maintained during G2 and early mitosis. Cells expressing an *orp2* (ORC2) allele that reduces binding of Cdc13 to replication origins are acutely prone to chromosomal reduplication. In synchronized endoreduplicating cells, following Cdc13 ablation, replication origins are coordinately licensed prior to each successive round of S phase with the same periodicity as in a normal cell cycle. Thus, ORC bound mitotic Cyclin B/Cdc2 kinase imposes the dependency of S phase on an intervening mitosis but not the temporal licensing of replication origins between each S phase.

Introduction

Thirty years ago, cell fusion studies established that only G1 but not G2 nuclei have the potential to replicate (Rao and Johnson, 1970). The ploidy of eukaryotic cells is therefore preserved by ensuring that S phase can occur only after a previous mitosis. A role for the mitotic cyclin-dependent kinase (Cdk) in maintaining the order of S phase and mitosis has been demonstrated in fission yeast (Broek et al., 1991; Hayles et al., 1994). In particular, mutational inactivation of either Cdc2 (Cdk1) or Cdc13, the major B type cyclin, causes cells to undergo chromosomal rereplication without an intervening mitosis (Broek et al., 1991; Hayles et al., 1994). In this situation, FACS analysis reveals distinct doublings of cellular DNA content, suggesting that cells lacking the Cdc13/Cdc2 complex undergo complete and separable rounds of S phase. In this manner, these rereplicating cells resemble the polyploid plant, arthropod, and mammalian cells that arise from natural endoreduplication cycles during development (reviewed in Edgar and Orr-Weaver, 2001). Intriguingly, downregulation of mitotic Cyclin B/Cdk1 activity correlates with and is required for the occurrence of endoreduplication cycles in both maize and fruit flies (Sigrist and Lehner, 1997). The precise mechanism by which the mitotic Cyclin B/Cdk1 complex suppresses the endoreduplication cycle is, however, unknown.

Replication in all eukaryotes is initiated at discrete chromosomal sites known as replication origins that are recognized by an evolutionarily conserved origin recognition complex (ORC) (Bell et al., 1993; Micklem et al., 1993; Leatherwood et al., 1996; Grallert and Nurse, 1996; Lygerou and Nurse, 1999). To ensure that chromosomes are duplicated only once each cell cycle and that no regions of DNA are overreplicated, replication forks must be initiated at individual origins only once every cell cycle. This has led researchers to propose the existence of a replication licensing factor (RLF) which ensures that origins are activated only once per cell cycle (Blow and Laskey, 1988; Diffley, 1996). As cells enter G1, origins are licensed by the step-wise recruitment of proteins to ORC to form a prereplicative complex (pre-RC) that is competent for the initiation of replication. These proteins include the Cdc18(CDC6) and Cdt1 proteins, which are in turn necessary for the recruitment of a heterohexameric complex of Mcm(2-7) proteins (Chong et al., 1995; Kubota et al., 1995; Madine et al., 1995; Nishitani and Nurse, 1995; Cocker et al., 1996; Ogawa et al., 1999; Kearsey et al., 2000; Maiorano et al., 2000; Nishitani et al., 2000). The Mcm(2-7) complex may have helicase activity required for the initiation and elongation of replication forks (Ishimi, 1997; Labib et al., 2000). As replication is initiated, the Mcm2-7 complex dissociates from the ORC. The reformation of prereplicative complexes on origins is then normally inhibited until sister chromatids are separated in mitosis. In fission yeast, this control can be subverted by either strong overexpression of *cdc18* alone or cooverexpression of both *cdc18* and *cdt1* in G2 cells (Nishitani and Nurse, 1995; Yanow et al., 2001). This causes cells to undergo continuous DNA synthesis, resulting in giant nuclei with up to 64C DNA content. However, in this case, no discrete doublings of cellular DNA content are observed by FACS analysis, suggesting that DNA replication is disordered or that only a subset of replication origins are repetitively activated.

The role of cyclin-dependent kinases in preventing the licensing of replicated DNA has been examined in a number of species (Wuarin and Nurse, 1996; Mahbubani et al., 1997; Noton and Diffley, 2000). A number of origin binding proteins including Cdc18(CDC6), Mcm4, Orc2, and Orc6 are targets for Cdk-dependent phosphorylation in vitro and in vivo (Hendrickson et al., 1996; Lopez-Girona et al., 1998; Vas et al., 2001; Nguyen et al., 2001). The function of Cdk-dependent phosphorylation of these proteins has been most extensively addressed in budding yeast. By itself, mutation of the phosphorylation sites on any one of these proteins has little influence on DNA replication, nor is ectopic DNA replication observed in cells in which the phosphorylation sites on both ORC2 and ORC6 has been mutated and which simultaneously express a constitutively nu-

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clear form of Mcm4. Only when these cells additionally overexpress a truncated, nondegradable form of Cdc6(Cdc18) do they enter a partial round of DNA synthesis equivalent to replication of approximately half the genome, possibly due to the repetitive activation of only a subset of origins (Nguyen et al., 2001). These results suggest that Cdks can block the formation of pre-RCs by multiple mechanisms. However, neither these nor previous studies distinguish whether phosphorylation of pre-RC components dictates the periodic formation of prereplicative complexes, or the preservation of cell ploidy, or both. Cdks have been reported to interact with the N terminus of Cdc18(CDC6) when immunoprecipitated from soluble extracts of cells in which Cdc18(CDC6) is overexpressed and with Orc2 by two-hybrid analysis or when cooverexpressed in insect cells (Leatherwood et al., 1996; Lopez-Girona et al., 1998; Romanowski et al., 2000; Weinreich et al., 2001). However, it is not known whether Cdc6(Cdc18) and Orc2 interact with the same or different Cyclin/Cdk complexes. Nor is it known precisely where in the cell these phosphorylations take place. In particular, no specific association of Cyclin or Cdk proteins with replication origins has been observed in vivo.

In this paper, we demonstrate that synchronized populations of fission yeast cells lacking the mitotic Cyclin B/Cdc2 kinase undergo complete, distinct, and successive rounds of S phase without an intervening mitosis. Importantly, in these rereplicating cells, all origins are coordinately and periodically licensed and replicated with the same timing as in a normal cell cycle. In this respect we have been able to experimentally distinguish the molecular mechanisms that impose the global dependency of S phase on mitosis from controls that ensure the correct order and timing of origin licensing and replication prior to and during each successive S phase. Furthermore, we show that stable association of mitotic Cyclin B/Cdc2 kinase to replication origins is responsible for ensuring that a subsequent round of S phase is not initiated until an intervening mitosis has taken place.

Results

Mitotic Cyclin B/Cdc2 Binds Replication Origins In Vivo

Several replication origins have been identified in fission yeast by their ability to act as autonomously replicating sequences (ARS) (Dubey et al., 1994; Okuno et al., 1999). These are composed of adenine- and thymidine-enriched regions of DNA clustered within a 1–2 kb region of DNA. The most efficient of these, ARS2004 and ARS3002, are utilized as replication origins in almost every cell cycle and bind components of both the ORC and MCM complexes. Although ORC binds constitutively to chromatin throughout the fission yeast cell cycle, the prereplicative complex only forms on origins in G1. We postulated that a factor may stably bind ORC during G2 and early M phase to prevent reinitiation of S phase. We reasoned that such a factor might be identifiable in an asynchronous culture, as 70% of rapidly growing wild-type cells are in G2. Since the Cdc13 protein resides in the chromatin domain of fission yeast nuclei and is required to prevent the endoreduplication cycle (Gallagher et al., 1993; Hayles et al., 1994), we examined whether this factor might be the Cdc13/Cdc2 complex itself. We used chromatin immunoprecipitation (ChIP) analysis to identify replication origin binding proteins in vivo. Two of the components of the fission yeast ORC complex, Orp1 and Orp2, were C-terminally tagged at their chromosomal locus with three tandem haemagglutinin (HA) epitopes. After crosslinking with formaldehyde, cell lysates were prepared and sonicated to shear the chromosomal DNA to 500–700 bp fragments. After immunoprecipitation of tagged proteins with an anti-HA antibody, associated chromosomal DNA was amplified using sets of oligos that recognize successive ~300 bp segments across the ARS2004 locus. We found that both Orp1 and Orp2 specifically bind to the region of ARS2004 that has been defined as the site of replication initiation (Figure 1A), in accordance with previous observations (Ogawa et al., 1999). Strikingly, in addition to Orp1 and Orp2, we found using specific antibodies that the major mitotic Cyclin B, Cdc13, associated with the same region of ARS2004 in log phase cultures (Figure 1A). Another region of chromosome III contains three sites of replication initiation, ARS3002, ARS3003, and ARS3004 (Dubey et al., 1994). We found that Orp1 and Cdc13 coincidently bind the strong ARS3002 origin and more weakly to ARS3003 and ARS3004 (Figure 1B). They also bound to other replication origins, including ARS1 (see below). We noted that the strength of Orp1, Orp2, and Cdc13 binding showed some correlation with the frequency of origin use, suggesting a functional relationship between ORC and Cdc13 at origins (Figure 1B). In fission yeast, the major cyclin-dependent kinase, Cdc2, binds not only Cdc13 but at least three other cyclins, Cig1, Cig2, and Puc1, which have an overlapping role in the initiation of S phase (Fisher and Nurse, 1996). We found using specific antibodies that Cdc2 but not the Cig1, Cig2, or Puc1 cyclins bound ARS2004 in log phase cultures (Figure 1C). Notably, association of Cdc2 to ARS2004 was abolished in cells lacking Cdc13 (Figure 1D), and conversely, the association of Cdc13 to ARS2004 was greatly diminished in *cdc2-33* cells when incubated at 42°C for 30 min (Figure 1E), a procedure that promotes endoreduplication in fission yeast (Broek et al., 1991). These results demonstrate that the major mitotic Cdc13/Cdc2 kinase complex specifically and stably binds to replication origins in fission yeast. To examine the relationship between ORC and the Cdc13/Cdc2 complex, the ability of Cdc13 to associate with replication origins was examined in cells bearing a temperature-sensitive *orp1-4* mutation. Although Cdc13 bound ARS2004 at the permissive temperature, binding was reduced by 80% after 30 min incubation at the restrictive temperature in *orp1-4* cells, but not in the wild-type control culture (Figure 1F). Loss of Cdc13 association was not due to proteolytic destruction of Cdc13 as cells accumulated in G1, since *orp1-4* cells retained a 2C DNA during this procedure (data not shown). A similar ORC dependence was observed in temperature-sensitive *orp2-2* cells (data not shown). We conclude Cdc13/Cdc2 binds indirectly to replication origins via association to ORC.

