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Multiple mechanisms prevent re-replication in budding yeast Saccharomyces cerevisiae

Author
Nguyen, Van Q.

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Multiple Mechanisms Prevent Re-replication in

Budding Yeast *Saccharomyces cerevisiae*

by

Van Q. Nguyen

DISSERTATION

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Dedicated to

Tinh T. T. Nguyen

Hiep Q. Nguyen

Duc Q. Nguyen

Hien D. Nguyen

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Chapters 2 and 5 of this dissertation are reprints of the material as they appear in the Current Biology, Nguyen, V. Q., Co, C., Irie, K., Li, J. J., (2000) **10**: 195-205.

Chapters 3 and 6 of this dissertation are reprints of the material as they appear in the Nature, Nguyen, V. Q., Co, C., Li, J. J., (2001) **411**: 1068-1073.

Contributions to Described Work

The work in Chapter 2 and 5 was a publication in Current Biology by myself, Carl Co, Kaoru Irie, and Joachim Li. I designed and executed all the experiments in Chapter 2 and 5, except for the experiments in Figures 1d, 2c, and 2d, which were carried out by Carl Co and Kaoru Irie.

The work in Chapter 3 and 6 was a publication in Nature by myself, Carl Co, and Joachim Li. I designed and executed all the experiments in Chapter 3 and 6, except for the experiments in Figures 1 and 3a, which were carried out by Carl Co and Joachim Li, respectively.

Multiple Mechanisms Prevent DNA Re-replication in

Budding Yeast *Saccharomyces cerevisiae*

Van Q. Nguyen

To stably propagate their genome, eukaryotic cells must replicate their DNA exactly once and only once per cell cycle. In eukaryotes, DNA replication initiates at multiple replication origins scattered throughout the genome. Re-initiation within a cell cycle must be prevented at each of these origins. Cyclin-dependent kinases (CDKs) have a central role in preventing re-initiation within a single cell cycle, but the molecular targets of the CDKs that mediate this inhibition are not known. My thesis project is to identify the critical targets of these kinases and to determine how phosphorylation of these targets inhibits re-initiation. The known pre-RC components at that time, ORC, Cdc6, and MCM are the most obvious candidates for inhibitory targets of the CDKs and the ones that I initially study. Cdc6 protein is periodically expressed during the cell cycle in G1 phase. Its absence at other points in the cell cycle is due to repression of *CDC6* transcription and promotion of Cdc6p degradation by CDKs. Deregulation of Cdc6 by constitutive overexpression of the protein, however, is not sufficient to trigger re-initiation. Mcm proteins are present in the nucleus in G1 and S phase, but exported to the cytoplasm in G2 and

M phase. I show that CDKs promote this export by directly phosphorylating Mcm3. Interestingly, I demonstrate that constitutive nuclear localization of the Mcm proteins is not sufficient to lead to re-initiation of DNA replication in G2/M cells, even if Cdc6, which requires to load Mcm proteins onto origins in G1, is ectopically expressed at the same time. I also show that ORC is phosphorylated in a cell cycle dependent manner by CDKs, but that disruption of this phosphorylation does not lead to re-initiation. Only when I simultaneously disrupt the regulation of ORC, Cdc6, and Mcms do I observe re-initiation and re-replication. This result demonstrates that each of these mechanisms is sufficient to block re-initiation and that CDKs use multiple overlapping mechanisms to ensure that the block is strictly maintained at every origin in the budding yeast genome. These results also provide one of the few examples where we have a mechanistic understanding of how CDKs regulate a key cell cycle event.

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University of California, San Francisco
Department of Microbiology and Immunology
513 Parnassus Avenue, Box 0414
San Francisco, CA 94143-0414
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CHAPTER 1

Introduction

Faithful duplication of genetic information is essential for all living organisms. This duplication is tightly coordinated with the progression of the cell cycle such that every segment of DNA is duplicated once and only once per cell cycle over many generations. This control requires that re-replication of DNA within a single cell cycle be prevented, and because replication is primarily controlled at the level of initiation, requires that re-initiation at replication origins be prevented within each cell cycle. In eukaryotes, this block to re-initiation must be maintained until the end of mitosis, at which time it is released to allow cells to prepare for replication initiation in next cell cycle. This thesis focuses on how cellular replication origins in eukaryotes are activated once and only once per cell cycle so as to maintain the precise duplication of the genome over many generations.

CONTROL OF DNA REPLICATION INITIATION IN *E. COLI*

Bacteria, bacteriophages, and animal viruses have provided the paradigms for understanding the initiation of DNA replication. In each of these systems, replication initiates at a single well-defined DNA sequence called the origin of DNA replication. Genetic and biochemical studies in these organisms have identified many molecular components required for replication initiation and provided an understanding of how these components work together to initiate DNA replication.

From these systems we know that there are several key tasks that must be executed in order to initiate DNA replication. First, initiator proteins must recognize and bind replication origins. Second, helicases must be loaded to separate the parental strands at the future replication forks. Third, the double-stranded DNA near the origin

must be unwound. Fourth, new daughter strand synthesis must be primed. Finally, the DNA synthetic machinery, which includes the DNA polymerases, must be loaded onto the nascent replication forks.

We understand most about the mechanism and control of replication initiation in the bacterium *Escherichia coli*. In this organism, DnaA is the initiator protein that recognizes and binds the replication origin, *oriC*. Approximately 20 DnaA molecules form a complex with *oriC* in which the DNA is wrapped around the proteins. DnaA is also responsible for inducing local unwinding of the double stranded DNA at the origin. DnaA then collaborates with DnaC to load the replicative helicase, DnaB, onto the single strands exposed by the local unwinding. Further unwinding by DnaB expands the single stranded region, which allows primase to initiate daughter strand synthesis. Finally, DNA polymerase III holoenzyme, which includes the core polymerase, a sliding ring-shaped clamp (β subunit), and a clamp loader (γ complex), loads onto the primed DNA to complete the assembly of the replication fork machinery (review in (Kornberg and Baker, 1992)).

In *E. coli*, there are at least three mechanisms that prevent re-initiation of origins within a single cell cycle. The first mechanism involves *oriC* sequestration, which makes replicated origins immediately inaccessible to DnaA binding. Central to this mechanism is a system for marking the DNA template so that replicated *oriC* can be distinguished from unreplicated *oriC*. *E. coli* replication origins contain an overrepresentation of GATC sequences, which are recognized and methylated by the enzyme Dam methyltransferase (Messer and Noyer-Weidner, 1988). When *oriC* is replicated, unmethylated nucleotides are incorporated into new DNA strands to form

hemimethylated DNA. Hence, the act of replication itself converts a fully methylated origin to two hemimethylated origins. A protein called SeqA strongly binds to hemimethylated *oriC* and appears to sequester the origin and prevent other proteins from recognizing it. By preventing DnaA from binding newly replicated origins, SeqA prevents re-initiation (Slater et al., 1995). SeqA mutants cannot sequester the origin and have a slight propensity to re-initiate DNA replication, confirming the role of origin sequestration in the block to re-initiation.

The second mechanism to prevent re-initiation involves the allosteric regulation of DnaA activity by ATP and ADP. DnaA protein binds both ATP and ADP with high affinity and is an ATPase that can slowly hydrolyze ATP to ADP. Both ATP and ADP bound forms of DnaA can recognize the origin, but only DnaA-ATP can unwind the origin and initiate replication (Sekimizu et al., 1987). The block to re-initiation is achieved by coupling the hydrolysis of ATP to the act of initiation. During initiation, the sliding clamp is loaded at the partially unwound origin. This loaded clamp acts with another protein Hda to stimulate the intrinsic hydrolytic activity of DnaA. This initiation induced hydrolysis converts DnaA-ATP to the inactive DnaA-ADP, thereby preventing re-initiation of DNA replication (Katayama et al., 1998). Mutations in DnaA that specifically disrupts hydrolysis of ATP cause the ATP bound form of DnaA to accumulate *in vivo* and make the cells susceptible to re-initiation. Similar results are obtained with Hda mutants. Thus, the initiation-coupled inactivation of DnaA by ATP hydrolysis is important in the block to re-initiation.

The third mechanism to prevent re-initiation at *oriC* involves the titration of free DnaA levels by binding to a high affinity site in the chromosome. The *datA* locus near

oriC has exceptional affinity and high capacity for binding to DnaA (Kitagawa et al., 1996). Replication of *datA* locus soon after initiation at *oriC* is thought to titrate out twice the amount of DnaA, therefore effectively reducing the level of free DnaA in the cell. As a result the amount of free DnaA available to bind *oriC* is greatly reduced, inhibiting re-initiation (Kitagawa et al., 1998). This model is supported by the observations that deletion of the *datA* promotes over-initiation, and additional copies of *datA* on a plasmid limit initiation or block it completely. Thus, *datA* is necessary and likely sufficient to inhibit re-initiation.

In summary, *E. coli* prevents re-initiation by down-regulating the levels or activity of the initiator protein that recognizes the origin, DnaA. This bacterium requires multiple mechanisms to prohibit re-initiation immediately after initiation. In the absence of any one mechanism, however, only a limited amount of re-initiation is observed, suggesting that the remaining mechanisms are still actively inhibiting (if not prohibiting) re-initiation. The simultaneous disruption of several mechanisms might be expected to result in a greater degree of re-initiation, but this experiment has not been done. Curiously, in several cases where re-initiation is observed, the resulting re-replication is restricted to a limited region surrounding the origin, suggesting that either re-elongation is inhibited or the forks assembled during re-initiation are defective.

Much remains to be discovered about how these inhibitory mechanisms are fully coordinated with the bacterial cell cycle. In particular, we do not understand how these mechanisms are turned off so that *oriC* can be initiated at the right time in the next cell cycle. It is not clear whether disengagement of these inhibitory mechanisms is sufficient

to allow the next round of initiation, or whether an additional activating or triggering signal is required.

EUKARYOTIC CELL CYCLE

The block to re-initiation is tightly coupled to the eukaryotic cell cycle. This was first illustrated by the classic experiments of Rao and Johnson. Synchronous HeLa cells at different stages of the cell cycle were fused to each other, and nuclear replication was examined. When S phase cells were fused to G1 phase cells, the G1 nuclei were stimulated to enter S phase and replicate their DNA. This result suggests that S phase cells contain a factor, SPF (S-phase promoting factor), which drives G1 cells into S phase. When G2 and S phase cells were fused, the S phase nuclei continued to replicate, but the G2 nuclei did not enter S phase. This result suggested that the G2 nuclei have lost the ability to respond to SPF. Thus, G1 phase cells but not G2 phase cells are in a replication-competent state. In order for cells to replicate again, they have to regain replication competence by passing through mitosis and completing the cell cycle. In this manner, replication of the eukaryotic genome is limited to one round per cell cycle (Rao and Johnson, 1970).

Further understanding of these competent states and their cell cycle regulation were hampered by the limited understanding of the molecular mechanisms that regulate the cell cycle and that initiate eukaryotic DNA replication. By the time I started this thesis work, however, significant progress had been made in understanding both of these mechanisms. In this section I will first summarize our molecular understanding of the

cell cycle. In the next, I will provide an outline of our understanding of the mechanism of eukaryotic replication initiation.

Cell cycle progression is driven by a family of protein kinases called cyclin-dependent kinases (CDKs). These kinases, which are composed of a cyclin regulatory subunit and a catalytic subunit, ensure that cell cycle events occur at the right time and in the right order. The regulation and function of these kinases is best understood in the budding yeast *Saccharomyces cerevisiae*. In this organisms, a single kinase catalytic subunit, Cdc28, can pair with one of nine different cyclin regulatory subunits: three G1 cyclins, Cln1-3, and six B-type cyclins, Clb1-6 (review in (Nasmyth, 1993)). Induction and association of Cln1-3 with Cdc28 in G1 phase allows cells to pass START, the point at which cells become committed to completing a round of DNA replication and cell division. The Cdc28-Cln kinases activate the next wave of CDKs, Cdc28-Clb, by inducing accumulation of Clbs and triggering the degradation of the Cdc28-Clb inhibitor Sic1 (Schwob et al., 1994; Verma et al., 1997). Clb5 and Clb6 are induced first, followed by Clb3 and Clb4, and then Clb1 and Clb2. Cdc28-Clb5 and Cdc28-Clb6 promote the initiation of DNA replication (Epstein and Cross, 1992; Schwob and Nasmyth, 1993), although in the absence of Clb5 and Clb6 later Clb kinases can perform the same function. Cdc28-Clb3 and Cdc28-Clb4 kinases have been implicated in proper spindle assembly (Segal et al., 2000), and Cdc28-Clb1 and Cdc28-Clb2 kinases are required for mitosis (Fitch et al., 1992). Finally, B-type CDKs must be inactivated by Clb destruction or Sic1 inhibition to allow exit from mitosis and entry into G1 phase of the next cell cycle.

Similar to budding yeast, fission yeast has a single kinase catalytic subunit, Cdc2/Cdk1, with multiple cyclin regulatory partners: puc1, and the B-type cyclins cig1, cig2, and cdc13. Puc1 is similar to Clns and promotes timely passage through G1. Cig1 and Cig2 are analogous to Clb5 and Clb6, and provide the primary means of triggering entry into S phase, although Cdc2-Cdc13 can substitute for them in their absence. Cdc2-Cdc13 is essential for entry into mitosis, and its inactivation is necessary for exit from mitosis (review in (Moser and Russell, 2000)).

In contrast to yeasts, metazoans have multiple kinase catalytic subunits (particularly cdk1/cdc2, cdk2, cdk4 and cdk6), which interact with different cyclin regulatory subunits (cyclin D, cyclin E, cyclin A, and cyclin B). Cyclin D in association with Cdk4 and Cdk6 in G1 may allow somatic cells to pass through the G1 commitment point by inducing activation of Cyclin E-Cdk2. The latter CDK has been implicated in the G1 to S transition and in some systems is thought to trigger replication initiation, similar to Cdc28-Clb5 and –Clb6. In other systems, Cyclin A-Cdk2 is thought to be required for initiation or at least proper passage through S phase. Finally, Cyclin A-Cdk1 and Cyclin B-Cdk1 are important for mitotic entry (review in (Obaya and Sedivy, 2002)).

Although the regulation of CDKs has been extensively studied, much less is known about how they promote specific cell cycle events. In most cases, we have little understanding of what the key protein targets of these kinases are, and how phosphorylation of these proteins induces various cell cycle events. Moreover, we do not understand what determines which kinases are capable of triggering which events.

CDK CONTROL OF EUKARYOTIC DNA REPLICATION

A number of different model systems have been used to study eukaryotic DNA replication. Prominent among these are two genetic systems provided by the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*, and a biochemical system, provided by *Xenopus laevis* egg extracts. The two yeast systems have pioneered the identity of replication initiation factors in eukaryotes and the characterization of their regulation during the cell cycle. Almost all the molecular components identified in these two systems have been shown to be evolutionarily conserved in other eukaryotes, suggesting that the basic mechanisms of initiation will also be conserved. One advantage of these systems is that replication origins are well defined in yeast (particularly for *S. cerevisiae*), allowing the interaction of initiation proteins with origins to be studied. The *Xenopus* extract system has allowed biochemical dissection of the initiation reaction. Specific sequences do not function as origins in this system, as any DNA will replicate, but the once-and-only-once regulation of initiation is still maintained.