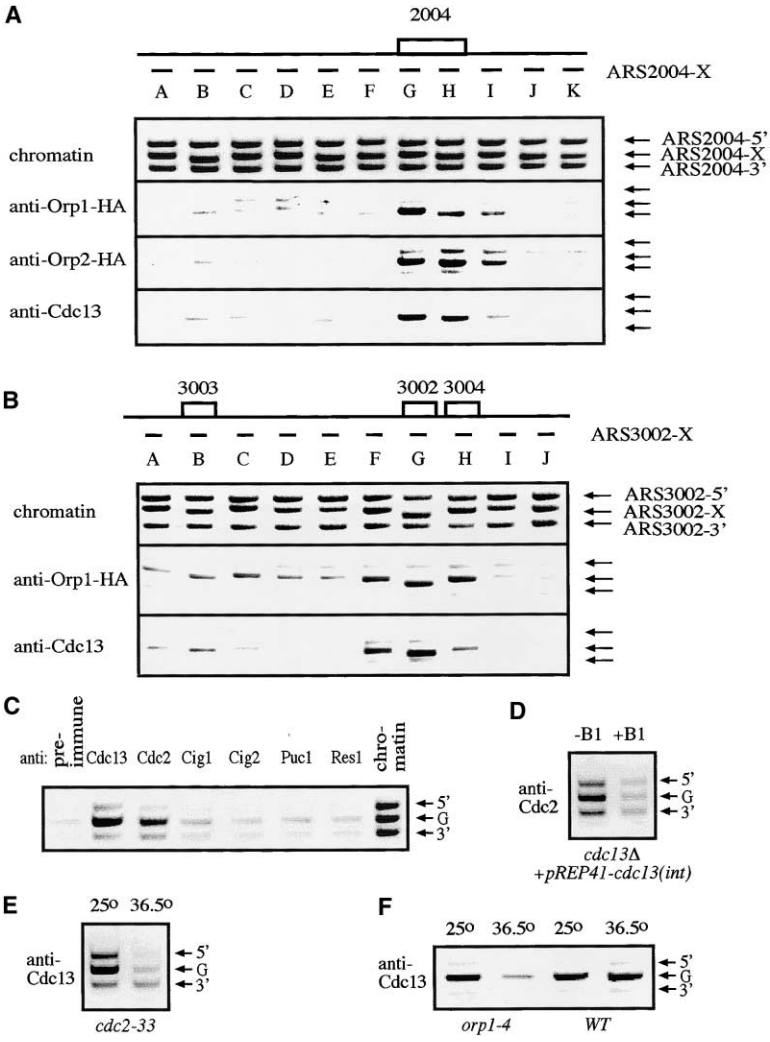

Figure 1. Mitotic Cyclin B/Cdc2 Kinase Binds to Replication Origins In Vivo

(A) Lysates from log phase cultures of *orp1-3HA*, *orp2-3HA*, or wild-type cells were subjected to ChIP analysis using either anti-HA or anti-Cdc13p antisera. Total chromatin from cell lysates (chromatin) or DNA associated with immunoprecipitates was amplified using primers that amplify consecutive ~300 bp fragments (A–K) along the ARS2004 locus (ARS2004-X). Control primers were included that amplify a 382 bp region located 5 kb 5′ of ARS2004 (ARS2004-5′) or a 280 bp region located 5 kb 3′ of ARS2004 (ARS2004-3′).

(B) Lysates were prepared from log phase *orp1-3HA* or wild-type cells and ChIP analysis performed with either anti-HA or anti-Cdc13 antibodies, respectively. Total chromosomal DNA from cell lysates (chromatin) or DNA purified from immunoprecipitates was amplified using a set of primers that amplify consecutive ~300 bp fragments (A–J) along the ARS3002 locus (ARS3002-X) and control primers as above.

(C) Wild-type cells were subjected to ChIP analysis using preimmune serum, anti-Cdc13, anti-Cdc2, anti-Cig1, anti-Cig2, anti-Puc1, or anti-Res1 antibodies. Coprecipitated DNA was amplified using oligos that amplify the ARS2004-5′ (5′), ARS2004-G (G), or ARS2004-3′ (3′) regions.

(D) *cdc13::ura4 nmt41-cdc13* cells were grown in the absence (−B1) or in the presence (+B1) of thiamine and ChIP performed using anti-Cdc2 serum. Purified DNA was amplified as before.

(E) *cdc2-33* cells were grown to log phase at 25°C and then either kept at 25°C or heat shocked at 42°C for an additional 30 min. Lysates were immunoprecipitated with anti-Cdc13 antibody and purified DNA amplified as before.

(F) Wild-type or *orp1-4* cells were grown to log phase at 25°C and then either kept at 25°C or shifted to 36°C for an additional 30 min. ChIP analysis was performed using anti-Cdc13 antibodies as described above.

Mutually Exclusive Association of Cdc13/Cdc2 and Mcm4 to Replication Origins

In growing cultures of fission yeast, recruitment of Mcm proteins to chromatin occurs transiently as cells pass through G1. A culture enriched for G1 cells was prepared by synchronous release of a G2-arrested *cdc25-22* strain to the permissive temperature. After crosslinking and immunoprecipitation with an anti-Mcm4 antibody, associated chromosomal DNA was amplified using the same set of oligos as described in Figure 1, which recognize successive ~300 bp segments across the ARS2004 locus. We found that Mcm4 binds to the same region of ARS2004 as Cdc13/Cdc2 and ORC complex (Figure 2A). To examine the relative timing of Cyclin B and Mcm4 association, temperature-sensitive *cdc25-22* cells were arrested in late G2 and synchronously released into the next cell cycle by rapid cooling to the permissive temperature. We found that Cdc13 bound the ARS2004 origin in late G2, transiently dissociated as cells passed through anaphase, and then reappeared as cells entered the next S phase (Figure 2B). Conversely, Mcm4 was absent in G2 and only associated with ARS2004 as cells entered G1, consistent with previous observations (Figure 2B). However, since G1 phase is short in these cells, we could not tell whether reassociation of Cdc13 occurred before, simultaneous to, or after Mcm4 dissociation. To answer this question, the experiment was repeated using cells bearing a temperature-sensitive mutation in the Cdc10 transcription factor (*cdc10-V50*), which arrest in G1 at the restrictive temperature. Since the expression of Cdt1 and Cdc18, which act as loading factors for Mcm4, are dependent on Cdc10, no association of Mcm4 to ORC was observed in *cdc10-V50* cells at 36°C (Figure 3A). Likewise, since Cdc13 Cyclin B is targeted for degradation in G1 by the $APC^{Ste9/Srw1}$ E3 ubiquitin ligase, no binding of Cdc13 to ARS2004 was observed in these cells (Figure 3A). However, on release to the permissive temperature, Mcm4 bound ARS2004

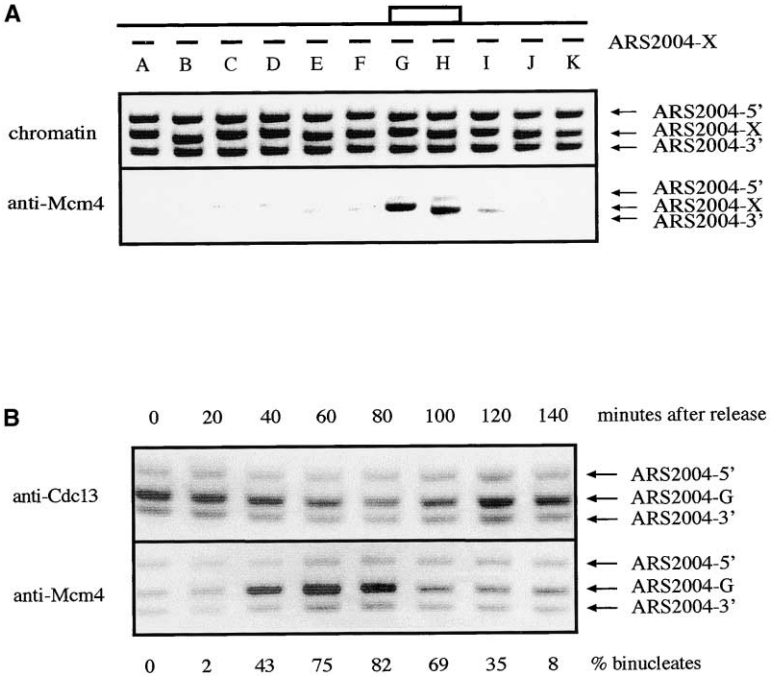

Figure 2. Association of Cdc13 and Mcm4 to Replication Origins Is Mutually Exclusive

(A) G1 cells collected after synchronous release of a G2-arrested *cdc25-22* strain to the permissive temperature for 60 min were subjected to ChIP analysis using anti-Mcm4 antibodies. Total chromatin (chromatin) or DNA associated with immunoprecipitates was amplified using primers that amplify consecutive ~300 bp fragments (A–K) along the ARS2004 locus (as in Figure 1).

(B) *cdc25-22* cells were synchronized in late G2 by incubation at 36.5°C for 4 hr. After release to 25°C, association of Cdc13 and Mcm4 to the ARS2004 origin was determined by ChIP analysis at the time points indicated. Cell cycle position was determined by assessing the percentage of binucleate cells by DAPI staining.

prior to S phase initiation and then dissociated coincidentally with the association of Cdc13 (Figures 3A and 3B). These data suggest that the Cdc13/Cdc2 complex associates with replication origins only in G2 and early mitosis and that binding of Cdc13/Cdc2 and Mcm4 binding are mutually exclusive.

Association of Mitotic Cyclin B/Cdc2 to ORC Prevents Diploidization

It has previously been shown that overexpression of Cdc13 in G1 prevents entry into S phase and triggers an aberrant mitosis without nuclear division (Fisher and Nurse, 1996). To examine the state of replication origins in this situation, *cdc10-V50 nmt41-cdc13 cdc13::ura4* cells were arrested in G1 and then released either in the presence or absence of thiamine. In the presence of thiamine, which suppresses expression from the *nmt41* promoter, Mcm4 bound to ARS2004 prior to a round of DNA replication. However, ectopic expression of *cdc13* caused binding of Cdc13 to replication origins, displacement of Mcm4, and inhibition of S phase initiation (Figures 3C and 3D). These data suggest that association of the mitotic Cyclin B/Cdc2 kinase with replication origins in G1 prevents replication licensing and the onset of S phase. Since Orp2 was initially identified by two-hybrid analysis as a protein that interacts with Cdc2, we reasoned it may act as a "docking site" for Cdc13/Cdc2 at replication origins. We postulated that cells expressing a mutant Orp2 protein to which Cdc13 is unable to bind may be susceptible to chromosomal reduplication. During the course of these experiments, we identified such a mutant. We noted that *orp2-3HA* cells underwent spontaneous diploidization at high frequency. Phloxin B, which is a stain that accumulates in dead cells, was used to differentiate between haploid and diploid colonies on plates. Diploid colonies contain 1%–5% dead cells and stain dark red, whereas haploid colonies contain fewer dead cells and stain light pink. When plated on Phloxin B-containing media, 15% of colonies stained dark red and were found to have a 4C DNA content by FACS analysis, whereas the remainder stained light red and were found to have a 2C DNA content (Figure 4A). Such diploidization has previously been associated with rereplication of DNA. A similar tendency to diploidize was observed in *orp2-protA* cells but not in wild-type cells, *orp1-3HA* cells, or the temperature-sensitive *orp1-4* or *orp2-2* strains (data not shown). Although the Orp2-3HA protein readily cross-links to replication origins (Figure 1A), association of Cdc13 to origins was reduced by between 65% and 85% in *orp2-3HA* cells depending on the origin analyzed (Figures 4B and 4C), an effect that was fully restored by ectopic expression of wild-type *orp2* (Figure 4D), as was the tendency of *orp2-3HA* cells to diploidize (data not shown).

Orp2 is phosphorylated on four Cdk-consensus sites in vivo (Vas et al., 2001). Importantly, no spontaneous diploidization or loss of Cdc13 binding to replication origins was observed in *orp2-T4A* cells, in which all four Cdk-consensus sites had been mutated to non-phosphorylatable residues (Figures 4E and 4F). This prompted us to examine the ability of *orp2-3HA* cells to restrain replication licensing. In wild-type cells, high overexpression of *cdc18* alone causes constitutive association of Mcm4 to replication origins and overreplication, whereas mild overexpression of *cdc18* from the pREP41 promoter has little or no effect unless *cdt1* is simultaneously overexpressed (Nishitani and Nurse, 1995; Nishitani et al., 2000; Yanow et al., 2001). However, mild overexpression of *cdc18* causes association of Mcm4 to ARS2004 and the formation of giant nuclei with up to 16C DNA content in *orp2-3HA* cells (Figures 4G

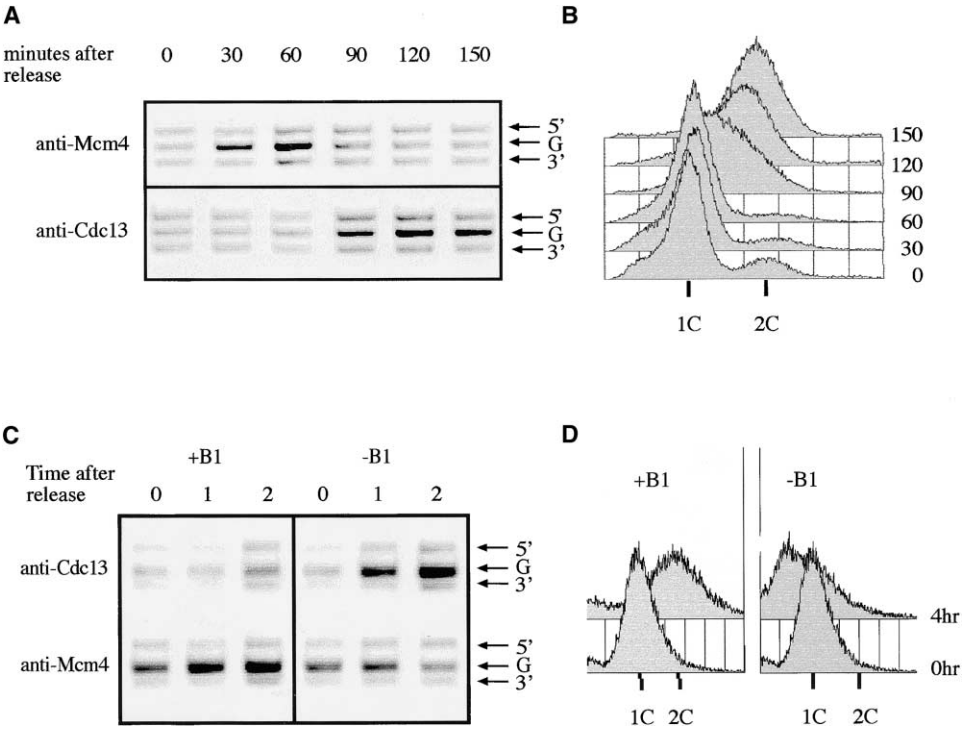

Figure 3. Cyclin B/Cdc2 Prevents Association of Mcm4 to Replication Origins

(A) *cdc10-V50* cells were synchronized in G1 by incubation at 36.5°C for 4 hr and release to 25°C. Association of Cdc13 and Mcm4 to ARS2004 was determined by ChIP analysis using oligos described above.

(B) DNA content of *cdc10-V50* cells was measured by FACS analysis. S phase occurs at 90 min.

(C) *cdc10-V50 cdc13::ura4 nmt41-cdc13* cells were arrested in G1 by incubation at 36.5°C for 4 hr and release to 25°C in the presence (+B1) or absence (−B1) of thiamine. Association of Cdc13 and Mcm4 to ARS2004 was determined by ChIP analysis as above.

(D) DNA content of G1-arrested *cdc10-V50 cdc13::ura4 nmt41-cdc13* cells was measured by FACS analysis before (0 hr) or after 4 hr (4 hr) incubation in the presence (+B1) or absence of thiamine (−B1) at 25°C.

and 4H). Since ORC is constitutively bound to replication origins throughout the fission yeast cell cycle, these data strongly argue that association of the mitotic Cdc13/Cdc2 kinase to ORC prevents reinitiation of DNA replication during G2 and early M phase, by a more complex mechanism than simply phosphorylation of Orp2.

Ablation of Cdc13 Causes Mcm4 Association to Chromatin Only in a Subset of Cells

Destruction of the Cdc13 Cyclin B/Cdc2 kinase causes cells to undergo chromosomal rereplication, resulting in highly elongated cells with giant nuclei. In this situation FACS analysis reveals distinct doublings of cellular DNA content, suggesting that cells undergo complete rounds of endoreduplication. By contrast, no such integral peaks of cellular DNA content are observed when *cdc18* is overexpressed. To examine the state of replication origins in these two situations, *cdc13::ura4 nmt41-cdc13* or *nmt1-cdc18* cells were grown either in the presence or absence of thiamine (Figures 5A and 5B). Since only 10% of exponentially growing cells are in G1, association of Mcm4 to ARS2004 is barely detectable in *cdc13::ura4 nmt41-cdc13* cells in the absence of thiamine or in *nmt1-cdc18* in the presence of thiamine, whereas Cdc13 is readily detectable. Suppression of *cdc13* expression or overexpression of *cdc18* causes the disappearance of Cdc13 from and binding of Mcm4 to ARS2004 in asynchronous rereplicating cells (Figures 5A and 5B). To examine association of Mcm4 to chromatin in individual rereplicating cells, Mcm4 was tagged with GFP and visualized using a previously described in situ chromatin binding assay (Kearsey et al., 2000). When *cdc18* is overexpressed, Mcm4-GFP associates to nuclei in all cells examined (Figure 5C). By contrast, in cells rereplicating after *cdc13* repression, only 60% of the nuclei stained positively with Mcm4-GFP (Figure 5C). These data indicate that inactivation of *cdc13* and overexpression of *cdc18* have qualitatively distinct effects on Mcm loading and thus pre-RC formation.