Studies in these systems has defined two major stages of initiation which are coupled to progression through the cell cycle (Diffley, 1996). In the first stage, which occurs in early G1 phase, a pre-replicative complex (pre-RC) is formed at origins by the sequential binding of the initiation proteins: ORC, Cdc6, Cdt1, and Mcm2-7. Assembly of the pre-RC makes origins competent for subsequent initiation later in S phase, and the presence of these competent origins corresponds to the replication-competent state observed by Rao and Johnson in G1 phase. In the second stage, two kinases Cdc7-Dbf4 and an S-phase promoting CDK (e.g. Cdc28-Clb5 or Cdc2-Cig2) promote the triggering

of initiation at competent origins. This triggering corresponds to the action of the SPF proposed by Rao and Johnson and involves the ordered binding of additional replication proteins, the unwinding of the origin, and the assembly of the DNA synthetic machinery at the replication fork. In the process of triggering initiation the pre-RC is disassembled, leaving the origins in a post-replicative state that is not competent for initiation. Hence, re-initiation of replication at these origins requires re-assembly and re-triggering of pre-RCs. In principle, blocking either step could prevent re-initiation.

In addition to acting as positive regulators that promote initiation, CDKs have also been implicated as negative regulators that prevent re-initiation. This inhibitory role was first observed by experiments showing that inactivation of S- and/or M-phase promoting CDK activity enables cells to undergo a second round of S phase in the absence of mitosis (Dahmann et al., 1995; Nishitani and Nurse, 1995). These cells enter G1 phase of the next cell cycle and re-assembly pre-RCs, suggesting that the presence of CDKs could directly or indirectly prevent pre-RC re-assembly. Similar conclusions were drawn from experiments showing that ectopic expression of Cdc28-Clb in G1 phase prevents the assembly of the pre-RC in budding yeast (Detweiler and Li, 1998) and that premature activity of Cyclin E- and Cyclin A- Cdk2 prevents pre-RC formation in *Xenopus* extracts (Hua et al., 1997). Thus, CDKs have opposite effects on the two different stages of initiation. They inhibit the first stage, assembly of pre-RCs, but once pre-RCs assemble, they activate the second stage, triggering initiation.

These opposing effects on different stages of initiation can couple each round of initiation to a single round of the cell cycle. Shortly after mitosis when S- and M-phase promoting CDKs are inactivated, the cell enters a state of low CDK activity where pre-

RCs can assemble, but cannot be triggered. Later, upon passing the G1 commitment point and activation of CDKs, the cell enters state where assembled pre-RCs can be triggered to initiate, but new pre-RC assembly cannot occur. Hence, at every origin each round of replication initiation requires that the cell pass through a complete cell cycle, ensuring once and only once initiation.

What was not understood when I began my thesis was exactly how CDKs prevent the assembly of the pre-RC. The key CDK substrates that have to be phosphorylated in order to inhibit pre-RC assembly were not known, although the obvious candidates were components of the pre-RC. To determine whether any of these components were critical targets, the first step would be to determine if they are regulated during the cell cycle in a manner that could prevent re-initiation. Next one would want to know if CDKs were responsible for this regulation. Finally, the key experiment would be to disrupt this regulation and see whether re-replication occurred. Re-replication would indicate that CDK regulation of this protein was important for preventing re-initiation. Below, I describe the different proteins that participate in the initiation of eukaryotic DNA replication and discuss what is known about their regulation across the cell cycle.

COMPONENTS OF PRE-REPLICATIVE COMPLEX

Origins of DNA replication

Eukaryotic genomes contain 10^7 to 10^9 base pairs and are organized into multiple chromosomes. To efficiently replicate their whole genome within a short period of time, eukaryotic cells initiate DNA replication at multiple origins of replication. There are around 400 active origins in budding yeast and an estimated 100,000 origins in somatic

human cells. This large number of origins compounds the problem of preventing re-replication, because each of these origins must be prohibited from re-initiating within a single cell cycle every cell cycle.

The best-characterized eukaryotic origins are those of budding yeast. These origins were first isolated as sequences that allow maintenance of an autonomous plasmid and, hence, became known as autonomously replicating sequences (*ARS*s). Subsequent studies established that *ARSs* act as origins on plasmids and, in many cases, function as origins in chromosomes. *ARSs* are approximately 100-150 bp long and can be divided into two regions called A and B that are each necessary but not sufficient for origin activity. The A region contains an 11 base pair AT-rich degenerate *ARS* consensus sequence (5'-[A/T]TTTAT[A/G]TTT[A/T]-3') referred to as the ACS. The less conserved B region lies 3' to the T rich strand of the ACS and contains a number of sub-domains: B1, B2, and B3 in *ARS1* and B1 and B2 in *ARS307* (Marahrens and Stillman, 1992; Van Houton and Newlon, 1990). Although no single B sub-domain is essential, together they are required for efficient origin activity and are functionally conserved among the budding yeast origins (Rao et al., 1994; Theis and Newlon, 1994). The regions A and B1 form the recognition site of the origin recognition complex (see below). B3 in *ARS1* is a binding site for the transcription factor Abf1 (*ARS1* binding factor 1), which helps to keep the origin clear of nucleosomes (Diffley and Stillman, 1988). The function of B2 is not clear.

The characterization of DNA replication origins in other eukaryotes is more complicated than budding yeast. In fission yeast *S. pombe*, origins of DNA replication were mapped to segments of 500 to 1500 base pairs, but essential sub-elements within

these segments have been difficult to define. In metazoans, site of replication initiation has been identified by physical mapping techniques, but these regions are spread over tens to hundreds of kilo-bases. In metazoan embryos, on the other hand, initiation sites are spaced only four to ten kilo-bases apart, and there is little or no sequence specificity for origins. Hence, metazoan origins have been difficult to characterize (review in (Kelly and Brown, 2000)).

Origin Recognition Complex (ORC)

Proteins recognizing origins were first identified in the budding yeast and called Origin Recognition Complex (ORC). In this organism, ORC recognizes regions A and B1 in an ATP dependent manner (Bell and Stillman, 1992). ORC is composed of six subunits, Orc1-Orc6, the first five of that, Orc1-Orc5, are necessary and sufficient for origin recognition (Lee and Bell, 1997). Genes encoding all six proteins have been identified, and the proteins have been shown to conserve from yeast to humans. Genetic and biochemical studies suggest that ORC is required for initiation of DNA replication. Temperature sensitive mutations in the budding yeast genes disrupt replication initiation (Loo et al., 1995). In the fission yeast *S. pombe*, deletion of *ORC1* or *ORC2* results in cell cycle arrest with unreplicated DNA (Grallert and Nurse, 1996; Leatherwood et al., 1996). In *Xenopus* egg extracts, immunodepletion of ORC prevents chromosomal replication (Romanowski et al., 1996; Rowles et al., 1996). And mutation in Drosophila *ORC2* reduces incorporation of BrdU in larvae (Pflumm and Botchan, 2001).

Analysis of ORC binding to endogenous replication origins in budding and fission yeasts has been performed using genomic footprinting, chromatin immunoprecipitation (chIP), or chromatin association. These experiments show that ORC binds origins

throughout the cell cycle (Aparicio et al., 1997; Diffley et al., 1994; Liang and Stillman, 1997; Tanaka et al., 1997), suggesting that the regulation of this binding is not critical for the regulation of replication initiation in these yeast. In contrast, there is evidence that ORC and Orc1 dissociate from metaphase chromosomes in *Xenopus* egg extracts and human cells, respectively (Natale et al., 2000; Romanowski et al., 1996). Although this regulation could prevent pre-RC re-assembly in M phase, it is not clear how important this regulation is to a re-initiation block that must be imposed in S phase and continue through G2 and M phase.

Like DnaA in *E. coli*, ORC has been shown to exist in an ATP and an ADP bound form and to convert the former to the latter by hydrolysis. Orc1 and Orc5 can both bind ATP or ADP, but only binding to Orc1 seems to regulate ORC activity (Klemm et al., 1997). ATP binding to Orc1 is essential for origin recognition and viability (Bell et al., 1995). Moreover, Orc1 (and not Orc5) can slowly hydrolyze this ATP to ADP, but not when ORC is bound to origins. Mutant ORC complexes that are specifically impaired for ATP hydrolysis appear to have enhanced binding to another pre-RC component, Cdc6 (see below) (Klemm and Bell, 2001). This suggests that ATP promotes ORC binding to origins and that this association stabilizes the ATP bound form of Orc1. Stabilizing this ATP bound state of ORC, in turn, facilitates subsequent steps in the assembly of the pre-RC. Binding to single-stranded DNA stimulates ATP hydrolysis (Lee and Hurwitz, 2000), raising the possibility that unwinding the origin converts ORC to the ADP bound form, releasing Cdc6 and allowing progression of the initiation reaction. Unlike DnaA, however, this hydrolysis is unlikely to prevent re-initiation, as a molecule of ORC (presumably bound to ATP) is still associated with origins after initiation has occurred.

CDC6

ORC binding to origins allows additional initiation proteins to load onto origins. One of these proteins, Cdc6, has been shown to bind ORC directly (Mizushima et al., 2000). Its binding is necessary for origin loading of the putative replicative helicase Mcm2-7 (see below) (Aparicio et al., 1997; Tanaka et al., 1997).

Cdc6 homologs have been identified in many eukaryotes and all have Walker A and B motifs suggestive of the ability to bind and hydrolyze ATP (Walker et al., 1982). These activities have only been demonstrated for human Cdc6, but are presumed to exist in Cdc6 from other species. The importance of ATP binding and hydrolysis for replication initiation has been demonstrated by microinjection of mutant proteins lacking these activities into human cells, but the exact role of these activities in initiation is not known (Herbig et al., 1999). In budding and fission yeast, mutations in the Walker A motif of Cdc6 that are expected to prevent nucleotide binding have defects in S phase entry and in the case of budding yeast have been shown to prevent pre-RC formation (Perkins and Diffley, 1998). Thus, Cdc6 is likely to require ATP binding to carry out its initiation function. Mutations in the Walker B motif of budding yeast Cdc6 do not give uniform results; one mutation has been shown to perturb pre-RC formation, while another has no effect on replication (Perkins and Diffley, 1998; Weinreich et al., 1999). The role of Cdc6 ATP hydrolysis, therefore, is less clear, and there is no evidence suggesting that this hydrolysis has a role in preventing re-initiation.

Cdc6 is regulated in a number of other ways during the cell cycle in a manner that could inhibit re-initiation. In both budding and fission yeasts, the protein is expressed primarily in G1 phase shortly after mitosis, and its absence later in the cell cycle could

prevent pre-RC re-assembly (Detweiler and Li, 1997; Nishitani and Nurse, 1995; Piatti et al., 1995). The periodic expression of Cdc6 (or its fission yeast homolog, Cdc18) is due to cell cycle regulated transcription in early G1 phase and instability of the protein. Both *CDC6* transcription and Cdc6 stability are negatively regulated by CDKs. CDK phosphorylation and inactivation of the transcriptional activator Swi5 is responsible for CDK inhibition of *CDC6* transcription (Moll et al., 1991). CDK phosphorylation of Cdc6 stimulates Cdc6 ubiquitination and subsequent degradation (Drury et al., 1997). In human cells, Cdc6 protein is constitutively expressed throughout the cell cycle, but its localization is regulated such that it is nuclear in G1 phase and cytoplasmic for the remainder of the cell cycle (Jiang et al., 1999; Petersen et al., 1999). This redistribution to the cytoplasm is dependent on CDK phosphorylation of Cdc6 and could, in principle, prevent re-replication in human cells.

Demonstrating the significance of these Cdc6 regulatory mechanisms, however, has been difficult. Constitutive overexpression of wild type or non-phosphorylatable mutant Cdc6 in budding yeast does not lead to re-replication (Drury et al., 1997). Similar negative results were obtained with overexpression of non-phosphorylatable and constitutively nuclear Cdc6 in human cells (Petersen et al., 1999). The one exception is in fission yeast where constitutive overexpression of the Cdc6 homolog, Cdc18, does lead to re-replication. Interpretation of this result, however, has been complicated by the ability of overexpressed Cdc6/Cdc18 to inhibit CDK activity (Greenwood et al., 1998) and potentially push cells into G1 phase of a new cell cycle. Such cells would be undergoing endoreduplication (i.e. bypassing mitosis) instead of re-replication in the same cell cycle.

CDT1

Cdt1 was initially identified in fission yeast as a protein that loads onto ORC-bound origins and is required, along with Cdc6/Cdc18, for loading of Mcm2-7 (Nishitani et al., 2000). Cdc6/Cdc18 and Cdt1 appear to load onto ORC independently of each other. Homologs with similar roles in loading Mcm2-7 have been identified in budding yeast (Tanaka and Diffley, 2002), *Drosophila melanogaster* (Whittaker et al., 2000), *Xenopus laevis* (Maiorano et al., 2000), and humans (Wohlschlegel et al., 2000). Unlike ORC and Cdc6, Cdt1 has no Walker A and B nucleotide binding motifs and there is no evidence that it binds any nucleotides.

Cdt1 protein is periodically expressed in G1 phase in fission yeast and humans although the periodicity is not as tight as it is for budding and fission yeast Cdc6/Cdc18 (Nishitani et al., 2000; Wohlschlegel et al., 2000). Moreover, constitutive overexpression of Cdt1 in fission yeast does not lead to re-replication indicating that regulation of Cdt1 levels is not required for the block to re-replication. Budding yeast Cdt1, in contrast, is expressed at constant levels, but its localization is regulated during the cell cycle (Tanaka and Diffley, 2002). The protein is nuclear in G1 phase and excluded from the nucleus later in the cell cycle, potentially preventing re-initiation of DNA replication. Demonstrating such a regulatory role, however, has been difficult, as constitutive nuclear localization of Cdt1 does not lead to re-replication in budding yeast.

Cdt1 can also be controlled by binding to a replication inhibitor called geminin (Tada et al., 2001; Wohlschlegel et al., 2000). This binding prevents Mcm2-7 loading (see below) and replication initiation in *Xenopus* egg extracts, but does not affect ORC and Cdc6 loading. Microinjection of geminin into *Xenopus* embryos can prevent S phase

(McGarry and Kirschner, 1998). Geminin is expressed in human cells in late S and G2 phase and is destroyed during mitosis by the Anaphase Promoting Complex (APC) (Wohlschlegel et al., 2000). The combination of its inhibitory activity and its presence in the later part of the cell cycle has led to the notion that geminins participates in the block to re-initiation. This role of geminins has been difficult to establish in *Xenopus*, as simple depletion of geminins in *Xenopus* extracts or antisense disruption in *Xenopus* embryos does not lead to re-replication (McGarry, 2002; Tada et al., 2001). However, antisense depletion of geminins in *Drosophlia* Schneider D2 (SD2) cell lines or P element disruption of geminins in *Drosophila* embryos leads to partial over replication, supporting a role for geminins in the block to re-replication in this organism (Mihaylov et al., 2002; Quinn et al., 2001). Furthermore, depletion of geminins in addition to inactivation of CDKs in *Xenopus* metaphase extracts leads to inappropriate assembly of pre-RCs, suggesting that, at least in this extract system, geminins and CDKs cooperate to block re-initiation in metaphase (Tada et al., 2001). Difference in the relative importance of geminins versus CDKs in the block to re-replication may account for the different results observed in *Drosophila* and *Xenopus*.