Replication Origins Are Periodically Licensed in the Absence of Cdc13

To examine whether replication origins may be periodically licensed in the absence of Cdc13, *cdc2-33 cdc13::ura4 nmt41-cdc13(LEU2) mcm4-GFP* cells were arrested in G1 by nitrogen starvation for 12 hr, refed with a nitrogen source at high temperature to inactivate Cdc2, and then released to the permissive temperature. This procedure allowed cells to undergo a synchronized endoreduplication cycle, as judged by FACS analysis (Figure 6C). We found that during the first G1 phase,

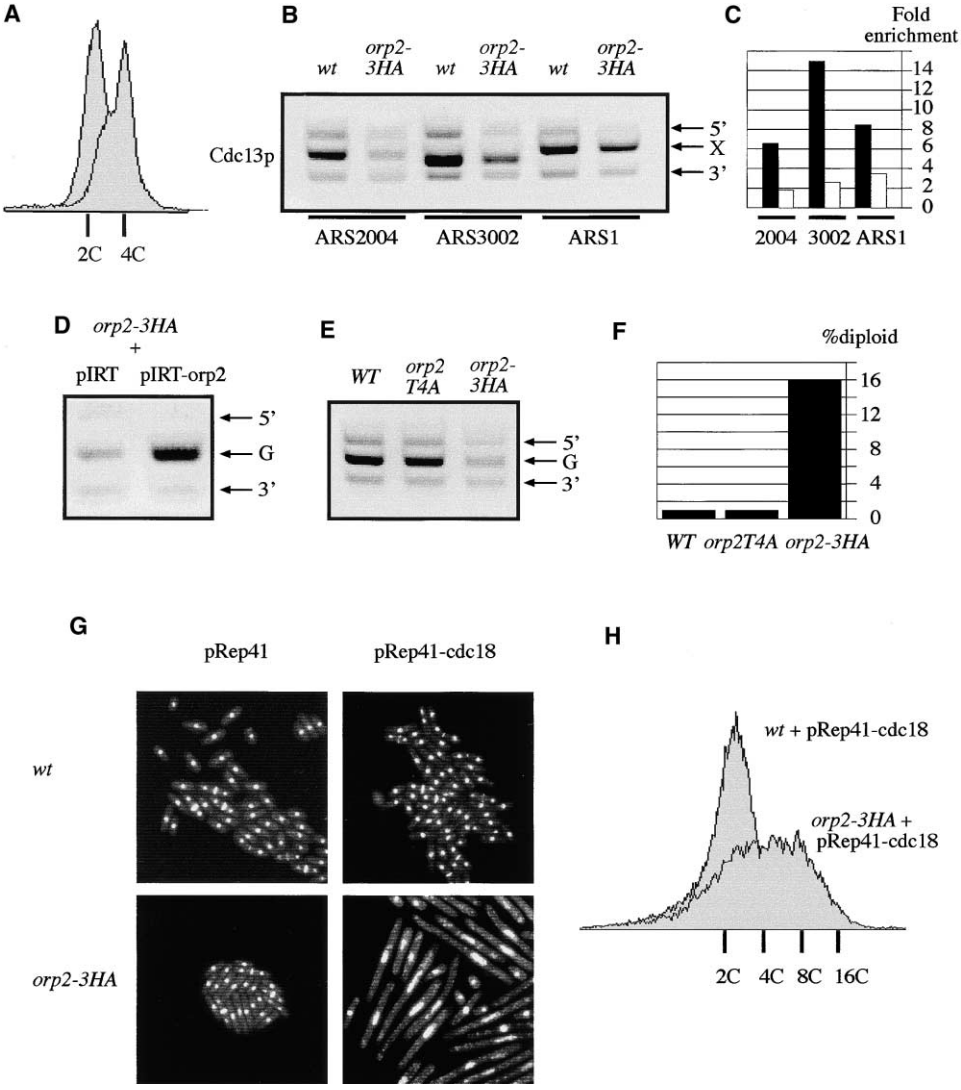

Figure 4. Association of Cyclin B/Cdc2 to ORC Prevents Endoreduplication

(A) A freshly isolated colony of *orp2-3HA* cells was inoculated into fresh YES medium and grown to exponential phase. Cells were diluted and plated onto fresh YES medium containing phloxin B (500 cells/plate) and incubated for an additional 2 days at 30°C. 15% of plated colonies stained dark red and the remainder light red. Cells from one dark red and one light red colony were inoculated into fresh YES medium and grown to mid-log phase. DNA content was analyzed by FACS analysis.

(B) Association of Cdc13 to the ARS2004, ARS3002, and ARS1 origins in log phase wild-type and *orp2-3HA* cells was determined by ChIP analysis as described above. Oligos were used that amplify the ARS2004 origin (ARS2004-G), ARS3002 origin (ARS3002-G), or the ARS1 (ARS1) origin. Oligos that amplify regions outside the ARS2004 locus (ARS2004-5′ and ARS2004-3′) were used as controls.

(C) Cdc13 binding signal to each origin was normalized with the signal from the corresponding control fragments. Bars indicate the fold enrichment in Cdc13 binding in either wild-type cells (black bars) or *orp2-3HA* cells (white bars) as an average from three independent experiments.

(D) Log phase *orp2-3HA* cells transformed with either empty vector (pIRT) or a plasmid expressing wild-type *orp2* from its own promoter (pIRT-orp2) were fixed with formaldehyde and association of Cdc13 to ARS2004 determined by ChIP analysis as above.

(E) Binding of Cdc13 to ARS2004 in wild-type, *orp2-T4A*, or *orp2-3HA* cells was determined by CHIP analysis as above.

(F) Quantitation of the number of colonies containing diploid cells in wild-type, *orp2-T4A*, and *orp2-3HA*. Cells were grown in liquid medium (YES) to stationary phase and then 500 cells were plated on phloxin-B-containing plates. Diploid colonies were scored.

(G) Wild-type or *orp2-3HA* cells transformed with either empty plasmid (pRep41) or a plasmid expressing *cdc18* from the medium-strength nmt41 promoter (pRep41-cdc18) were grown to early log phase in minimal medium containing thiamine, washed, and then reinoculated into fresh minimal medium in the absence of thiamine. After 18 hr, cells were fixed and stained with DAPI and calcofluor to visualize nuclei and septa, respectively.

(H) Wild-type or *orp2-3HA* cells overexpressing *cdc18* from the pRep41 promoter (pRep41-cdc18) were fixed and DNA content was analyzed by FACS analysis.

Mcm4-GFP stains unreplicated nuclei and disappears as cells progress through S phase, reappears in the following pseudo-G1 phase, and disappears concomitant with the second round of replication (Figure 6A). Note that both cell length and the size of the nuclei increase during the period of this experiment (Figure

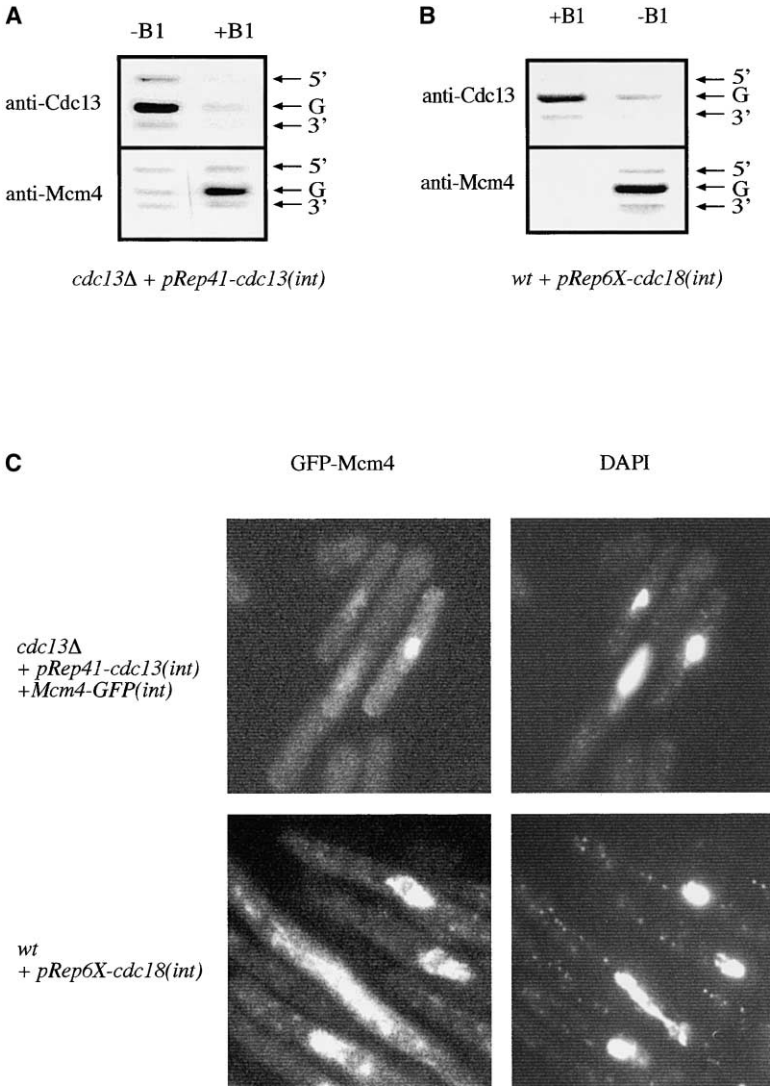

Figure 5. Mcm4 Association to Chromatin during Rereplication Induced by Cdc18 Overexpression or by Cdc13 Ablation

(A) Log phase *cdc13::ura4 nmt41-cdc13* cells were grown for 14 hr in the absence (−B1) or presence (+B1) of thiamine. Association of Cdc13 and Mcm4 to the ARS2004 origin was analyzed as above.

(B) Log phase *nmt1-cdc18* cells were grown in the absence (−B1) or presence (+B1) of thiamine for 18 hr. Association of Cdc13p and Mcm4p to the ARS2004 origin was analyzed by ChIP as described above.

(C) *cdc13::ura4 nmt41-cdc13 mcm4-GFP* cells (top) and *pRep6X-cdc18 mcm4-GFP* cells (bottom) were grown in the presence (*cdc13::ura4 nmt41-cdc13 mcm4-GFP* cells, 14 hr) or absence (*pRep6X-cdc18 mcm4-GFP* cells, 24 hr) of thiamine. Chromatin-associated Mcm4 was visualized (left) as described in the Experimental Procedures. Nuclei were visualized by DAPI staining (right).

6A). To examine licensing on individual origins, *cdc2-33 cdc13::ura4 nmt41-cdc13(LEU2)* cells were synchronized by an identical procedure and association of Mcm4 to the ARS2004 origin was examined by ChIP analysis. We observe Mcm4 binding and dissociation to the ARS2004 origin during each round of S phase with exactly the same periodicity as that observed above (Figure 6B). We conclude that all origins are coordinately licensed during an endoreduplication cycle lacking the Cdc13/Cdc2 kinase. The periodicity of S phase initiation and fluctuations in Mcm4 association to chromatin are similar to that observed in a normal cell cycle.

Orp2 Is Transiently Phosphorylated in Endoreduplicating Cells

Overexpression of *rum1* in fission yeast is thought to cause cells to arrest with overreplicated giant nuclei both by directly inhibiting the activity of the Cdc13/Cdc2 kinase and by promoting its destruction (Moreno and Nurse, 1994; Correa-Bordes and Nurse, 1995; Correa-Bordes et al., 1997). To examine whether Orp2 is phosphorylated in rereplicating cells, the Rum1 Cdk inhibitor was overexpressed in *orp2-3HA* cells and cell extracts analyzed by Western blot using Orp2-specific antibodies. Under repressive conditions, Orp2 remains primarily in its phosphorylated state and Cdc13 is detected on replication origins (Figures 7A and 7B). By contrast, overexpression of *rum1* causes loss of Cdc13 association to ARS2004 and dephosphorylation of Orp2, although some slower migrating forms were also observed (Figures 7A and 7B). To examine the status of Orp2 phosphorylation in endoreduplicating cells in more detail, *cdc2-33 cdc13::ura4 nmt41-cdc13(LEU2)* cells were arrested in G1 and released into a synchronized endoreduplication cycle as described above and Orp2 was analyzed at various times after release by Western blot. As shown in Figure 7, Orp2 undergoes a clear shift to a slower migrating form as the cells pass through each successive S phase (Figure 7C). These results suggest that although Cdc13/Cdc2 is primarily responsible for phosphorylation of Orp2 in wild-type cells, a kinase that is distinct from Cdc13/Cdc2 phosphorylates Orp2 during each successive S phase of an endoreduplication cycle.

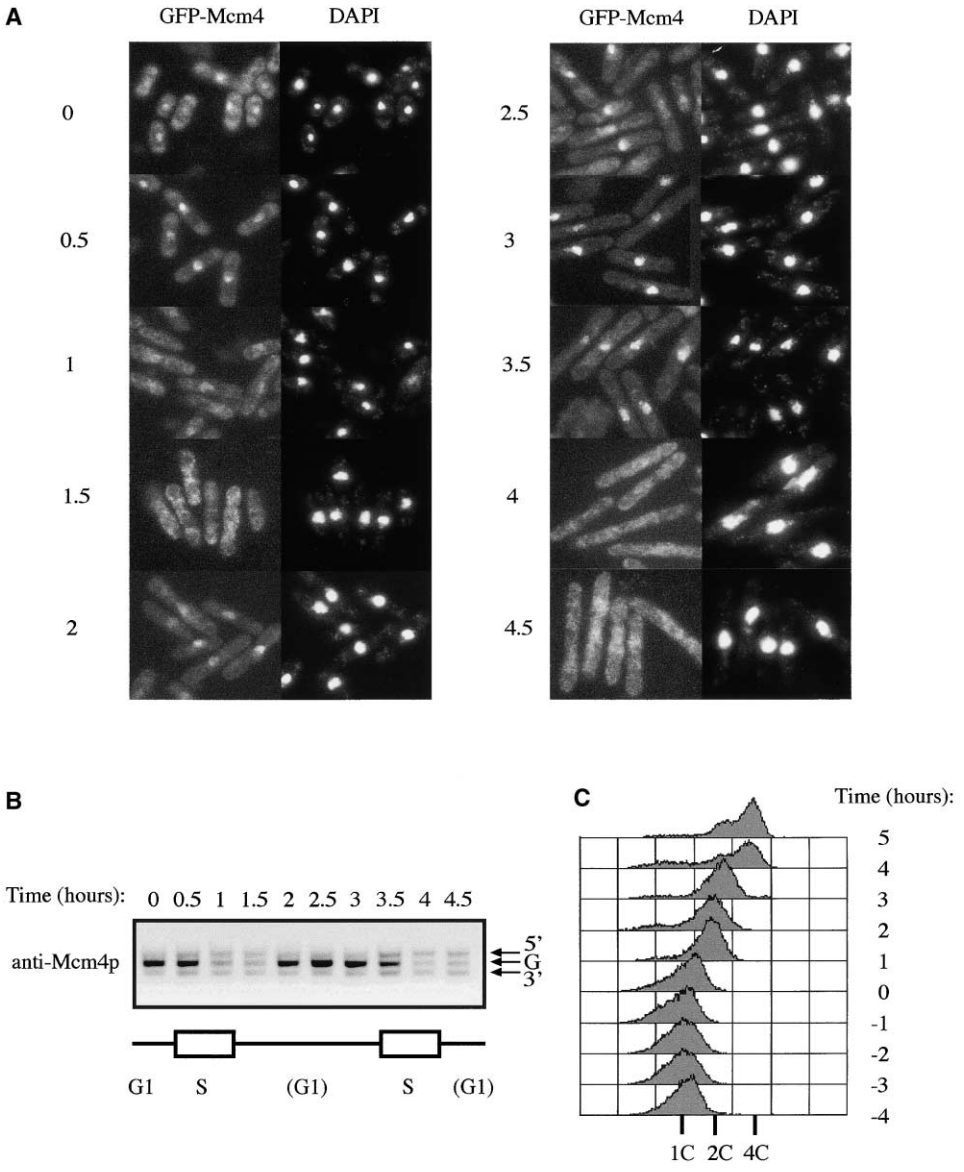

Figure 6. Periodic Association of Mcm4 to Replication Origins during Endoreduplication

(A) *cdc2-33 cdc13::ura4 nmt41-cdc13 mcm4-GFP* cells were arrested in G1 phase and released into a synchronous endoreduplication cycle as described in the Experimental Procedures. Cells were collected at the indicated time points and chromatin-associated Mcm4-GFP visualized by fluorescence microscopy. Nuclei were visualized by DAPI staining.

(B) *cdc2-33 cdc13::ura4 nmt41-cdc13* cells were induced to undergo synchronous endoreduplication as in (A). Cells were collected every 30 min and ChIP analysis performed using anti-Mcm4 antibodies. Immunoprecipitated DNA was amplified with primers amplifying ARS2004-G.

(C) DNA content of *cdc2-33 cdc13::ura4 nmt41-cdc13* cells was measured using FACS analysis during the time course described in (B) above.

Cig2 Transiently Binds the *cdc18, cdt1*, and *cig2* Promoters but not Replication Origins

Fission yeast cells lacking both *cig2* and *cdc13* are unable to undergo rereplication, suggesting that Cig2 controls S phase initiation (Mondesert et al., 1996). In addition, Cig2/Cdc2 binds and phosphorylates Res1 in vitro, a component of the Cdc10-dependent transcription complex (also known as MBF) to suppress transcription of the *cig2*, *cdt1*, and *cdc18* genes (Ayté et al., 2001). For these reasons, we wished to examine whether Cig2 associates with replication origins or the promoters of genes under the control of MBF. Transient accumulation of Cig2 at the G1-S border is dictated both by MBF and by the combined actions of the APC and SCF ubiquitin ligases (Yamano et al., 2000). We find that, in synchronized *cdc25-22* cells, Cig2 transiently associates with the *cdc18* promoter for a 4 min period prior to S phase initiation, coincident with the peak of *cig2* and *cdc18* expression but not to ARS2004 or other replication origins (see Supplemental Figures S1A and S1B at http://www.cell.com/cgi/content/full/111/3/419/DC1; data not shown). Cdc10 remained bound to the *cdc18* promoter throughout the cell cycle (see Supplemental Figure S1B). To map the site of Cig2 association to the *cdc18* gene, we employed a chromatin scanning protocol. This revealed that Cig2 binds the *cdc18* promoter

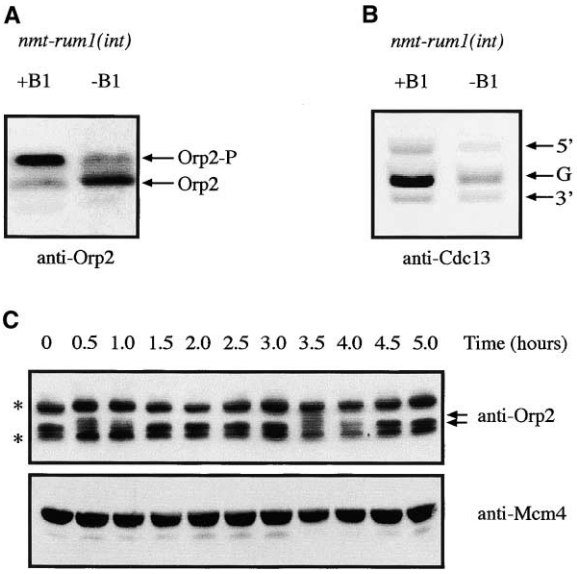

Figure 7. Periodic Orp2 Phosphorylation during Synchronous Endoreduplication

(A) *nmt1-rum1* cells were grown in the presence (+B1) or absence (−B1) of thiamine for 24 hr. Cell lysates were prepared and proteins analyzed by Western blot. Orp2 was detected using anti-Orp2 antibodies.