MCM2-7

The final components implicated in the assembly of the pre-RC component are the six minichromosome maintenance proteins Mcm2- Mcm7. These highly conserved proteins share significant identity in an approximately 200 amino acid "Mcm Box" that contains Walker A and Walker B motifs (Walker et al., 1982). Chromatin association and chromatin immunoprecipitation (ChIP) assays performed in several organisms indicate that these proteins associate with chromosomal DNA (specifically origins in

budding yeast) during G1 phase in a manner dependent on ORC, Cdc6, and Cdt1 (Aparicio et al., 1997; Maiorano et al., 2000; Nishitani et al., 2000; Tanaka et al., 1997). Genetic studies in fission and budding yeasts have demonstrated that each Mcm protein is essential for viability and is required for the initiation DNA replication (Dalton and Whitbread, 1995; Hennessy et al., 1990; Labib et al., 2000; Miyake et al., 1993; Takahashi et al., 1994; Yan et al., 1993). Thus, although the six Mcm proteins have similar sequences and functions, each performs a unique and essential function in the cell.

Mcm2-7 complex is the leading candidate for the eukaryotic replicative helicase. Purified complexes from fission yeast, humans, and mice have been shown to bind and hydrolyze ATP (Ishimi, 1997; Lee and Hurwitz, 2000; You et al., 1999). These proteins are thought to form a heterohexameric complex, with the six subunits arranged in a ring around a central cavity, reminiscent of the DnaB helicase. Consistent with this analogy, a sub-complex composed of Mcm4, Mcm6, and Mcm7 (arranged in a double heterotrimeric complex) has been shown to have a weak 3' to 5' helicase activity, that can be stimulated by the addition of a 5' tail on the displaced strand (Lee and Hurwitz, 2001). In addition, ChIP experiments in budding yeast show that during replication elongation, the Mcm proteins move bi-directionally away from origins at a rate roughly comparable to that of polymerase ε, a replication fork component (Aparicio et al., 1997). Moreover, genetic experiments in budding yeast demonstrate that the Mcm proteins are required for fork movement during S phase (Labib et al., 2000). Finally, a single Mcm homolog has been identified in several archaebacteria, and in each case a homohexamer of these homologs has been shown to have helicase activity (Shechter et al., 2000).

Understanding exactly how the eukaryotic Mcm proteins could provide helicase activity is complicated by the absence of detectable helicase activity in the full Mcm2-7 complex. In fact, addition Mcm2 or Mcm3-Mcm5 sub-complex to the Mcm4-6-7 complex inhibits helicase activity (Ishimi, 1997; Lee and Hurwitz, 2000). This has led to the suggestion that Mcm2, Mcm3, and Mcm5 act to negatively regulate the activity of the Mcm4-6-7 helicase. Such a simple model, however, does not account for the requirement of Mcm2 and Mcm3 for fork elongation. It is possible that the helicase activity of full Mcm2-7 complex is tightly regulated and only activated in the context of the other proteins at the replication fork.

Biochemical studies suggest that CDKs can negatively regulate Mcm helicase activity. Cyclin A-Cdk2 was shown to phosphorylate the Mcm4-6-7 complex in vitro and inhibit its helicase activity (Ishimi and Komamura-Kohno, 2001). This phosphorylation was shown to occur primarily on Mcm4 and was mapped to six serine and threonine residues in the N-terminus of the protein. Mutation of these six sites prevented both phosphorylation of Mcm4 and Cyclin A-Cdk2 inhibition of kinase activity. Moreover, Mcm4-6-7 purified from mitotic cells contains phosphorylated Mcm4 and has reduced helicase activity compared to Mcm4-6-7 purified from G1 cells. These results have led to the suggestion that CDKs may inhibit Mcm helicase activity in vivo and that this inhibition may be one way in which CDKs could block re-replication. However, how this inhibition would specifically inhibit re-replication without inhibiting elongation during the first round of replication is not clear. Moreover, there is no direct in vivo evidence showing that this phosphorylation is important in the block to re-replication.

Mcm phosphorylation has also been suggested to prevent re-replication by regulating the association of Mcm proteins with chromatin. After loading onto chromatin in G1 phase, Mcm proteins gradually dissociate from chromatin during S phase and remain dissociated until the exit from mitosis. Studies in *Xenopus laevis* and human have demonstrated that CDKs phosphorylate Mcm2 and Mcm4 (and possibly other subunits) in vitro and in vivo in a cell cycle dependent manner. Mcm4 hyper-phosphorylation has been temporally correlated with the release of Mcm proteins from chromatin (Fujita et al., 1998; Hendrickson et al., 1996; Pereverzeva et al., 2000). However, there are no direct experiments showing that this modification can regulate Mcm chromatin association.

A final potential mode of regulating Mcm proteins has been observed in budding yeast, but not other organisms. In budding yeast, the sub-cellular distribution of several Mcm proteins was shown by indirect immunofluorescence to be regulated during the cell cycle (Hennessy et al., 1990; Yan et al., 1993). Mcm2, Mcm3, and Mcm5 were observed to accumulate in the nucleus during G1, gradually disperse to the cytoplasm during S phase, and appear distributed throughout the cell in G2/M phase. Clearly, reducing Mcm levels in the nucleus could potentially inhibit re-replication. At the time I began my thesis, however, this model was called into question by fractionation experiments that showed the constitutive presence of Mcm proteins in the nucleus (Young and Tye, 1997). As part of my thesis, I reexamined this regulation to reassess its role in the block to re-replication.

COMPONENTS ACTING AFTER PRE-REPLICATIVE COMPLEX

Although the inhibitory effect of CDKs on pre-RC assembly has focused attention on this event as a regulatory step in the block to re-replication, current experiments cannot rule out a role for CDK inhibition of later initiation or elongation steps in this block. Hence, replication proteins that act after pre-RC assembly are still possible inhibitory targets of the CDKs. These proteins are discussed below.

It should be noted that, if these proteins are inhibited by CDK phosphorylation to prevent re-initiation or re-elongation, there must be a way to prevent this inhibition from disrupting the first round of replication. Since CDK activity is required to recruit many of these proteins to the origin, it would appear to be difficult for CDKs to distinguish between proteins that are promoting replication and those that are promoting re-replication. A distinction can be made, however, if the proteins can perform some key step before the CDKs are activated that make them refractory to CDK inhibition, for example, a conformational change or an association with another protein. This would allow the first round of replication to occur unimpeded. At the completion of their replication function, these proteins must be restored to their inhibitable state so that the second round of replication is blocked.

MCM10/DNA43

Characterization of temperature sensitive allele of *MCM10*, *mcm10-1*, suggests that Mcm10 protein is involved in the initiation of DNA replication (Merchant et al., 1997). ChIP experiments performed in the budding yeast show that Mcm10 loads onto origins of replication (Homesley et al., 2000). What is not clear is the time and

dependence of this loading on other proteins, as experiments in budding yeast and *Xenopus* give conflicting results.

In budding yeast, Mcm10 has been reported to associate with chromatin throughout the cell cycle (Homesley et al., 2000). Although ORC and Mcm10 interact genetically and physically, their association with chromatin was reported to occur independently of each other. Budding yeast Mcm10 has also been shown to interact genetically and physically with Mcm2-7. Because Mcm10 associates with chromatin throughout the cell cycle, it is presumed that its association is independent of Mcm2-7, which only associates in G1 and parts of S phase. The maintenance of Mcm2 loading, on the other hand, was reported to depend on Mcm10. In *Xenopus* egg extracts, however, Mcm10 chromatin association was shown to be dependent on prior loading of Mcm2-7 (and thus indirectly dependent on ORC loading). It was also independent of the activity of CDK and Cdc7-Dbf4. Mcm10 loading, in turn, was shown to be required for Cdc45 loading (see below) (Wohlschlegel et al., 2002). Thus, Mcm10 appears to have a role in the triggering of initiation, but its loading onto chromatin is not fully understood.

Part of the confusion about Mcm10 chromatin loading may be attributable to problems with the chromatin association assays that were used. In budding yeast and *Xenopus*, confirmation that the association was DNAse I sensitive, and thus was DNA dependent, was not performed. This test has been performed in studies on the human homolog of Mcm10. In these experiments Mcm10 has been shown to interacts with a DNAse I resistant nuclear structure in S and G2 phase (Izumi et al., 2000), raising the possibility that previous reports of chromatin association were actually looking at these structures.

Several lines of evidence suggest that Mcm10 also has a role in elongation during DNA replication. In the budding yeast, a *mcm10-1* mutation causes DNA replication forks to pause near replication origin (Merchant et al., 1997) and shows genetic interactions with components of the replication fork, including DNA polymerases _ and _ (Homesley et al., 2000; Kawasaki et al., 2000). Full inactivation of the *mcm10-1* mutation at the restrictive temperature after cells have entered an early S phase arrest, disrupts subsequent replication elongation (Kawasaki et al., 2000).

There is no evidence of cell cycle regulation of Mcm10. First, the protein is present throughout the cell cycle. Second, although there is one full ((S/T)-P-X-(K/R)) and one partial ((S/T)-P) consensus CDK sites on Mcm10, there is no evidence that the protein is phosphorylated in a cell cycle regulated manner and mutations of these sites have no phenotypic consequence (personal communication, M Lei and R Morreale).

Ccd7-Dbf4 kinase (DDK)

Cdc7 is a serine-threonine kinase that is activated by binding to a regulatory subunit Dbf4 (Jackson et al., 1993). Cdc7-Dbf4 kinase activity is required for replication initiation, but the key target for this kinase is not known (Diffley et al., 1994). Genetic and biochemical evidence suggests Mcm2-7 could be the key target. Bypass suppression of *cdc7* and *dbf4* deletions by the *mcm5-bob1* mutant is consistent with the mutant Mcm complex becoming independent of activation by Cdc7-Dbf4 (Hardy et al., 1997). An additional genetic connection is provided by the suppression of *mcm2-1* by a *dbf4* mutation (Lei et al., 1997). Moreover, purified Cdc7-Dbf4 from budding yeast, fission yeast, and mouse all phosphorylate Mcm2-7 in vitro, most notably on Mcm2 (Brown and Kelly, 1998; Lei et al., 1997; Masai et al., 2000). The significance of this

phosphorylation is unknown, however, and awaits the mapping of the Mcm2 phosphorylation sites. Cdc45 (see below) and the catalytic subunit of DNA polymerase _ have also been implicated as possible substrates of Cdc7-Dbf4, but again further analysis requires mapping the sites of phosphorylation and mutating them (Nougarede et al., 2000; Weinreich and Stillman, 1999).

Several lines of evidence suggest that Cdc7-Dbf4 kinase interacts with origins of replication. Indirect one-hybrid analysis suggests that Dbf4 binds to origins of replication in budding yeast (Dowell et al., 1994). More direct analysis by chromatin association shows that Cdc7 is bound to chromatin throughout the cell cycle and that Dbf4 associates when it is present in the cell from late G1 phase to M phase (see below) (Weinreich and Stillman, 1999). Both budding yeast and *Xenopus* egg extracts indicate that Cdc7-Dbf4 loading onto chromatin is required for replication initiation, but they reach different conclusion about the dependence of this loading on other initiation proteins. In budding yeast, the loading of Dbf4 was reported to be dependent on ORC but independent of Cdc6 and the Mcm2-7 complex (Pasero et al., 1999). In *Xenopus* egg extracts, Cdc7 loading was reported to be dependent on prior loading of Mcms onto chromatin (Walter, 2000).

The activity of Cdc7-Dbf4 kinase is regulated during the cell cycle (Cheng et al., 1999; Ferreira et al., 2000; Oshiro et al., 1999). The kinase activity is low in G1 before START, peaks at the G1/S transition, remains high until G2 phase, and then falls as cells enter anaphase. Cdc7 protein levels are constant throughout the cell cycle, and the fluctuation of kinase activity correlates closely with levels of Dbf4. This level is not controlled by gene expression, as mRNA levels remain relatively constant throughout the

cell cycle. Instead Dbf4 levels appear to be regulated by protein degradation mediated by the anaphase-promoting complex (APC) (Cheng et al., 1999; Ferreira et al., 2000; Oshiro et al., 1999), which is active primarily in G1 when Dbf4 levels are low.

Because both the chromatin association and kinase activity of Cdc7-Dbf4 remain high during S, G2, and early M phase, the block to re-initiation does not appear to involve the down regulation of Cdc7-Dbf4 kinase. In fact, the persistence of this kinase during the later parts of the cell cycle would be expected to promote re-initiation if pre-RCs are allowed to reassemble.

CDK

As discussed above CDKs are required for both triggering initiation and blocking re-initiation. My thesis work focused on understanding the block to re-initiation, but my experiments also investigated the importance of CDK phosphorylation of several pre-RC components for triggering of initiation. Because mutations that disrupt CDK consensus sites in ORC, Cdc6, and Mcm2-7 do not impair replication, I was not able to demonstrate that CDK phosphorylation of pre-RC components are required for triggering initiation. Recent studies in budding and fission yeast have implicated Sld2/Drc1, an initiation protein acting after pre-RC formation, as an important CDK substrate for replication initiation (Masumoto et al., 2002; Noguchi et al., 2002) (see below).

Studies in a number of different organisms show that CDKs can physically associated with components of the pre-RC, such as ORC, Cdc6, and Mcm2-7. In the fission yeast, Cdc2/Cdk1, has been shown to associate with Orc2 by two-hybrid and co-immunoprecipitation (co-IP) assays (Leatherwood et al., 1996). Similarly, biochemical studies in *Xenopus* egg extracts show that Orc1 and Orc2 co-purify and co-

immunoprecipitate with cyclin A-Cdk1 (Romanowski et al., 2000). In both budding and fission yeasts, CDKs have been shown to interact with Cdc6/Cdc18 *in vivo* and *in vitro*, an interaction that is dependent on the N terminus of Cdc6 (Brown et al., 1997; Elsasser et al., 1996; Lopez-Girona et al., 1998). These associations may help bring CDKs to pre-RCs assembled at chromosomal origins so that they can activate DNA replication. In *Xenopus* egg extracts, cyclin E-Cdk2 associates with chromatin in an ORC and Cdc6 dependent manner and this binding appears to be required for efficient replication initiation (Furstenthal et al., 2001). This binding could promote initiation by bringing the kinase closer to its key substrates. It is also possible that the physical association itself helps to promote initiation.

CDC45-SLD3

Cdc45 becomes tightly associated with chromatin in a manner dependent on pre-RC assembly, Mcm10, Cdc7-Dbf4, and CDKs (Wohlschlegel et al., 2002; Zou and Stillman, 2000; Zou and Stillman, 1998). In budding yeast, ChIP experiments demonstrate that this association occurs at origins (Aparicio et al., 1997), although there is some controversy over exactly when Cdc45 associates with origins. Some experiments suggest that a weaker form of association may occur in G1 phase before the kinases are active, specifically at origins that initiate early in S phase (Aparicio et al., 1999; Kamimura et al., 2001; Zou and Stillman, 2000). Whether this weak association occurs, it is clear that Cdc7-Dbf4 and CDKs induce a much tighter association with origins around the time each origin initiates DNA replication in S phase.

Experiments in *Xenopus* egg extracts show that Cdc45 association with plasmid templates correlates with a topological change in the plasmids consistent with DNA

unwinding (Walter and Newport, 2000). This result suggests that Cdc45 is necessary for the critical unwinding step in initiation. It may not be sufficient to trigger this step, as there are many other proteins that load onto chromatin around the same time. It is tempting to speculate that this unwinding event allows Cdc45 to become tightly associated with chromatin.