(B) *nmt1-rum1* cells were grown in the presence (+B1) or absence (−B1) of thiamine for 24 hr and association of Cdc13 to the ARS2004 origin determined by ChIP analysis as described above.

(C) *cdc2-33 cdc13::ura4 nmt41-cdc13* cells were induced to undergo synchronous rereplication as described above. Cells were collected every 30 min and Orp2 protein detected by Western blot using anti-Orp2 antibodies. The asterisks indicate two bands resulting from nonspecific interaction of proteins with the anti-Orp2 antibody.

in a region that overlaps the binding site for MBF (see Supplemental Figure S1C). Cig2 also associates to its own promoter and to the promoter of the *cdt1* gene at this time (see Supplemental Figure S1D). These results suggest that in a normal cell cycle, the Cig2/Cdc2 kinase specifically and transiently binds to the promoters of MBF-dependent genes in late G1 but not to replication origins. We next examined if Cig2/Cdc2 dictates replication licensing in the absence of Cdc13. On release of *cdc2-33 cdc13::ura4 nmt41-cdc13* cells into a endoreduplication cycle, we were also unable to detect association of Cig2 to ARS2004 as Mcm4 dissociated from the origin (see Supplemental Figure S2A), even though Cig2 transiently associated to the *cdc18* promoter during this period (see Supplemental Figure S2B). Thus, Cig2/Cdc2 is unlikely to be responsible for the phosphorylation of Orp2 in the absence of Cdc13. To assess whether Cig2/Cdc2 may regulate replication licensing by catalyzing the destruction of nonchromatin bound Cdc18 protein, we examined the expression of Cig2 and Cdc18 during an endoreduplication cycle. We found that both *cdc18* and *cig2* mRNAs and their corresponding proteins are both periodically expressed in synchronous endoreduplicating cells lacking Cdc13 (see Supplemental Figures S2C and S2D). We noted that the Cdc18 protein is degraded and MBF-dependent transcription is repressed coincidently with the peak of Cig2 protein expression during each endoreduplication cycle. Together, these results suggest that during an endoreduplication cycle, Cig2/Cdc2 kinase prevents relicensing of origins both by catalyzing the periodic destruction of Cdc18 and by regulating its own synthesis.

Discussion

In the work described in this paper, we demonstrate that, in fission yeast, replication origin bound mitotic cyclin B/Cdc2 kinase is the critical component of the postreplicative complex that ensures the maintenance of cell ploidy. These results suggest that the Cdc13/Cdc2 complex acts locally at replication origins to inhibit the formation of pre-RCs by phosphorylation of pre-RC components. These data support a simple model to explain why only G1 but not G2 nuclei have the potential to replicate. Namely, activation of the anaphase-promoting complex (APC), a specialized E3 ubiquitin ligase, during mitosis leads both to the dissolution of cohesion between sister chromatids and the removal of the mitotic Cyclin B/Cdc2 kinase from the origins of replicated nuclei. In this manner, licensing of replication origins and sister chromatid separation may be temporally coordinated. In addition we show that, in endoreduplicating cells lacking Cdc13, replication origins are periodically and coordinately licensed prior to each successive S phase with the same timing as in a normal cell cycle. Thus, the mechanisms that determine the *temporal* licensing of replication origins can be experimentally distinguished from the controls that maintain the *dependency* of S phase on an intervening mitosis. Furthermore, since cells lacking Cdc13 undergo complete genome duplications, we reason that the controls that maintain the correct order of origin firing within S phase and those that ensure each origin fires only once each rereplicative S phase must remain intact in the absence of Cdc13 (Cimbora and Groudine, 2001; Heun et al., 2001). In this respect, the experimentally induced rereplication cycle in fission yeast described here closely resembles the naturally occurring endoreduplication cycles observed in plants, flies, and certain human tissues.

Since ORC is constitutively bound to replication origins throughout the fission yeast cell cycle and Orp2 interacts with Cdc2 by two hybrid analysis, our data suggest Orp2 provides a docking site for Cdc13/Cdc2 at replication origins (Leatherwood et al., 1996; Lygerou and Nurse, 1999). Furthermore, mutational analysis of Cdc2-dependent phosphorylation sites has suggested that phosphorylation of Orp2 by Cdc2 contributes to suppression of pre-RC formation during a normal cell cycle (Vas et al., 2001). Importantly, cells that express a mutant Orp2 protein which substantially abrogates binding of Cdc13 to replication origins are acutely prone to diploidization, but this is not observed in cells in which all of the known Cdk phosphorylation sites in Orp2 have been abolished. We propose that ORC bound Cdc13/Cdc2 kinase acts locally at the replication origin to prevent pre-RC formation by phosphorylation of additional proteins such as Orc6, Cdc18, and possibly other components of the pre-RC complex. To formally verify this prediction, it would be necessary to simultaneously mutate all the phosphorylation sites on each of these pro-

teins, as has been attempted previously in budding yeast (Nguyen et al., 2001). However, we need first to consider whether the mechanisms that impose the dependency of S phase on an intervening mitosis are distinct from the controls that dictate the temporal licensing of replication origins during and between each S phase. Importantly, we show that origins are periodically licensed, Orp2 is periodically phosphorylated, and Cdc18 is periodically degraded in the absence of Cdc13/Cdc2. This may be due to the action of a distinct Cyclin/Cdk complex, such the Cig1/Cdc2 or Cig2/Cdc2 kinases. Importantly, cells lacking both *cdc13* and *cig1* undergo distinct rounds of S phase, indicating that the Cig1/Cdc2 kinase is not required for periodic licensing of origins in the absence of Cdc13/Cdc2. Our preliminary data suggest that Orp2 is not phosphorylated in the absence of Cdc13 and Cig1 (data not shown), suggesting that phosphorylation of Orp2 is not necessary for replication licensing in endoreduplicating cells, although we have failed to observe association of Cig1 to replication origins in either synchronized wild-type or rereplicating cells.

One possibility is that periodic licensing of replication origins in the absence of *cdc13* and *cig1* may be driven by the periodic activation of Cig2/Cdc2 kinase. This is consistent with the observation that overexpression of *cig2* blocks rereplication in Δ*cdc13* cells (Lopez-Girona et al., 1998). This might be analogous to the situation in endoreplicating *Drosophila* larval salivary glands, in which Mcm proteins are observed to exist in two states: either associated with or dissociated from chromosomes. Since fluctuations in cyclin E expression are needed for the endoreduplication cycles, it has been suggested that dissociation of MCM proteins from chromatin in these cells may be driven by periodic activation of cyclin E/Cdk2 (Weiss et al., 1998). However, we failed to observe association of Cig2 cyclin to replication origins in cycling normal or rereplicating cells, even though Cig2 associates to other specialized regions of chromatin, namely the promoters of genes regulated by the Cdc10 transcription factor (see Supplemental Figure S1 at http://www.cell.com/cgi/content/full/111/3/419/DC1). Thus, Cig2/Cdc2 may prevent the licensing of origins solely by catalyzing the phosphorylation-dependent destruction of nonchromatin bound Cdc18 as cells enter S phase. This is consistent with the observation that destruction of Cdc18 coincides with the peak of Cig2 expression and that temperature-sensitive *cdc18* mutants are suppressed by deletion of *cig2* (see Supplemental Figure S2; Lopez-Girona et al., 1998). An alternative possibility is that Cig2 may block endoreduplication by suppressing transcription through the Cdc10/Res1/Res2 complex rather than or, in addition to, causing destruction of Cdc18 (Ayté et al., 2001). Although periodic Cdc10-dependent transcription is not necessary for a normal cell cycle, its importance during an endoreduplication cycle has not been assessed. Regardless, these data suggest that the mechanisms that impose the dependency of S phase on an intervening mitosis and control licensing during an endoreduplication cycle are distinct and are regulated by distinct Cdk/cyclin complexes.

Previous studies have implied that Cyclin/Cdk complexes can act at replication origins to regulate DNA replication. Cyclin A/Cdk2 locates to replication foci in mammalian tissue culture cells and interacts with ORC in cell lysates. Second, ubiquitination of the CDK inhibitor XIC1 by Cyclin E/Cdk2 requires the formation of pre-RCs on chromatin (Cardoso et al., 1993; Romanowski et al., 2000; Furstenthal et al., 2001). This study provides the first direct demonstration that distinct Cyclin/Cdk complexes can bind to chromatin in a temporally and spatially restricted manner. More particularly, it demonstrates that stable association of mitotic Cyclin B/Cdc2 to replication origins inhibits rather than activates DNA replication. These data raise a number of important questions. First, is origin bound Cdc13/Cdc2 complex catalytically active during G2 or does it inhibit pre-RC formation by steric hinderance? We favor the former possibility, since Orp2 is hyperphosphorylated in the presence of Cdc13/Cdc2. This raises an intriguing paradox. Inactivation of the Cdc25 tyrosine phosphatase or overexpression of the Wee1 or Mik1 tyrosine kinases causes cells to arrest in G2 with inactive Cdc13/Cdc2 kinase, but does not induce rereplication (Russell and Nurse, 1986, 1987). One possibility that merits further investigation is that origin bound Cdc13/Cdc2 kinase is not accessible for tyrosine phosphorylation and thus remains active throughout G2 and can only be inactivated by proteolytic destruction in anaphase. If so, does the anaphase promoting complex act locally at origins to ubiquitinate Cdc13 or does the Cdc13/Cdc2 complex dissociate from origin prior to its ubiquitination and destruction? A third important question is whether binding of Cdc13 to origins merely reflects its abundance or whether accessory factors, other than ORC, are needed for loading of Cdc13/Cdc2 to chromatin. Lastly, it will be of interest to examine whether the mitotic Cyclin B/Cdc2 kinase associates to each individual origin as it is replicated or binds coordinately to all origins when S phase is complete. Careful analysis of Mcm4 and Cdc13 binding to early and late origins will be necessary to answer this question.