The tight association of Cdc45 with origins allows additional proteins to be recruited to chromatin. Cdc45 is required to load the single-stranded DNA binding proteins, RPA, onto chromatin in *Xenopus* and onto chromosomal origins in budding yeast (Mimura et al., 2000; Zou and Stillman, 2000). The exact relationship between Cdc45 and RPA loading is not entirely clear, as Cdc45 loading has been reported to be dependent on RPA in budding yeast but independent of RPA in *Xenopus*. Experiments in *Xenopus* egg extracts also indicate that Cdc45 associates with DNA polymerase ___a component of the DNA priming complex, and is required to load this polymerase onto chromatin prior to initiation (Mimura and Takisawa, 1998). Similarly, ChIP and chromatin association experiments in *S. cerevisiae* demonstrate that Cdc45 is required to load both DNA polymerase __and the potential replicative polymerase, DNA Polymerase ___onto chromosomal origins(Aparicio et al., 1999; Zou and Stillman, 2000).

In addition to helping unwind the DNA and load the DNA synthetic machinery, Cdc45 also has a role in the elongation of DNA replication. ChIP experiments suggest that Cdc45, like Mcm proteins, moves away from origins as if they are associated with the replication fork. This association could be mediated through its ability to interact with Mcm proteins and DNA polymerases (Aparicio et al., 1997). Genetic study of a temperature sensitive "degron" allele of Cdc45 in budding yeast, which induces Cdc45

degradation at non-permissive temperatures, demonstrated that Cdc45 is required for the progression of replication fork after initiation has occurred (Tercero et al., 2000).

Cdc45 may perform all its activities in conjunction with another protein Sld3. Biochemical studies in the budding yeast show that Cdc45 complexes with Sld3 throughout the cell cycle (Kamimura et al., 2001). ChIP experiment demonstrates that both Cdc45 and Sld3 associate with origins and that each protein is dependent on the other for its association. Although the role of Sld3 in origin unwinding, DNA polymerase loading, and replication elongation has not been carefully documented, it is possible that the Cdc45-Sld3 complex is responsible for all these functions.

Although protein levels of Cdc45 and Sld3 appear to be constant in all species examined, there are hints that these proteins may be regulated during the cell cycle in fission yeast (Nakajima and Masukata, 2002). First, association of Cdc45 and Sld3 is periodic with peak association occurring around S phase. Second, Sld3 appears to be phosphorylated in M phase and hypo-phosphorylated in S phase. Whether the regulation of Sld3 phosphorylation is related to the regulation of Cdc45-Sld3 association, and whether either regulation is important for the control of initiation will require further analysis.

Sld2-Dpb11

The gene encoding Dpb11 was first isolated in budding yeast as a high copy suppressor of a mutation in the gene for the DNA polymerase ε subunit, Dpb2 (Araki et al., 1995). Physical association between Dpb11 and DNA polymerase ε can be detected by co-IP, but only after chemical cross-linking, suggesting that the interaction is transient or labile. Potential homologs in fission yeast (Cut5/Rad4) and *Xenopus* (xMus101) have

been identified (Saka and Yanagida, 1993; Van Hatten et al., 2002). Dpb11 and xMus101 have been shown to be required for replication initiation.

ChIP experiments suggest that Dpb11 is involved with other initiation proteins in a web of dependencies for origin loading. Mcms and RPA are required for Dpb11 loading onto origins (Masumoto et al., 2000). Dpb11 and DNA polymerase ε appear to be mutually dependent for origin loading. And DNA primase from the priming complex is dependent on Dpb11 (and DNA polymerase ε) to load onto origins. Thus, like the Cdc45-Sld3 complex, Dpb11 acts during the triggering of initiation to help complete the assembly of the replication fork. Dpb11 has also been indirectly implicated in replication elongation; conditional *dpb11* mutants shifted to the restrictive temperature in S phase but not G2/M phase cannot complete the cell cycle. The role of Dpb11 in elongation, however, is much less understood.

A screen for mutations that are synthetically lethal with *dpb11-1* led to mutations in the genes for Sld2/Drc1, Sld3, Sld5, Cdc45, and Dpb3 (the third largest subunit of the DNA polymerase ε complex) (Kamimura et al., 1998). Budding yeast Sld2/Drc1 was shown to physically associate with Dpb11 in a cell cycle dependent manner. The association coincides with Cdc28-Clb activity, which is present from late G1 phase to the end of mitosis. This regulation appears to be mediated by phosphorylation of Sld2/Drc1 by Cdc28-Clb. Mutation of all five CDK consensus sites ((S/T)-P-X-(K/R)) and one degenerate site ((S/T)-P) on Sld2/Drc1 prevents its phosphorylation by Cdc28-Clb and its association with Dpb11. These mutations also disrupt replication (Masumoto et al., 2002), leading to a model in which CDK phosphorylation of Sld2/Drc1 promotes replication initiation in part by facilitating the association of Sld2/Drc1 with Dpb11.

How this association then facilitates replication initiation is not known, but it could somehow facilitate Dpb11 loading of Polymerase e at origins.

Although Sld2/Drc1 appears to be a critical target for CDKs in the triggering of initiation, it is not known if phosphorylation of Sld2/Drc1 is sufficient to bypass the requirement for CDKs in this triggering. Hence, it is possible that there are other targets of CDKs required for triggering initiation.

Sld2/Drc1 protein levels also appear to be regulated during the budding yeast cell cycle (Masumoto et al., 2002). After the initial phosphorylation by Cdc28-Clb, the protein levels seem to drop at the end of S phase and remain low until late G1 phase of the next cell cycle. This periodicity may be attributable in part to the cell cycle regulation of *SLD2* expression, which peaks in late G1 phase (Kamimura et al., 1998). The disappearance of Sld2/Drc1 protein cannot explain the block to re-replication during S phase, but could contribute to maintaining this block after S phase. Dpb11, on the other hand, does not appear to undergo any changes in its protein levels or modification states during the cell cycle.

RPA

RPA is a conserved three-protein complex (Rpa1/RPA70, Rpa2/RPA32, and Rpa3/RPA14) that binds single-stranded DNA, and participates in DNA replication, repair, and recombination. During replication, the protein facilitates the initial unwinding of the duplex DNA at origins of replication and promotes the continual unwinding of double-stranded DNA at the replication fork. Its recruitment to origins of replication is suggestive of DNA unwinding, but this connection has never been rigorously established.

Human, *Xenopus*, and yeast Rpa12/RPA32 have been shown to be hypo-phosphorylated in G1 phase and hyper-phosphorylated upon entry into S phase. Although CDKs can phosphorylate RPA32 *in vitro*, it is not clear whether CDKs directly phosphorylate the protein in vivo. Moreover, the significance of the cell cycle regulated phosphorylation remains unknown (review in (Wold, 1997)).

DNA Polymerases

There are three highly conserved replicative DNA polymerase complexes in eukaryotes: DNA polymerase α-primase, DNA polymerase δ, and DNA polymerase ε. The loading of DNA polymerases α and ε at replication origins has been studied in budding yeast. Loading of both proteins requires prior loading of the pre-RC components, Sld2-Dpb11, and Cdc45-Sld3 (Aparicio et al., 1999; Masumoto et al., 2000; Zou and Stillman, 2000). Interestingly, although DNA polymerase α–primase must synthesize a primer before polymerase ε can commence DNA synthesis, DNA polymerase α-primase is dependent on DNA polymerase ε for origin loading (Masumoto et al., 2000). Because the synthesis of primers is the true start of DNA replication, it is possible that the cell wants to ensure that the entire DNA synthetic machinery is in place assembled onto replication forks before this replication begins.

Only DNA polymerase α-primase has been examined for its cell cycle regulation. In budding yeast, protein levels, assembly, and activity of DNA polymerase α-primase is constant during the cell cycle (Ferrari et al., 1996). However, the second largest subunit (Pol12) is phosphorylated when Cdc28-Clb activity is present. In addition, DNA polymerase α has been shown to become associated with chromatin after mitosis and to release from chromatin at the end of S phase. This association is correlated with de-

phosphorylation of Pol12 at the end of mitosis, and inactivation of Clb-Cdc28 kinase activity promotes both association and de-phosphorylation (Foiani et al., 1995). The exact nature of this chromatin association is not known. It is clearly distinct from the origin association observed by ChIP assay. Origin association depends on an elaborate assembly of initiation proteins at replication origins and occurs only in S phase, whereas the chromatin association is independent of Cdc6 and occurs as soon as cells exit mitosis. It is possible that the chromatin association is required before DNA polymerase can be loaded at origins, but this has not been established. Nonetheless, this regulation provides a potential way in which Cdc28-Clb kinases could prevent re-replication during the cell cycle.

The Thesis

My thesis project was to identify the critical targets of CDKs and to determine how phosphorylation of these targets inhibits re-initiation. My basic strategy was to identify initiation proteins that are regulated by CDKs and to determine whether disrupting this regulation would lead to re-initiation.

I began the project by confirming that Mcm nuclear localization is regulated during the budding yeast Saccharomyces cerevisiae cell cycle as described in Chapter 2. I showed that Mcm proteins are actually excluded from the nucleus by net nuclear export. Moreover, I demonstrated that CDKs promote this export and that Mcm2-7 co-localizes as a complex. Interestingly, I showed that constitutive nuclear localization of the Mcm proteins is not sufficient to lead to re-initiation of DNA replication in G2/M cells.

In Chapter 3, I examined the regulation of ORC during the cell cycle. I showed that ORC is phosphorylated in a cell cycle dependent manner by CDKs, but that disruption of this phosphorylation does not lead to re-initiation. Only when I simultaneously disrupted the regulation of ORC, Cdc6, and Mcm proteins do I observe re-initiation and re-replication. This result demonstrates that each of these mechanisms is sufficient to block re-initiation and that CDKs use multiple overlapping mechanisms to ensure that the block is strictly maintained at every origin in the budding yeast genome. In the concluding section, I briefly summarize my work and discuss its relevance to other eukaryotes in understanding the block to re-initiation of DNA replication.

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CHAPTER 2

Clb/Cdc28 kinases promote nuclear export of the replication initiator proteins Mcm2-7

ABSTRACT

Background: In *Saccharomyces cerevisiae*, the cyclin dependent kinases Clb/Cdc28 restrict the initiation of DNA replication to once per cell cycle by preventing the re-assembly of pre-replicative complexes (pre-RCs) at replication origins that have initiated replication. This assembly involves the Cdc6-dependent loading of six replication initiator proteins, Mcm2-7, onto origins. How Clb kinases affect the initiation machinery to prevent pre-RC assembly is not understood.

Results: We demonstrate in living cells that the Mcm proteins colocalize in a cell cycle regulated manner. Mcm2, 3, 4, 6, and 7 were concentrated in the nucleus in G1 phase, gradually exported to the cytoplasm during S phase, and excluded from the nucleus by G2 and M phase. Tagging any single Mcm protein with the SV40 nuclear localization signal (NLS) made all Mcm proteins constitutively nuclear. In the absence of functional Cdc6, Clb/Cdc28 kinases were necessary and sufficient for efficient net nuclear export of Mcm7-GFP, while inactivation of these kinases at the end of mitosis coincided with the net nuclear import of Mcm7-GFP. In contrast, in the presence of functional Cdc6, which loads Mcm proteins onto chromatin, S phase progression as well as Clb kinases was required for Mcm-GFP export.

Conclusions: We propose that Clb kinases prevent pre-RC reassembly in part by promoting the net nuclear export of Mcm proteins. We further propose that Mcm proteins become refractory to this regulation when they load onto chromatin and must be

dislodged by DNA replication before they can be exported. Such an arrangement could ensure that Mcm proteins complete their replication function before they are removed from the nucleus.

INTRODUCTION

The faithful transmission of genetic information during cell division requires that the entire genome be replicated once and only once. In eukaryotic cells, DNA replication initiates at multiple replication origins scattered throughout the genome. To ensure that each genomic segment is duplicated exactly once, re-initiation within a cell cycle must be prevented at every origin.

The initiation of eukaryotic DNA replication can be divided into two fundamental stages that are best characterized in the budding yeast *Saccharomyces cerevisiae* (Diffley et al., 1994; Owens et al., 1997; Tanaka and Nasmyth, 1998; Zou and Stillman, 1998). In the first stage, which occurs as cells enter G1 phase, pre-replicative complexes (pre-RCs) assemble at origins [2], making them competent to initiate replication [3, 4]. A six-protein origin recognition complex (ORC) [5] binds origins throughout the cell cycle [6, 7]. During pre-RC assembly the initiator protein Cdc6 is thought to load a family of six minichromosome maintenance proteins, Mcm2-7, onto the ORC-bound origins [7, 8]. The second stage of initiation occurs as cells enter S phase and involves the triggering of initiation by two kinase complexes: the cyclin-dependent kinase (CDK) Cdc28 in association with the B-type cyclins, Clb1-6 (Schwob et al., 1994), and Cdc7 kinase in association with its regulatory subunit Dbf4 (Jackson et al., 1993). During this stage, origins are unwound and additional proteins required for DNA synthesis are thought to assemble into the elongation machinery at nascent replication forks (Aparicio et al., 1997; Tanaka and Nasmyth, 1998). Importantly, initiation results in disassembly of pre-RCs, leaving only ORC bound to origins for the remainder of the cell cycle (Aparicio et al.,

1997; Diffley et al., 1994; Tanaka et al., 1997). The Mcm proteins appear to be incorporated into the elongation machinery and to remain associated with chromatin at moving replication forks until replication terminates (Aparicio et al., 1997).

In addition to triggering initiation, Clb/Cdc28 kinases can prevent pre-RC assembly (Detweiler and Li, 1998; Piatti et al., 1996). This does not interfere with the initiation of DNA replication in S phase, as these kinases are activated well after pre-RCs assemble in G1 phase. However, after initiation these kinases remain poised to inhibit the re-assembly of pre-RCs and are believed to block re-initiation at origins. This block is maintained until the end of mitosis, whereupon inactivation of Clb kinases allows pre-RC assembly in the next cell cycle. Clearly, identifying the relevant inhibitory targets of these kinases is critical for understanding the block to re-initiation.

Cdc6 and its *S. pombe* homolog Cdc18 have been implicated as likely inhibitory targets for cyclin-dependent kinases (Jallepalli et al., 1997; Liang and Stillman, 1997; Nishitani and Nurse, 1995). Another possible target is the Mcm family of proteins. Mcm2-7 are six sequence-related and evolutionarily conserved proteins, each of which is essential for replication initiation (reviewed in Tye, 1999). Indirect immunofluorescence of Mcm2, 3, 5, and 7 indicates that the subcellular distribution of Mcm proteins in *S. cerevisiae* is regulated during the cell cycle. These proteins appear to concentrate in the nucleus in G1 phase, gradually disperse during S phase, and predominantly reside in the cytoplasm during G2 and M phase (Dalton and Whitbread, 1995; Hennessy et al., 1990; Yan et al., 1993). Given that only a two-fold reduction in Mcm2 levels can impair the activity of some origins (Lei et al., 1996), a more drastic reduction of all Mcm levels in the nucleus during S, G2, and M phase is likely to severely inhibit re-initiation. Hence,

regulation of Mcm localization provides a potential mechanism for Clb/Cdc28 kinases to prevent re-initiation during the cell cycle. This regulation, however, has recently been called into question by a report that Mcm2 and Mcm3 are in fact constitutively present in the nucleus when examined by subcellular fractionation (Young and Tye, 1997).

To clarify these issues, we investigated the regulation of Mcm localization in living cells by studying Mcm proteins fused to green fluorescent protein (GFP). We show that the subcellular distribution of these Mcm-GFP fusions is regulated during the cell cycle, confirming the results obtained by indirect immunofluorescence. We further demonstrate that these proteins are actually excluded from the nucleus in G2/M phase and that they are dependent on each other for their proper localization, suggesting that they colocalize together as a complex. Mcm7-GFP accumulated in the nucleus in G1 phase even in the absence of Cdc6 loading onto chromatin. This finding indicates that the nuclear accumulation is independent of Mcm chromatin association and most likely results from a change in Mcm nucleocytoplasmic transport. Moreover, Mcm7-GFP was stable during its disappearance from the nucleus in S phase, arguing that this disappearance is due to net nuclear export. In the absence of Cdc6, Clb/Cdc28 kinases were necessary and sufficient for efficient export of the Mcm proteins. However, in the presence of Cdc6, which loads Mcm proteins onto chromatin, S phase progression was also needed to induce export. These results support a model in which Clb/Cdc28 kinases promote the net nuclear export of Mcm proteins only after S phase has led to their dissociation from chromatin. By such a mechanism Clb kinases could help block re-initiation without interfering with the function of Mcm proteins during S phase.

RESULTS

Subcellular distribution of Mcm-GFP fusions is regulated during the cell cycle.

To examine the localization of Mcm proteins in living cells, Mcm2, 3, 4, 6, and 7 were fused to GFP, and the fusion constructs under the control of the endogenous *MCM* promoters were substituted for the corresponding wild-type *MCM* genes by two-step gene replacement. The resulting strains all grew at wild-type rates and displayed normal flow cytometry profiles during log phase growth (data not shown), indicating that these Mcm-GFP fusions could functionally substitute for the wild-type proteins. Appending GFP to either 5' or 3' end of Mcm5 destroyed Mcm function, precluding an examination of Mcm5 localization in living cells.

Localization of GFP in the *MCM-GFP* strains was monitored by fluorescence microscopy. In asynchronous populations the Mcm-GFP fusions exhibited a continuous range of subcellular distributions (Fig. 1A) roughly correlating with cell cycle position. GFP fluorescence was primarily nuclear in unbudded cells (100% nuclear, n>100), partially nuclear in small budded cells (35-60% nuclear, n>100) and primarily cytoplasmic in large budded cells (<15% nuclear, n>90). Similarly, in cells synchronously released from an alpha factor arrest in G1 phase (Fig. 1B), Mcm7-GFP was nuclear in G1 phase (0 min), partly dispersed in S phase (60 min), predominantly cytoplasmic in G2/M phase (100min), and nuclear again in the next cell cycle (data not shown). Thus, the Mcm proteins concentrate in the nucleus at the beginning of each cell cycle and gradually disappear from the nucleus during S phase.

To determine more precisely when Mcm proteins change their subcellular distribution, *MCM7-GFP* cells were arrested at various points in the cell cycle (Fig. 1C). In early S phase cells (HU), Mcm7-GFP was predominantly nuclear, indicating that exit from G1 phase is not sufficient to trigger the disappearance of Mcm proteins from the nucleus; instead, progression through S phase and possibly DNA replication is also required. In G2/M phase cells (NOC) or in late anaphase at (*dbf2*) Mcm7-GFP was primarily cytoplasmic, suggesting that Mcm proteins are fully dispersed after S phase and that reaccumulation in the nucleus requires the completion of mitosis. This reaccumulation was observed within 20 min after release from a *dbf2* arrest when nuclei were still at opposite poles of the cell, indicating that Mcm proteins rapidly reenter the nucleus at telophase or early G1 phase. The localization of Mcm2-GFP, GFP-Mcm3, Mcm4-GFP, and Mcm6-GFP exhibited a temporal pattern similar to that of Mcm7-GFP (data not shown). Previous microscopic studies of Mcm localization did not observe exclusion of Mcm proteins from the nucleus (Dalton and Whitbread, 1995; Hennessy et al., 1990; Yan et al., 1993). Similarly, in our haploid cells we could not reliably observe nuclear exclusion of Mcm-GFP proteins in G2 or M phase cells. However, in *MATa/_* and *MATa/a* diploid cells, which have larger nuclei, we readily saw nuclear exclusion of Mcm2-GFP, GFP-Mcm3, and Mcm7-GFP (Fig. 1D, data not shown) in G2 and M phase.

In summary, our analysis of Mcm-GFP localization in living cells corroborates and extends the analysis of Mcm localization determined by indirect immunofluorescence of Mcm2, Mcm3, Mcm5 and Mcm7 in fixed cells. Taken together these results demonstrate that all six Mcm proteins share the same cell cycle localization pattern as schematized in Fig. 1E.

The Mcm proteins colocalize as a complex.

Complexes containing homologs of all six Mcm proteins have been isolated *from S. pombe* (Adachi et al., 1997), *X. laevis* (Thommes et al., 1997), and humans (Fujita et al., 1997). Although a comparable complex has yet to be isolated from budding yeast, numerous genetic and biochemical interactions between the budding yeast Mcm proteins (Dalton and Hopwood, 1997; Hennessy et al., 1991; Hopwood and Dalton, 1996; Lei et al., 1996) suggest that these proteins do associate with each other *in vivo*. We therefore investigated whether Mcm proteins might co-localize as a complex by asking whether perturbing the localization of one Mcm protein affects the localization of the others. To perturb the localization of an Mcm protein we fused it to two tandem copies of the SV40 nuclear localization signal ($SVNLS_2$). To monitor the effect of this perturbation on the localization of an Mcm protein we fused it to GFP.

When GFP and $SVNLS_2$ were attached to the same Mcm protein, the resulting Mcm-GFP-$SVNLS_2$ fusion protein was constitutively nuclear (Fig. 2A, 2B). In contrast, the Mcm-GFP-svnls3A_2, which contains a mutant NLS, was localized normally (Fig. 2B, data not shown). Hence, the $SVNLS_2$ tag was capable of overriding the nuclear export of Mcm proteins observed in S, G2, and M phase. Mcm-GFP fusion proteins were also constitutively nuclear when the $SVNLS_2$ tag was placed on a different Mcm protein. Fig. 2B shows two strains expressing Mcm2-GFP arrested with nocodazole at the G2/M phase boundary. Mcm2-GFP was nuclear when Mcm4 was fused to $SVNLS_2$ but not when Mcm4 was fused to the mutant svnls3A_2. Hence, constitutive nuclear localization of Mcm4 resulted in constitutive nuclear localization of Mcm2-GFP.

Fig. 2C summarizes the G2/M phase localization of Mcm-GFP for all the strains constructed with GFP and $SVNLS_2$ attached to different Mcm proteins. As discussed earlier, strains containing Mcm5-GFP could not be constructed because this fusion is nonfunctional. In virtually all cases, the Mcm-GFP fusion was nuclear (in contrast to the control series constructed with the mutant $svnls3A_2$ tag where Mcm-GFP was consistently cytoplasmic). The only exceptions were the strains expressing Mcm7-$SVNLS_2$/GFP-Mcm3 and Mcm7-$SVNLS_2$/GFP-Mcm6. We suspect that the $SVNLS_2$ and GFP tags in these strains are positioned on the Mcm complex in such a way that the $SVNLS_2$ tag is masked or inaccessible. Consistent with this idea, the equivalent strains in which the positions of the tags were switched (Mcm7-GFP/$SVNLS_2$-Mcm3 and Mcm7-GFP/$SVNLS_2$-Mcm6) displayed constitutive nuclear localization of the Mcm-GFP fusions. Thus, we conclude that the localization of any two Mcm proteins is coupled during S, G2, and M phase in budding yeast, and propose that all six Mcm proteins co-localize together as a complex.

Nuclear accumulation of Mcm7-GFP in G1 phase is not dependent on Cdc6 or Cdc45 function.

The nuclear influx of Mcm-GFP proteins during the M to G1 phase transition could be due to a change in Mcm nucleocytoplasmic transport or to regulated association with a nuclear anchor such as chromatin. To investigate whether Mcm chromatin association, might be responsible for this influx, we asked whether the influx is dependent on Cdc6. When Cdc6 was thermally inactivated in *MCM7-GFP cdc6-1^{ts}* cells before the M to G1 phase transition, Mcm7-GFP accumulated in the nucleus to the same

extent as the *MCM7-GFP CDC6* control (Fig. 3A). If allowed to proceed through G1 phase, these cells failed to replicate their DNA as monitored by flow cytometry (data not shown), confirming that Cdc6 was effectively inactivated. Thus, thermal inactivation of Cdc6 did not prevent Mcm7 nuclear accumulation in G1 phase. Similar results were obtained when Cdc6 was inactivated by transcriptional repression of the *CDC6* gene (Fig. 5A, 60 min). Previous experiments have demonstrated that both thermal inactivation and transcriptional depletion of Cdc6 prevent Mcm association with replication origins and chromatin (Donovan et al., 1997; Tanaka et al., 1997). We therefore conclude that the nuclear accumulation of Mcm7 in G1 phase does not require its association with chromatin.

Cdc45 is another potential nuclear anchor for Mcm proteins, as it is constitutively nuclear and has been shown to physically associate with several Mcm proteins (Dalton and Hopwood, 1997; Hopwood and Dalton, 1996). However, we have also shown that Cdc45 is not required for the accumulation of Mcm7 in G1 nuclei (see Supplementary Material). In summary, we have found no evidence to support an anchoring model for the nuclear accumulation of Mcm proteins. It is formally possible that residual Cdc6 or Cdc45 activity was facilitating nuclear accumulation in our experiments or that association with some other nuclear component is responsible for this accumulation. Nonetheless, our data is most consistent with a model in which regulation of Mcm nucleocytoplasmic transport is responsible for the nuclear influx of Mcm proteins at the end of mitosis.

Mcm7-GFP is exported from the nucleus.

As first pointed out by Hennessy et. al. (Hennessy et al., 1990), the disappearance of Mcm proteins from the nucleus during S phase could be due to either (1) net export of Mcm proteins from the nucleus or (2) concommitant nuclear degradation and cytoplasmic synthesis of Mcm proteins (with the newly synthesized Mcms remaining in the cytoplasm). The latter model predicts that Mcm levels should fall during S phase, if protein synthesis is blocked, and that Mcm-GFP protein should still disappear from the nucleus, even if fused to an SV40 NLS.

To test these predictions, *MCM7-GFP- SVNLS$_2$* and control *MCM7-GFP-svnls3A$_2$* cells were arrested in early S phase with HU, then released into medium containing cycloheximide to block protein synthesis and nocodazole to catch cells in G2/M phase (Fig. 4). Immunoblot analysis showed that Clb2 failed to accumulate during the release (data not shown) confirming that protein synthesis was blocked. Despite this block, both strains completed S phase and acquired a 2C DNA content within 120 min (Fig. 4A & 4B). During this period, *MCM7-GFP7- SVNLS$_2$* cells retained high concentrations of nuclear GFP fluorescence (Fig. 4A), and immunoblot analysis showed that they maintained total cellular levels of Mcm7-GFP protein (Fig. 4C). Thus, nuclear Mcm proteins are not degraded to any significant extent during S phase. While GFP fluorescence did disappear from the nucleus of *MCM7-GFP- svnls3A$_2$* cells during S phase (Fig. 4B), as expected, immunoblot analysis demonstrated that Mcm7-GFP-svnls3A$_2$ was still stably maintained in these cells (Fig.4D). We conclude that the Mcm proteins are stable during S phase and that their disappearance from the nucleus is due to net nuclear export rather than degradation.

Clb/Cdc28 kinases are required for efficient nuclear export of Mcm7-GFP.

What cell cycle signals are responsible for inducing and maintaining the net export of Mcm proteins from the nucleus? We initially investigated these signals in the absence of chromatin association by monitoring the localization of Mcm7-GFP in cells depleted for Cdc6 protein (Fig. 5A). *pMET-CDC6 MCM7-GFP dbf2-2^{ts}* cells growing in medium containing dextrose and lacking methionine were blocked in late anaphase at a *dbf2* arrest, exposed to methionine for 30 min to repress Cdc6 expression, then synchronously released from the arrest at the permissive temperature. Within 60 min after the release, cells had entered G1 phase and concentrated Mcm7-GFP in the nucleus. By 100 min, however, the cells had passed START (>80% small budded, n=100) and lost their nuclear concentration of Mcm7-GFP. These cells failed to replicate their DNA (Fig. 5B), establishing that Cdc6 had been effectively depleted and that S phase progression is not intrinsically required for the nuclear exit of Mcm proteins. Instead, this exit is dependent on passage through START, since cells released from the *dbf2* block into medium containing _ factor retained Mcm7-GFP in the nucleus (data not shown).

One possible signal for the net nuclear export of Mcm proteins is the activation of Clb/Cdc28 kinases, which is dependent on and occurs soon after START. These kinases were apparently activated during the course of our experiment, as a congenic *CDC6* strain treated in parallel to the *pMET-CDC6* strain initiated and completed S phase within 100 min after release from the *dbf2* block (Fig. 5B). To determine if the nuclear exit of Mcm7-GFP was dependent on Clb/Cdc28 kinases, we examined this exit in the presence of the Clb kinase-specific inhibitor, Sic1 (Schwob et al., 1994) (Fig. 5C). *pMET-CDC6 pGAL-sic1-9A MCM7-GFP dbf2-2^{ts}* and *pMET-CDC6 pGAL MCM7-GFP dbf2-2^{ts}* cells

were subjected to the same experimental protocol described above, except cells were grown in raffinose instead of dextrose and galactose was added 10 min after the release from mitotic arrest to induce sic1-9A, a hyperstabilized form of Sic1 (Mendenhall, 1999) (Fig. 5C). In the absence of sic1-9A, nuclear fluorescence of Mcm7-GFP began to disappear at 100 min and was completely gone by 120 min, similar to what was seen in Fig.5A. In the presence of sic1-9A, nuclear fluorescence of Mcm7-GFP began to diminish at 120 min, but did not completely disappear and was still faintly detectable in most cells at 160 min. Hence, sic1-9A delayed the onset of Mcm7-GFP disappearance from the nucleus and made it less efficient. To confirm that the induction of sic1-9A was effective in inhibiting Clb/Cdc28 kinases, this experiment was repeated except congenic strains containing wild-type *CDC6* were used instead of *pMET-CDC6* and the cells were analyzed by flow cytometry to see if they could replicate their DNA. The expression of sic1-9A completely prevented DNA replication (Fig. 5D), demonstrating that sic1-9A could effectively inhibit Clb/Cdc28 kinase in our experimental protocol. Thus, we conclude that Clb/Cdc28 kinases are required for efficient nuclear export of Mcm7-GFP in Cdc6-depleted cells.

The requirement for Clb/Cdc28 kinases to efficiently export Mcm7-GFP from the nucleus does not preclude other cell cycle signals from sharing a major role in this process. Cdc7/Dbf4 kinase and Cdc45 also act soon after START (Diffley et al., 1994; Owens et al., 1997; Tanaka and Nasmyth, 1998; Zou and Stillman, 1998) and have been implicated in the regulation of Mcm function. Cdc7/Dbf4 kinase phosphorylate several Mcm proteins *in vitro* (Lei et al., 1997; Oshiro et al., 1999) and interact genetically with Mcm5 and Mcm2 to trigger initiation (Hardy et al., 1997; Lei et al., 1997). Cdc45

interacts genetically and physically with multiple Mcm proteins (Dalton and Hopwood, 1997; Hardy, 1997; Hennessy et al., 1991; Hopwood and Dalton, 1996; Zou et al., 1997). Thermal inactivation of Cdc7 or Cdc45, however, does not block the nuclear export of Mcm7-GFP after START in Cdc6 depleted cells (see Supplementary Material). Hence, Cdc7/Dbf4 kinase and Cdc45 do not appear to be required for this export.