Recent evidence suggests metazoans have evolved additional mechanisms to prevent relicensing of origins to those found in the yeasts. Whereas the ORC complex remains bound to replication origins throughout the fission yeast cell cycle, in human cells the Orc1 protein undergoes phosphorylation-induced degradation after initiation of DNA replication (Lygerou and Nurse, 1999; Méndez et al., 2002). This may be an additional event catalyzed by a Cyclin/Cdk complex bound to the remaining ORC subunits at replication origins in human cells. More particularly, human and frog cells contain a protein called geminin that inhibits the activity of the Cdt1 loading factor and that, like cyclin B, is destroyed at anaphase (McGarry and Kirschner, 1998; Wohlschlegel et al., 2000; Tada et al., 2001). Geminin may operate to prevent ectopic formation of prereplicative complexes in G2. However, immunodepletion of geminin from cycling *Xenopus* extracts does not cause the repetitive initiation of S phase, suggesting that other mechanisms are involved. Fission yeast, which also replicates its chromosomes only once per cell cycle, does not contain an obvious geminin homolog. This argues that stable association of the mitotic Cyclin B/Cdc2 kinase to replication origins may be the primary mechanism by which the order of

S phase and M phase is maintained during the mitotic cell cycle of eukaryotes.

Experimental Procedures

Cell Culture, FACS Analysis, and Microscopy

All protocols for the culture, analysis of DNA content, and microscopic analysis of *Schizosaccharomyces pombe* can be found at http://www.bio.uva.nl/pombe/handbook/.

Cell Synchronization

A synchronous endoreduplication cycle was imposed in *cdc2-33 cdc13::ura4 nmt41-cdc13(LEU2)* cells. Cells were incubated in minimal medium lacking a nitrogen source for 12 hr to arrest them in G1. After this time, cells were supplemented with a nitrogen source and thiamine to suppress *cdc13* expression at 36.5°C for 4 hr (from time −4 to 0 hr) to arrest them in late G1. At time 0 hr, cells were shifted to 25°C in the same medium containing thiamine, which led to rapid and synchronous entry into S phase (S phase was completed after 1 hr). At time 1 hr, cells were shifted back to 36.5°C for 2 hr (from time 1 hr to 3 hr) and then incubated at 25°C, which induced a second round of S phase.

Construction of *orp2-3HA* Allele

Three copies of the haemagglutinin (HA) epitope and the kanamycin drug resistance gene were introduced at the C terminus of the *orp2* gene by homologous recombination using a standard protocol available at http://pingu.salk.edu/users/forsburg/vectors.html.

Biochemistry

Western blots were performed as previously described (Hayles et al., 1994). Anti-HA antibody (12CA5) (Babco) was used at 1:1000 dilution. Anti-Orp2 antibody (a generous gift of Dr. Paul Russell) was used at 1:1000 dilution. Anti-Cig2 antibody (Ayté et al., 2001) was used at 1:400 dilution, anti-Cdc18 (Nishitani and Nurse, 1995) was used at 1:400 dilution, and anti-Mcm4 antibody (Lygerou and Nurse, 1999) at 1:500 dilution.

Chromatin Immunoprecipitation (ChIP) Analysis

ChIP was performed as described previously with some modifications (Ogawa et al., 1999). Fission yeast cells (5×10^8) were fixed in 1% formaldehyde for 15 min at room temperature and then in 125 mM glycine for 5 min. After being washed twice with TBS and resuspended in 0.4 ml of lysis buffer (50 mM HEPES-KOH [pH 7.4], 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin), the cells were disrupted with glass beads by using a bead beater for 1 min. The broken cells were sonicated four times for 15 s each until chromatin DNA was sheared into 500 to 700 bp fragments. Supernatant obtained by centrifugation at 12,000 × g for 10 min was used for immunoprecipitation with either anti-Cdc13, anti-Cdc2, anti-Mcm4, anti-Cig1, anti-Cig2, anti-Puc1, anti-Res1 (J.W., unpublished), or anti-HA antibodies. Immunoprecipitates were washed and DNA was purified as described (Ogawa et al., 1999). PCR amplification by ampli-Taq Gold (Perkin-Elmer) was performed in 50 μl of supplemented buffer with a 1/50 dilution of immunoprecipitated DNA or a 1/3300 dilution of total DNA and a mixture of three sets of primers. All primers were used at a concentration of 0.3 μM. An initial incubation for 9 min at 95°C to activate Taq polymerase was followed by 29 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 53°C, and elongation for 2 min at 72°C and a final extension for 7 min at 72°C. PCR products were separated in 2.3% agarose gels and visualized with 0.5 μg/ml ethidium bromide. The gel images obtained with a charge-coupled device camera (Epi-UV FA1100; Aisin Cosmos) were processed by using Photoshop (Adobe).

Oligonucleotides Used in ChIP Analysis

The essential elements for *ARS2004* function locate between nucleotide 894 and 1552 using previously described nomenclature (Okuno et al., 1999). The amplified fragments A to K used to scan *ARS2004* locus are positioned at the following sites: fragment A, −2809 to −2466; B, −2170 to −1847; C, −1573 to −1244; D, −981 to −641; E, −364 to −45; F, 250 to 571; G, 828 to 1161; H, 1268 to 1588; I, 1869 to 2195; J, 2474 to 2791; and K, 3071 to 3390. Fragments G and H cover the elements that have been genetically characterized as being essential for *ARS2004* function. Using previously described nomenclature, the *ARS3003* replication origin is located between position 1 to 542, *ARS3002* at position 2763 to 3123, and *ARS3004* at position 3342 to 4804 (Dubey et al., 1994). Using this nomenclature, the fragments used to scan this locus are positioned at the following sites: fragment A, −649 to −308; B, −57 to 275; C, 575 to 918; D, 1133 to 1469; E, 1766 to 2105; F, 2363 to 2699; G, 2942 to 3254; H, 3462 to 3793; I, 4166 to 4494; and J, 4739 to 5077. The site of replication initiation of *ARS3002* is covered by fragments F and G, *ARS3003* by fragments B and C, and *ARS3004* by fragments H and I. The amplified fragments A to F scanning *cdc18* promoter region are positioned at the following sites (A from the initial ATG is +1): fragment A, −1878 to −1570; B, −1187 to −879; C, −691 to −386; D, −310 to −5; E, 210 to 524; and F, 903 to 1210. The control fragment 5′ of *cdc18* (*cdc18-5′*) is located at position −2668 to −2286, and the control fragment 3′ of *cdc18* (*cdc18-3′*) at position 3771 to 4050. The amplified fragment for *cig2* promoter is positioned at −1497 to −1177 from the ATG. Cdt1 promoter fragment is located at position −380 to −63 from the ATG.

Northern Analysis

Northern assays were performed as previously described (Aves et al., 1985). For detecting *cdc18* mRNA, the Northern blot was probed with a PCR fragment (1200 to 1582) of *cdc18* ORF. The probe for detecting *cig2* mRNA was a PCR fragment covering the entire *cig2* ORF.

In Situ Chromatin Binding Assay for Mcm4-GFP

In situ chromatin binding assay for Mcm4-GFP was as previously described with the following modifications (Kearsey et al., 2000). Briefly, cells (5×10^8) were collected, washed in 1.2 M sorbitol, and digested at room temperature in 500 μl 1.2 M sorbitol containing 2 μg/ml zymolyase until cells appeared phase dark. 500 μl 2% TritonX-100 was then added and cells were incubated for an additional 5 min at room temperature. Permeabilized cells were fixed by incubation in precooled methanol, rehydrated in PBS, spread onto polylysine-coated slides, and mounted in Vectashield mounting medium (Vector Laboratories) containing DAPI (0.4 μg/ml). Fluorescence microscopy was performed on a Deltavision system containing a Photometrics CH350L liquid-cooled CCD camera and Olympus IX70 inverted microscope with a 100× objective. Cell images were captured and manipulated using SoftWoRx software (Applied Precision Inc., Issaquah, WA) and Adobe Photoshop version 6.0 (Adobe Systems Incorp., Mountain View, CA).

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References

Aves, S.J., Durkacz, B.W., Carr, A., and Nurse, P. (1985). Cloning, sequencing and transcriptional control of the *Schizosaccharomyces pombe cdc10* 'start' gene. EMBO J. *4*, 457–463.

Ayté, J., Schweitzer, C., Zarzov, P., Nurse, P., and DeCaprio, J.A. (2001). Feedback regulation of the MBF transcription factor by cyclin Cig2. Nat. Cell Biol. *3*, 1043–1050.

Bell, S.P., Kobayashi, R., and Stillman, B. (1993). Yeast origin recognition complex functions in transcription silencing and DNA replication. Science *262*, 1844–1849.

Blow, J.J., and Laskey, R.A. (1988). A role for the nuclear envelope

in controlling DNA replication within the cell cycle. Nature *332*, 546–548.

Broek, D., Bartlett, R., Crawford, K., and Nurse, P. (1991). Involvement of $p34^{cdc2}$ in establishing the dependency of S phase on mitosis. Nature *349*, 388–393.

Cardoso, M.C., Leonhardt, H., and Nadal-Ginard, B. (1993). Reversal of terminal differentiation and control of DNA replication: cyclin A and Cdk2 specifically localize at subnuclear sites of DNA replication. Cell *74*, 979–992.

Chong, J.P., Mahbubani, H.M., Khoo, C.Y., and Blow, J.J. (1995). Purification of an MCM-containing complex as a component of the DNA replication licensing system. Nature *375*, 418–421.

Cimbora, D.M., and Groudine, M. (2001). The control of mammalian DNA replication: a brief history of space and timing. Cell *104*, 643–646.