Ectopic induction of Clb2 or Clb5 can promote the net nuclear export of Mcm7-GFP.

To determine whether Clb kinase activity is sufficient to promote the net nuclear export of Mcm proteins, we ectopically expressed Clb2 or Clb5 in G1 phase before START and asked whether the resulting induction of Clb/Cdc28 kinase activity (Amon et al., 1994) could cause Mcm7-GFP to disappearance from the nucleus (Fig. 6A). *pMET-CDC6 pGAL-CLB2ΔDB MCM7-GFP dbf2-2^{ts}* or *pMET-CDC6 pGAL-CLB5ΔDB MCM7-GFP dbf2-2^{ts}* cells growing in medium containing raffinose and lacking methionine were blocked in late anaphase at a *dbf2* arrest and exposed to methionine for 30 min to repress Cdc6 expression. Effective depletion of Cdc6 was confirmed by releasing a portion of the cells from the arrest and showing by flow cytometry that the cells fail to replicate their DNA in the next cell cycle (data not shown). The remainder of the cells was released into medium containing _ factor, which traps them in the following G1 phase. After 60 min, when most of the cells were in G1 phase and had accumulated Mcm7-GFP in their nuclei (Fig. 6A, 0 min), galactose was added to induce Clb2$^{\Delta db}$ or Cbl5$^{\Delta db}$ (stabilized forms of Clb2 and Clb5 lacking their N-terminal destruction boxes (Amon et al., 1994; Cross et al., 1999)). Loss of GFP nuclear fluorescence could be seen

in approximately 20% of cells (n=100) by 120 min and nearly 100% of cells (n=100) by 180 min (Fig. 6A), correlating with the increase in Clb levels (Fig. 6B). If galactose was not added so that $Clb2^{\Delta db}$ or $Clb5^{\Delta db}$ were not induced, Mcm7-GFP was strongly retained in the nucleus (data not shown). Moreover, control *pMET-CDC6 pGAL MCM7-GFP dbf2-2^{ts}* cells that do not induce any stabilized Clb also retained Mcm7-GFP in the nucleus (Fig. 6A). We conclude that ectopic activation of Clb2/Cdc28 or Clb5/Cdc28 kinase in G1 phase can induce the net nuclear export of Mcm proteins. The resulting nuclear exclusion of Mcm proteins likely contributes to the inhibition of pre-RC formation that we previously observed when $Clb2^{\Delta db}$ was ectopically induced before START (Detweiler and Li, 1998). In summary, the data from both ectopic and endogenous activation of ClbCdc28 kinases indicate that, in the absence of Cdc6, these kinases are both necessary and sufficient for the efficient export of Mcm proteins from the nucleus.

Clb/Cdc28 kinase activity is not sufficient to induce Mcm disappearance from the nucleus.

The data implicating Clb/Cdc28 kinases in the net nuclear export of Mcm7-GFP were obtained in the absence of Cdc6 to remove any potential influence of chromatin association on this export. When these same studies were repeated in the presence of Cdc6 by using congenic *CDC6* strains no nuclear export of Mcm7-GFP was observed (Fig. 5A & 6C). Hence, when Cdc6 functions normally, activation of Clb/Cdc28 kinases is not sufficient to induce net nuclear export of Mcm7-GFP; passage through S phase is now also required (Fig. 1C). The simplest interpretation of these data is that chromatin

association promoted by Cdc6 makes Mcm proteins refractory to the nuclear export induced by Clb/Cdc28 kinases and S phase progression (and possibly DNA replication) is required to dissociate Mcm proteins from chromatin before they can be exported.

Regulation of Mcm localization is not essential for the preventing re-initiation.

Exclusion of Mcm proteins from the nucleus during S, G2 and M phase may be sufficient to inhibit re-initiation of DNA replication during the cell cycle. However, given the need to strictly enforce this block at hundreds of origins in the budding yeast genome, it is unlikely to be the sole mechanism for preventing re-initiation. Consistent with this notion, *MCM7-GPF-SVNLS*$_2$ cells, in which all six Mcm proteins are constitutively nuclear (Fig. 2C), showed no signs of re-replicating. The cells displayed a normal distribution of 1C and 2C DNA content during exponential growth and maintained a stable 2C DNA content (matching DNA content of *MCM7-GFP* control cells) when arrested at the G2/M boundary with nocodazole (Fig.2D). These cells, moreover, divided and maintained plasmids at wild-type rates (data not shown), indicating that the initiation of DNA replication was normal in these cells. We propose that the exclusion of Mcm proteins from the nucleus is only one of several overlapping mechanisms employed by the cell to insure that re-initiation does not occur within a cell cycle.

DISCUSSION

Cell cycle regulation of Mcm localization in budding yeast

In this study we show that the subcellular distribution of budding yeast Mcm2, Mcm3, Mcm4, Mcm6, and Mcm7 is regulated during the cell cycle as schematized in Fig 1E. Our data confirm and extend previous immunofluorescence analyses of Mcm2, Mcm3, Mcm5, and Mcm7 localization in fixed cells (Dalton and Whitbread, 1995; Hennessy et al., 1990; Yan et al., 1993) and finally demonstrate that the localization of all six Mcm proteins share the same cell cycle regulation. In addition, we show that these proteins are actually exported from the nucleus and that this export results in nuclear exclusion of Mcm proteins in G2 and M phase. This exclusion makes the control of Mcm localization particularly compelling as a means of regulating initiation (see below). Our data also suggest that the six Mcm proteins co-localize as a complex. They not only share identical localization during the cell cycle but are also dependent on each other for this localization. The simplest interpretation of these findings is that the Mcm proteins are transported through the nuclear pore as a complex comprised of all six Mcm proteins. Our results, however, do not rule out the possibility that the Mcm proteins are transported across the nuclear envelope as individual proteins or subcomplexes and then assembled into a full complex on either side. Nonetheless, because Mcm proteins co-localize, we believe that our more detailed analysis of Mcm7 localization can be extended to the other Mcm proteins.

Regulation of Mcm localization by Clb/Cdc28 kinase and chromatin association.

Our data suggest that cell cycle regulation of Mcm localization is driven by Clb kinase modulation of Mcm nucleocytoplasmic transport. In the absence of Cdc6 function, induction of these kinases is both necessary and sufficient to induce efficient net export of Mcm proteins from the nucleus. Moreover, a drop in Clb kinase activity at the end of mitosis (after the *dbf2* arrest point) appears to be necessary for the rapid entry of Mcm proteins into G1 nuclei.

While our manuscript was undergoing review it was reported that Cln kinases promote the nuclear exit of Mcm4 based on the observation that Clb5 and Clb6 are not essential for Mcm4 nuclear exit during the passage through START (Labib et al., 1999). We also observed some Clb kinase-independent nuclear export of Mcm proteins during the passage through START (Fig. 5C), but this export was both slow and incomplete, as evidenced by the persistence of Mcm protein in the nucleus (which we could see in live but not fixed cells). It is tempting to speculate that Cln/Cdc28 kinases induce this export because of a weak overlap in substrate specificity with Clb/Cdc28 kinases, but a role for Cdc7/Dbf4 or Cdc45 has not been ruled out (these components have not been inactivated in the absence of Clb kinase activity). The inefficiency of this Clb kinase-independent export suggests that it may not be potent enough to exclude Mcm proteins from the nucleus and may not be a major determinant in the block to re-initiation. This view is consistent with the previously published observation that Clb kinase activity is required for the block to pre-RC assembly that is induced after START (Piatti et al., 1996; Tanaka et al., 1997). The view is also consistent with the observation that the Clb kinase-independent (but not the Clb kinase-dependent) export of Mcm4 can be overridden by

expression of Cdc6 (Labib et al., 1999), which favors the nuclear accumulation of Mcm proteins by loading them onto chromatin. Hence, we believe that Clb kinases are required for the efficient and effective exclusion of Mcm proteins from the nucleus.

Our experiments do not address the mechanism by which Clb kinases promote Mcm nuclear exit. We do not know whether the kinases phosphorylate the Mcm proteins and/or other proteins that mediate Mcm transport. In addition, we do not know whether Clb kinases promote net nuclear export by inhibiting Mcm import rates, stimulating Mcm export rates, or both. Identification of the transport signals and transport receptors for the Mcm complex will be important for addressing these questions in the future.

Although Clb/Cdc28 kinases can promote net nuclear export of Mcm proteins, the activation of these kinases is normally not sufficient for this export to occur. Once Cdc6 loads Mcm proteins onto chromatin, their nuclear export also requires progression through S phase. Previous work has shown that the association of Mcm proteins with chromatin is regulated during the cell cycle in budding yeast (Aparicio et al., 1997; Donovan et al., 1997; Tanaka et al., 1997) and other eukaryotes (reviewed in Tye, 1999). Mcm proteins are tightly associated with chromatin in G1 phase, gradually dissociate during S phase, and remain unassociated in G2 and M phase. These observations raise the possibility that chromatin association prevents Mcm nuclear export and that Mcm dissociation from chromatin during S phase is required before Clb/Cdc28 kinases can expel Mcm proteins from the nucleus.

We propose the following model for the regulation of Mcm localization during the cell cycle (Fig. 7). In G2 and M phases, Clb/Cdc28 kinases maintain Mcm proteins in the cytoplasm by promoting their net nuclear export. At the end of mitosis, the

precipitous drop in Clb/Cdc28 kinase activity allows the Mcm proteins to revert to a default state of net nuclear import. Once the Mcm proteins enter the nucleus, Cdc6 loads them onto chromatin in preparation for replication initiation. Although the resulting chromatin association is not necessary for the accumulation of Mcm proteins into the nucleus, it does affect their subsequent relocalization to the cytoplasm by making the Mcm proteins refractory to nuclear export. Consequently, Mcm proteins are not exported as soon as Clb/Cdc28 kinases are activated in late G1 phase but instead remain on chromatin poised to initiate DNA replication. After initiation, Mcm proteins are still tightly associated with chromatin (possibly at replication forks) and are retained in the nucleus. During the course of S phase (and possibly DNA replication) Mcm proteins gradually dissociate from chromatin and finally become susceptible to nuclear export. Hence, both passage through S phase and Clb/Cdc28 kinase activation are required for Mcm proteins to be excluded from the nucleus.

Role of regulating Mcm localization in the block to re-initiation

Clb/Cdc28 kinases play a pivotal role in the block to re-initiation. We and others have shown that these kinases can block re-initiation by preventing the re-assembly of pre-replicative complexes at origins that have already fired (Detweiler and Li, 1998; Piatti et al., 1996; Tanaka et al., 1997). Exactly how these kinases prevent pre-RC assembly is not understood. In this report we present one likely mechanism—the exclusion of Mcm proteins from the nucleus. Given that a two-fold reduction of Mcm2 levels can measurably impair origin function (Lei et al., 1996), nuclear exclusion of all six Mcm proteins should cripple any attempts to re-initiate DNA replication in G2 and M

phase. This net export of Mcm proteins could also discourage re-initiation during S phase, despite the persistence of Mcm proteins in S phase nuclei. We suggest that this persistent population consists of Mcm proteins that are tightly associated with chromatin and that the free nucleoplasmic pool of Mcm proteins (which is likely the pool involved in pre-RC assembly) is significantly reduced in S phase by Clb kinase induced export.

Multiple overlapping mechanisms are used to ensure reliable, complete, and decisive execution of many key cell cycle events. For example, both Clb destruction and Sic1 mediated kinase inhibition ensure the exit from mitosis in budding yeast (Schwab et al., 1997; Visintin et al., 1997). Although neither mechanism is essential, since either can drive mitotic exit in the absence of the other, each is considered a crucial component of this pathway. It makes sense for Clb/Cdc28 kinases also to employ multiple overlapping mechanisms to guarantee the block to re-initiation, as this block must be strictly enforced at hundreds of origins scattered throughout the budding yeast genome. In support of this notion, we have shown that constitutive nuclear localization of all six Mcm proteins does not result in any detectable re-replication, indicating that the regulation of Mcm localization is not the sole mechanism for preventing re-initiation.

Another likely mechanism is the regulation of Cdc6 levels. Cdc6 is an unstable protein whose levels peak during G1 phase when pre-RC assembly occurs and plummet in S, G2 and M phase when pre-RC reassembly is prevented (Detweiler and Li, 1997; Drury et al., 1997; Piatti et al., 1995). Clb/Cdc28 kinases maintain these low levels in part by negatively regulating *CDC6* transcription (Piatti et al., 1995). The potential importance of this regulation for the block to re-initiation is highlighted by the observation that constitutive overexpression of the Cdc6 homolog, Cdc18, results in re-

replication in *Schizosaccharomyces pombe* (Jallepalli et al., 1997; Nishitani and Nurse, 1995). In *S. cerevisiae*, however, constitutive overexpression of a stabilized Cdc6 protein does not lead to re-replication (Drury et al., 1997; Tanaka et al., 1997, Detweiler and Li, unpublished data). We suspect that the regulation of Mcm localization can serve as a backup mechanism to prevent re-initiation in these cells. Interestingly, we do not observe any re-replication when both Mcm localization and Cdc6 expression are deregulated (Nguyen, V. and Li, J.J., unpublished data). This suggests that additional mechanisms, possibly acting on other initiator proteins like ORC, block re-initiation in budding yeast.

Multiple overlapping regulatory mechanisms are likely to be used to enforce the block to re-initiation in other eukaryotes. Although we believe that similar regulatory strategies will be employed, their precise implementation may differ. For example, in all eukaryotes other than budding yeast, Mcm proteins are constitutively nuclear (reviewed in Tye, 1999) so their localization cannot play a role in the block to re-initiation. On the other hand, in human cells Cdc6 is constitutively expressed and displays a pattern of cell cycle localization resembling that of budding yeast Mcm proteins (Fujita et al., 1999; Jiang et al., 1999; Petersen et al., 1999; Saha et al., 1998). Hence, the fundamental strategy of excluding initiator proteins from the nucleus to prevent re-initiation may be conserved in eukaryotes.

CONCLUSION

We investigated one mechanism by which CDKs can inhibit re-initiation of DNA replication within a cell cycle. Our results suggest that all six members of the Mcm2-7

family of initiator proteins in budding yeast accumulate in the nucleus in G1 phase, are gradually exported from the nucleus in S phase, and are finally excluded from the nucleus in G2/M phase (see Fig. 1E). Mcm proteins are dependent on each other for this regulated localization, consistent with the proteins co-localizing as a complex. Clb/Cdc28 kinases play a major role in this regulation by promoting the efficient nuclear export of Mcm2-7. Although this nuclear exclusion of Mcm proteins may be sufficient to prevent re-initiation, it is not necessary, suggesting that Clb/Cdc28 kinases employ additional overlapping mechanisms to ensure that origins fire at most once per cell cycle.

MATERIALS AND METHODS

Yeast media, growth, and arrest

YEP medium and synthetic complete medium lacking methionine (Owens et al., 1997) were supplemented with 2% dextrose (YEPD; SCD-Met), or 2% raffinose (YEPR; SCR-Met). The *GAL1* promoter (*pGAL*) was induced by addition of 2% galactose and the *MET* promoter (*pMET*) was repressed by the addition of 2 mM methionine.