Cocker, J.H., Piatti, S., Santocanale, C., Nasmyth, K., and Diffley, J.F. (1996). An essential role for the Cdc6 protein in forming the pre-replicative complexes of budding yeast. Nature *379*, 180–182.

Correa-Bordes, J., and Nurse, P. (1995). p25rum1 orders S phase and mitosis by acting as an inhibitor of the p34cdc2 mitotic kinase. Cell *83*, 1001–1009.

Correa-Bordes, J., Gulli, M.P., and Nurse, P. (1997). p25rum1 promotes proteolysis of the mitotic B-cyclin p56cdc13 during G1 of the fission yeast cell cycle. EMBO J. *16*, 4657–4664.

Diffley, J.F. (1996). Once and only once upon a time: specifying and regulating origins of DNA replication in eukaryotic cells. Genes Dev. *10*, 2819–2830.

Dubey, D.D., Zhu, J., Carlson, D.L., Sharma, K., and Huberman, J.A. (1994). Three ARS elements contribute to the *ura4* replication origin region in the fission yeast, *Schizosaccharomyces pombe*. EMBO J. *13*, 3638–3647.

Edgar, B.A., and Orr-Weaver, T. (2001). Endoreplication cell cycles: more for less. Cell *105*, 297–306.

Fisher, D.L., and Nurse, P. (1996). A single fission yeast mitotic cyclin B $p34^{cdc2}$ kinase promotes both S-phase and mitosis in the absence of G1 cyclins. EMBO J. *15*, 850–860.

Furstenthal, L., Swanson, C., Kaiser, B.K., Eldridge, A.G., and Jackson, P.K. (2001). Triggering ubiquitination of a CDK inhibitor at origins of DNA replication. Nat. Cell Biol. *3*, 715–722.

Gallagher, I.M., Alfa, C.E., and Hyams, J.S. (1993). $p63^{cdc13}$, a B-type cyclin, is associated with both the nucleolar and chromatin domains of the fission yeast nucleus. Mol. Biol. Cell *4*, 1087–1096.

Grallert, B., and Nurse, P. (1996). The ORC1 homolog orp1 in fission yeast plays a key role in regulating onset of S phase. Genes Dev. *10*, 2644–2654.

Hayles, J., Fisher, D., Woollard, A., and Nurse, P. (1994). Temporal order of S phase and mitosis in fission yeast is determined by the state of the $p34^{cdc2}$-mitotic B cyclin complex. Cell *78*, 813–822.

Hendrickson, M., Madine, M., Dalton, S., and Gautier, J. (1996). Phosphorylation of MCM4 by Cdc2 protein kinase inhibits the activity of the minichromosome maintenance complex. Proc. Natl. Acad. Sci. USA *93*, 12223–12228.

Heun, P., Laroche, T., Raghuraman, M.K., and Gasser, S.M. (2001). The positioning and dynamics of origins of replication in the budding yeast nucleus. J. Cell Biol. *152*, 385–400.

Ishimi, Y.A. (1997). DNA helicase activity is associated with an MCM4, -6, and -7 protein complex. J. Biol. Chem. *272*, 24508–24513.

Kearsey, S.E., Montgomery, S., Labib, K., and Lindner, K. (2000). Chromatin binding of the fission yeast replication factor Mcm4 occurs during anaphase and requires ORC and Cdc18. EMBO J. *19*, 1681–1690.

Kubota, Y., Mimura, S., Nishimoto, S., Takisawa, H., and Nojima, H. (1995). Identification of the yeast MCM3-related protein as a component of *Xenopus* DNA replication licensing factor. Cell *81*, 601–609.

Labib, K., Tercero, J.A., and Diffley, J.F. (2000). Uninterrupted MCM2–7 function required for DNA replication fork progression. Science *288*, 1643–1647.

Leatherwood, J., Lopez-Girona, A., and Russell, P. (1996). Interaction of Cdc2 and Cdc18 with a fission yeast ORC2-like protein. Nature *379*, 360–363.

Lopez-Girona, A., Mondesert, O., Leatherwood, J., and Russell, P. (1998). Negative regulation of Cdc18 DNA replication protein by Cdc2. Mol. Biol. Cell *9*, 63–73.

Lygerou, Z., and Nurse, P. (1999). The fission yeast origin recognition complex is constitutively associated with chromatin and is differentially modified through the cell cycle. J. Cell Sci. *112*, 3703–3712.

Madine, M.A., Khoo, C.Y., Mills, A.D., and Laskey, R.A. (1995). MCM3 complex required for cell cycle regulation of DNA replication in vertebrate cells. Nature *375*, 421–424.

Mahbubani, H.M., Chong, J.P., Chevalier, S., Thommes, P., and Blow, J.J. (1997). Cell cycle regulation of the replication licensing system: involvement of a Cdk-dependent inhibitor. J. Cell Biol. *136*, 125–135.

Maiorano, D., Moreau, J., and Mechali, M. (2000). XCDT1 is required for the assembly of pre-replicative complexes in *Xenopus laevis*. Nature *404*, 622–625.

McGarry, T.J., and Kirschner, M.W. (1998). Geminin, an inhibitor of DNA replication, is degraded during mitosis. Cell *93*, 1043–1053.

Méndez, J., Zou-Yang, X.H., Kim, S.Y., Hidaka, M., Tansey, W.P., and Stillman, B. (2002). Human origin recognition complex large subunit is degraded by ubiquitin-mediated proteolysis after initiation of DNA replication. Mol. Cell *9*, 481–491.

Micklem, G., Rowley, A., Harwood, J., Nasmyth, K., and Diffley, J.F. (1993). Yeast origin recognition complex is involved in DNA replication and transcriptional silencing. Nature *366*, 87–89.

Mondesert, O., McGowan, C.H., and Russell, P. (1996). Cig2, a B-type cyclin, promotes the onset of S in *Schizosaccharomyces pombe*. Mol. Cell. Biol. *16*, 1527–1533.

Moreno, S., and Nurse, P. (1994). Regulation of progression through the G1 phase of the cell cycle by the rum1+ gene. Nature *367*, 236–242.

Nguyen, V.Q., Co, C., and Li, J.J. (2001). Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms. Nature *411*, 1069–1073.

Nishitani, H., and Nurse, P. (1995). p65cdc18 plays a major role controlling the initiation of DNA replication in fission yeast. Cell *83*, 397–405.

Nishitani, H., Lygerou, Z., Nishimoto, T., and Nurse, P. (2000). The Cdt1 protein is required to license DNA for replication in fission yeast. Nature *404*, 625–628.

Noton, E., and Diffley, J.F. (2000). CDK inactivation is the only essential function of the APC/C and the mitotic exit network proteins for origin resetting during mitosis. Mol. Cell *5*, 85–95.

Ogawa, Y., Takahashi, T., and Masukata, H. (1999). Association of fission yeast Orp1 and Mcm6 proteins with chromosomal replication origins. Mol. Cell. Biol. *19*, 7228–7236.

Okuno, Y., Satoh, H., Sekiguchi, M., and Masukata, H. (1999). Clustered adenine/thymine stretches are essential for function of a fission yeast replication origin. Mol. Cell. Biol. *19*, 6699–6709.

Rao, P.N., and Johnson, R.T. (1970). Mammalian cell fusion: studies on the regulation of DNA synthesis and mitosis. Nature *225*, 159–164.

Romanowski, P., Marr, J., Madine, M.A., Rowles, A., Blow, J.J., Gautier, J., and Laskey, R.A. (2000). Interaction of *Xenopus* Cdc2/cyclin A1 with the origin recognition complex. J. Biol. Chem. *275*, 4239–4243.

Russell, P., and Nurse, P. (1986). cdc25+ functions as an inducer in the mitotic control of fission yeast. Cell *45*, 145–153.

Russell, P., and Nurse, P. (1987). Negative regulation of mitosis by wee1+, a gene encoding a protein kinase homolog. Cell *49*, 559–567.

Sigrist, S.J., and Lehner, C.F. (1997). *Drosophila* fizzy-related down-regulates mitotic cyclins and is required for cell proliferation arrest and entry into endocycles. Cell *90*, 671–681.

Tada, S., Li, A., Maiorano, D., Mechali, M., and Blow, J.J. (2001). Repression of origin assembly in metaphase depends on inhibition of RLF-B/Cdt1 by geminin. Nat. Cell Biol. *3*, 107–113.

Vas, A., Mok, W., and Leatherwood, J. (2001). Control of DNA rereplication via Cdc2 phosphorylation sites in the origin recognition complex. Mol. Cell. Biol. *21*, 5767–5777.

Weinreich, M., Liang, C., Chen, H.H., and Stillman, B. (2001). Binding of cyclin-dependent kinases to ORC and Cdc6p regulates the chromosome replication cycle. Proc. Natl. Acad. Sci. USA *98*, 11211–11217.

Weiss, A., Herzig, A., Jacobs, H., and Lehner, C.F. (1998). Continuous Cyclin E expression inhibits progression through endoreduplication cycles in *Drosophila*. Curr. Biol. *8*, 239–242.

Wohlschlegel, J.A., Dwyer, B.T., Dhar, S.K., Cvetic, C., Walter, J.C., and Dutta, A. (2000). Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. Science *290*, 2309–2312.

Yamano, H., Kitamura, K., Kominami, K., Lehmann, A., Katayama, S., Hunt, T., and Toda, T. (2000). The spike of S phase cyclin Cig2 expression at the G1-S border in fission yeast requires both APC and SCF ubiquitin ligases. Mol. Cell *6*, 1377–1387.

Yanow, S.K., Lygerou, Z., and Nurse, P. (2001). Expression of Cdc18/Cdc6 and Cdt1 during G2 phase induces initiation of DNA replication. EMBO J. *20*, 4648–4656.

Wuarin, J., and Nurse, P. (1996). Regulating S phase: CDKs, licensing and proteolysis. Cell *85*, 785–787.