To arrest cells, _-factor was added to 50 ng/ml (all strains were*bar1*), hydroxyurea (Schulte et al.,) was added to 0.2 M, and nocodazole (NOC) was added to 10 ug/ml. These cell cycle blocks were released by filtering the cells, washing them three times with an equal volume of resuspension medium prewarmed to the appropriate temperature, then resuspending them in the appropriate medium. In some cases, _-factor arrested cells were released by the addition of pronase to 100 µg/ml to degrade the alpha factor. To inhibit protein synthesis during S phase, cycloheximide was added to 100 µg/ml. This inhibition blocks entry into S phase and M phase but not progression through S phase.

Plasmids and Strains

See Chapter 5

Flow cytometry, and immunoblot analysis, and fluorescence microscopy

Flow cytometry was performed as described in (Detweiler and Li, 1997). Immunoblot analysis was performed as described in (Owens et al., 1997). Blots were probed with B34 monoclonal anti-GFP antibodies at a 1:40 dilution (a gift of E. O'Shea,

University of California, San Francisco), anti-Prp16 antibodies at a 1:5000 dilution (a gift of C. Guthrie, University of California, San Francisco), or anti-Clb2 antibodies at a 1:1200 dilution (a gift of D. Morgan, University of California, San Francisco).

For fluorescence microscopy of live cells, cells were rapidly washed with PBS and photographed within 3 min of sampling using a Leica DMLB fluorescence microscope with a 100X PL Fluotar oil immersion objective. Images were acquired with an Optronics DEI-750 CCD camera using the Scion Image Software program. Panels were assembled with Adobe Photoshop software.

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REF

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Figure 1. Mcm-GFP protein localization is regulated across the cell cycle. (**A**) Asynchronous cultures. YJL1969 (*MCM2-GFP*), YJL2160 (*GFP-MCM3*), YJL1973 (*MCM4-GFP*), YJL2163 (*GFP-MCM6*), and YJL1977 (*MCM7-GFP*) were grown exponentially in YEPD containing 2 μg/ml DAPI for 1 hr before being examined by fluorescence and bright-field microscopy. (**B**) Synchronous cultures. YJL1977 (*MAT***a** *MCM7-GFP*) growing exponentially in YEPD at 30° C was arrested in _-factor for 90 min and synchronously released from the arrest by addition of pronase at 22° C (0 min). Samples were taken at 0, 60, and 100 min and analyzed by fluorescence microscopy and flow cytometry. (**C**) Arrested cultures. YJL1977 was released from _ arrest as described in (B) and incubated for 90 min at 30° C with hydroxyurea (HU) or nocodazole (NOC). YJL1937 (*dbf2-2 MCM7-GFP*) growing exponentially in YEPD at 24° C was arrested in late anaphase by incubation at 37° C for 3 hr (dbf2) then released from the arrest at 22° for 20 min (dbf2 release + 20 min). (**D**)Mcm3-GFP is excluded from the nucleus in G2/M phase. YJL2756 (*MAT***a**/*MAT_GFP-MCM3/GFP-MCM3*) was grown to exponential phase in YEPD at 30° C. DAPI (5 μg/ml) was added to half the culture (LOG), and nocodazole and DAPI (5ug/ml) added to the other half (NOC). 90 min later, when the nocodazole treated cells had arrested in G2/M phase, cells from both cultures were examined by fluorescence microscopy. Arrows point our examples of nuclei excluding Mcm3-GFP. (**E**) Schematic of Mcm protein localization during the cell cycle. Mcm proteins rapidly accumulate in the nucleus in telophase or early G1 phase, gradually redistribute to the cytoplasm during S phase, and are excluded from the nucleus in G2 and M phase.

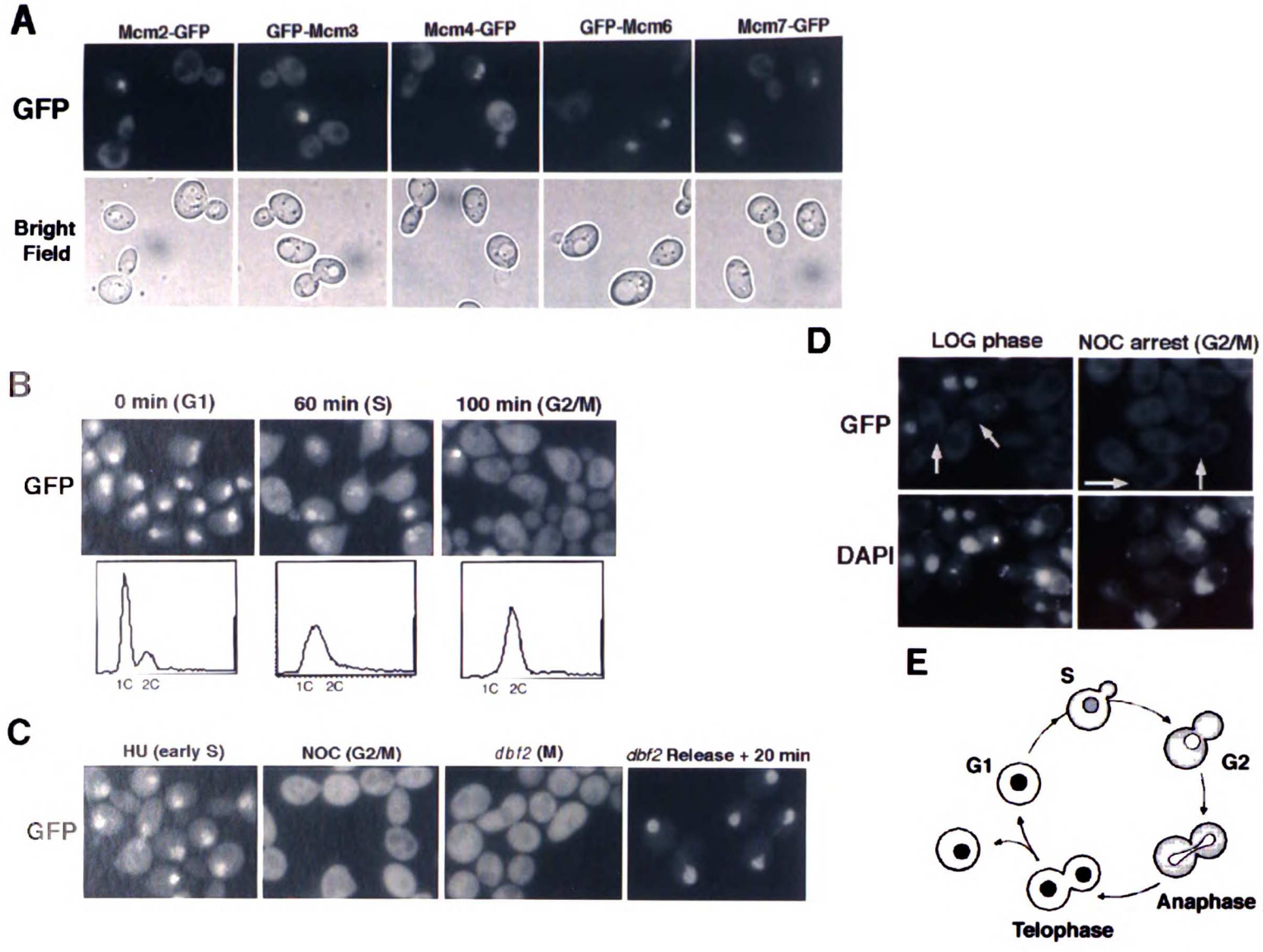

A
Mcm2-GFP
GFP-Mcm3
Mcm4-GFP
GFP-Mcm6
Mcm7-GFP
GFP
Bright Field
B
0 min (G1)
60 min (S)
100 min (G2/M)
GFP
1C 2C
1C 2C
1C 2C
C
HU (early S)
NOC (G2/M)
dbf2 (M)
dbf2 Release + 20 min
GFP
D
LOG phase
NOC arrest (G2/M)
GFP
DAPI
E
S
G1
G2
Anaphase
Telophase

Figure 2. Constitutive nuclear localization of one Mcm protein results in constitutive nuclear localization of all Mcm proteins. (**A**) Mcm-GFP proteins fused to tandem copies of the SV40 NLS ($SVNLS_2$) are constitutively nuclear. YJL1987 (*MCM2-GFP-$SVNLS_2$*), YJL2227 (*$SVNLS_2$-GFP-MCM3*), YJL2155 (*MCM4-GFP-$SVNLS_2$*), YJL1991 (*MCM5-GFP-$SVNLS_2$*), YJL2229 (*$SVNLS_2$-GFP-MCM6*), and YJL1981 (*MCM7-GFP-$SVNLS_2$*) were grown to exponential phase in YEPD at 30° C and examined by fluorescence microscopy. (**B**) Mcm4-$SVNLS_2$ directs Mcm2-GFP into the nucleus in G2/M phase. YJL2039 (*MCM4-GFP-$SVNLS_2$*), YJL2221 (*MCM4-GFP-$svnls3A_2$*), YJL2170 (*MCM4-$SVNLS_2$ MCM2-GFP*), and YJL2172 (*MCM4-$svnls3A_2$ MCM2-GFP*) were arrested in G2/M phase with nocodazole and examined by fluorescence microscopy. $svnls3A_2$, mutant SV40 NLS tag. (**C**) G2/M phase localization of Mcm-GFP in nocodazole arrested cells expressing a second Mcm fused to $SVNLS_2$. Strains used are listed in Supplementary Materials. Numbers (2-7) identify the Mcm proteins fused to either GFP or $SVNLS_2$ in each strain. N - Mcm-GFP predominantly nuclear; C - Mcm-GFP predominantly cytoplasmic; _ - strain not constructed or not part of this analysis. (**D**) Nocodazole was added (T=0 min) to exponentially growing cultures of YJL1981 (*MCM7-GFP-$SVNLS_2$*) and YJL1985 (*MCM7-GFP-$svnls3A_2$*) and samples were analyzed by flow cytometry at the indicated times. Cells were >95% budded by 120 min.

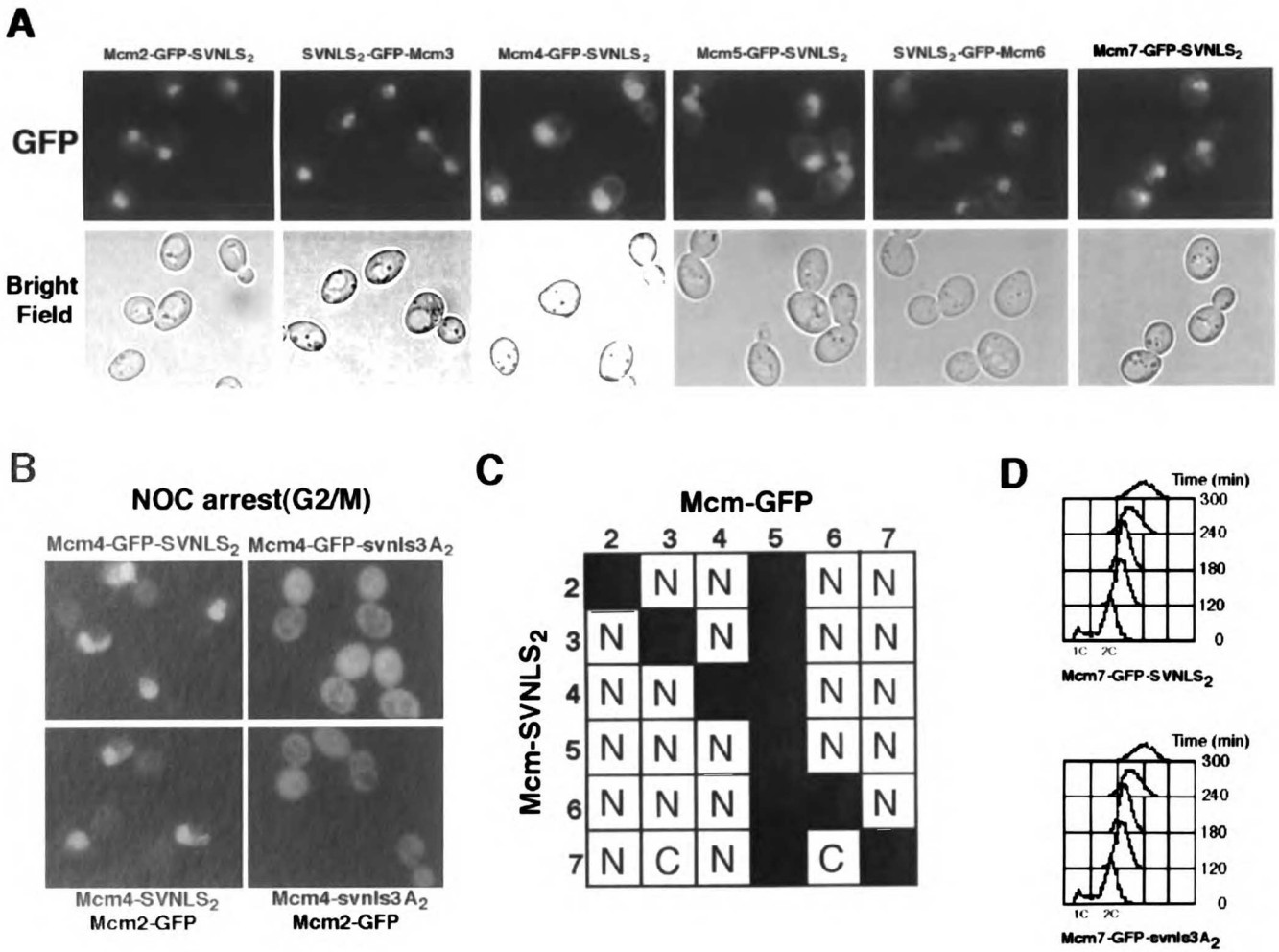

A
Mcm2-GFP-SVNLS2
SVNLS2-GFP-Mcm3
Mcm4-GFP-SVNLS2
Mcm5-GFP-SVNLS2
SVNLS2-GFP-Mcm6
Mcm7-GFP-SVNLS2
GFP
Bright Field
B
NOC arrest(G2/M)
Mcm4-GFP-SVNLS2
Mcm4-GFP-svnls3A2
Mcm4-SVNLS2 Mcm2-GFP
Mcm4-svnls3A2 Mcm2-GFP
C
Mcm-GFP
2 3 4 5 6 7
Mcm-SVNLS2
2 N N N N
3 N N N N
4 N N N N
5 N N N N
6 N N N N
7 N C N C
D
Time (min)
300
240
180
120
0
1C 2C
Mcm7-GFP-SVNLS2
Time (min)
300
240
180
120
0
1C 2C
Mcm7-GFP-svnls3A2

Figure 3. Nuclear accumulation of Mcm7-GFP in G1 phase is independent of Cdc6. *CDC6* (YJL1977, *MAT***a** *CDC45 CDC6 MCM7-GFP*) and *cdc6-1*^{ts} (YJL1925, *MAT***a** *cdc6-1 MCM7-GFP*) cells growing exponentially in YEPD at 23° C were arrested in G2/M phase by addition of nocodazole for 3 hr then shifted to 37° C to inactivate the ts mutant. After 30 min at 37° C, cells were released from the G2/M phase arrest by filtration and resuspended in prewarmed 37° C YEPD medium containing _ factor. Cells were examined by fluorescence microscopy just before release from the G2/M arrest (NOC) and 90 min later when they had fully arrested in G1 phase (_F arrest).

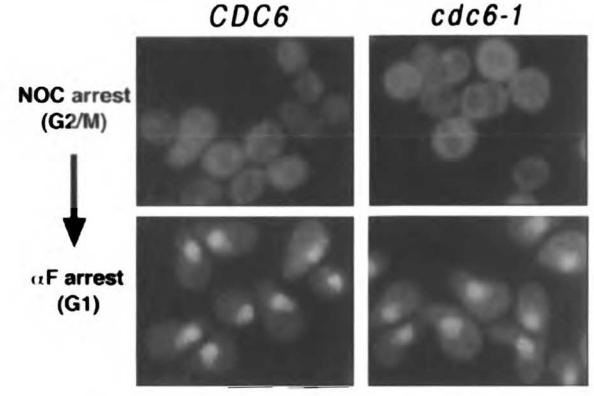

CDC6
cdc6-1
NOC arrest
(G2/M)
αF arrest
(G1)

Figure 4. Disappearance of Mcm7-GFP from the nucleus during S phase is not due to Mcm degradation. Cells growing exponentially in YEPD at 30° C were arrested in early S phase by treatment with _ factor for 1 hr followed by addition of pronase (to inactivate _ factor) and hydroxyurea for 90 min. Half of the cells were released from the early S phase arrest by filtering the cells and resuspending them in YEPD medium containing cycloheximide to block new protein synthesis. The other half were treated with cycloheximide without releasing them from the arrest. (**A, B**) Samples taken at the early S phase arrest (HU arrest) and 120 min after treatment with cycloheximide (HU release + CXH, HU arrest + CXH) were analyzed by fluorescence microscopy and flow cytometry. (**C, D**) Samples taken in exponential growth (log), after 1 hr _ factor treatment (_F), at the early S phase arrest (Schulte et al.,), and at 30 min intervals after cycloheximide addition (30-120) were immunoblotted with _-GFP monoclonal antibodies. Blots were reprobed with _-Prp16 antibodies as a loading control. (**A, C**) YJL1981 (*MAT***a** *MCM7-GFP-SVNLS*$_2$). (**B, D**) YJL1985 (*MAT***a** *MCM7-GFP-svnls3A*$_2$).

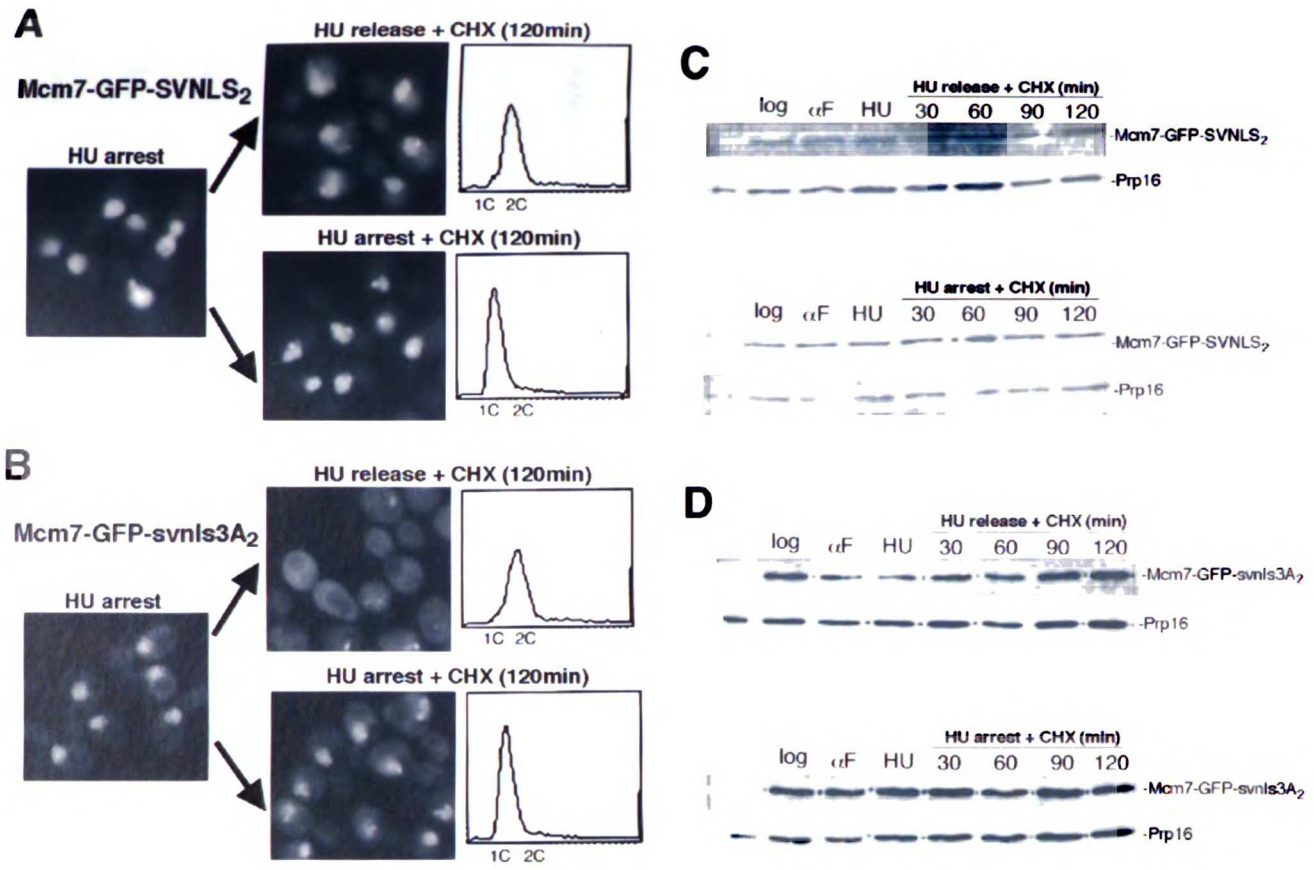

A
Mcm7-GFP-SVNLS2
HU arrest
HU release + CHX (120min)
HU arrest + CHX (120min)
1C 2C
B
Mcm7-GFP-svnls3A2
HU arrest
HU release + CHX (120min)
HU arrest + CHX (120min)
1C 2C
C
HU release + CHX (min)
log αF HU 30 60 90 120
Mcm7-GFP-SVNLS2
Prp16
HU arrest + CHX (min)
log αF HU 30 60 90 120
Mcm7-GFP-SVNLS2
Prp16
D
HU release + CHX (min)
log αF HU 30 60 90 120
Mcm7-GFP-svnls3A2
Prp16
HU arrest + CHX (min)
log αF HU 30 60 90 120
Mcm7-GFP-svnls3A2
Prp16

Figure 5. Clb/Cdc28 kinases are necessary for efficient net nuclear export of Mcm7-GFP in Cdc6 depleted cells. (**A**, **B**) Congenic strains YJL1945 (*MAT***a** *dbf2-2 pMET-CDC6 MCM7-GFP*) and YJL1937 (*MAT***a** *dbf2-2 CDC6 MCM7-GFP*) were grown to exponential phase in SDC-Met at 23° C and arrested in late anaphase by incubation at 37° C for 2 hr. The arrested cells were then filtered and resuspended in prewarmed 37° C YEPD medium containing methionine to repress *pMET-CDC6* transcription. After 30 min, the cells were released into the next cell cycle at 23° C and split into two cultures (T=0 min). (**A**) Hydroxyurea was added to one culture to prevent DNA replication and live cells were examined by fluorescence microscopy at the indicated times. Nocodazole was also added to prevent any inappropriate mitosis in the next cell cycle arising from Cdc6 depletion (Piatti et al., 1995). (**B**) Nocodazole was added to the second culture and samples were analyzed at the indicated times by flow cytometry. (**C**, **D**) Cells were arrested in late anaphase, treated with methionine to repress *pMET-CDC6*, and released into the next cycle (T=0 min) as described above except the medium contained raffinose instead of dextrose. At 0 min, _ factor was added to transiently block cells before START. After 10 min, galactose was added to induce sic1-9A. After 60 min, pronase was added to degrade the _ factor, allowing cells to proceed through START, and nocodazole was added to block any inappropriate mitosis (Piatti et al., 1995). Samples were analyzed at the indicated times by fluorescence microscopy or flow cytometry. (**C**) Fluorescence micrographs and % budding of congenic *pMET-CDC6* strains YJL2962 (*MAT***a** *dbf2-2 pMET-CDC6 MCM7-GFP pGAL*)and YJL2971 (*MAT***a** *dbf2-2 pMET-CDC6 MCM7-GFP pGAL-sic1-9A*). (**D**) Flow cytometry of congenic *CDC6* strains

YJL2951 (*MAT***a** *dbf2-2 CDC6 MCM7-GFP pGAL*) and YJL2959 (*MAT***a** *dbf2-2 CDC6 MCM7-GFP pGAL-sic1-9A*).

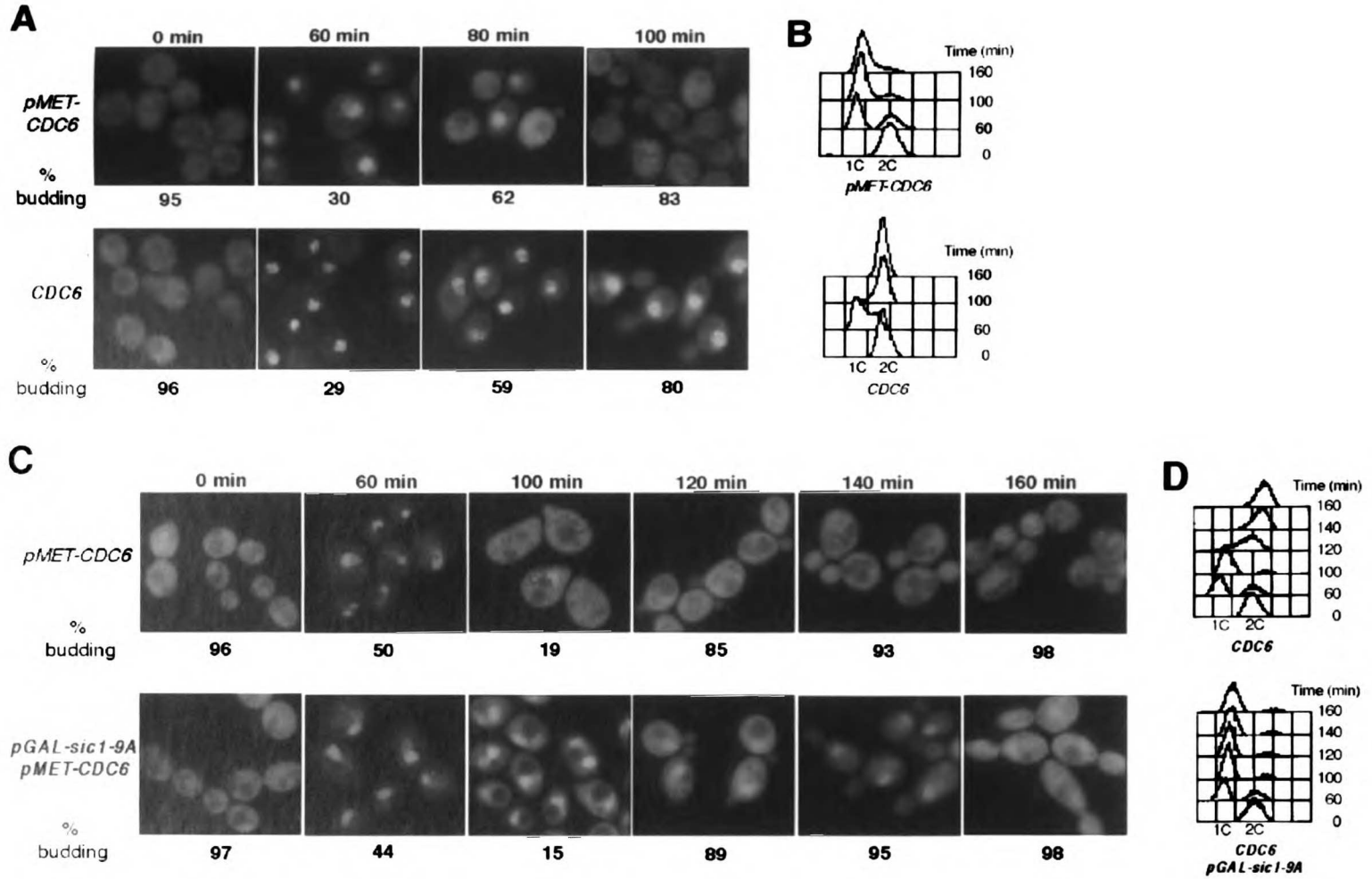

A
0 min
60 min
80 min
100 min
pMET-CDC6
% budding
95
30
62
83
CDC6
% budding
96
29
59
80
B
Time (min)
160
100
60
0
1C 2C
pMET-CDC6
Time (min)
160
100
60
0
1C 2C
CDC6
C
0 min
60 min
100 min
120 min
140 min
160 min
pMET-CDC6
% budding
96
50
19
85
93
98
pGAL-sic1-9A pMET-CDC6
% budding
97
44
15
89
95
98
D
Time (min)
160
140
120
100
60
0
1C 2C
CDC6
Time (min)
160
140
120
100
60
0
1C 2C
CDC6
pGAL-sic1-9A

Figure 6. Ectopic Clb2$^{\Delta db}$ and Clb5$^{\Delta db}$ induction before START can induce net nuclear export of Mcm7-GFP in Cdc6-depleted cells. (**A,B**) Congenic strains YJL1939 (*MAT***a** *dbf2-2 pMET-CDC6 MCM7-GFP pGAL-CLB2ΔDB*), YJL2964 (*MAT***a** *dbf2-2 pMET-CDC6 MCM7-GFP pGAL-CLB5ΔDB*), and YJL2962 (*MAT***a** *dbf2-2 pMET-CDC6 MCM7-GFP pGAL*) were grown to exponential phase in SRC-Met at 23° C and arrested in late anaphase by incubation at 37° C for 2 hr. The arrested cells were then filtered and resuspended in prewarmed 37° C YEPR medium containing additional methionine to repress *pMET-CDC6* transcription. After 30 min (T=0 min), the cells were released into the next cell cycle at 23° and _ factor was added to rearrest them before START of the next cell cycle. Hydroxyurea was also added to mirror the treatment in (C,D), where it was added to prevent S phase progression. After 60 min the cells had progressed into G1 phase and galactose was added to induce Clb2$^{\Delta db}$ or Clb5$^{\Delta db}$. Samples were taken at the indicated times for fluorescence microscopy (**A**) and (for YJL1939) immunoblot analysis of total Clb2 levels (with Prp16 serving as a loading control) (**B**). (**C,D**) Congenic strains YJL1935 (*MAT***a** *dbf2-2 CDC6 MCM7-GFP pGAL-CLB2ΔDB*), YJL2954 (*MAT***a** *dbf2-2 CDC6 MCM7-GFP pGAL-CLB5ΔDB*), and YJL2951 (*MAT***a** *dbf2-2 CDC6 MCM7-GFP pGAL*) were treated as described in (A,B). Hydroxyurea was added to prevent cells that were inducing Clb2$^{\Delta db}$ and Clb5$^{\Delta db}$ from replicating their DNA (Amon et al., 1994). Samples were taken at the indicated times for fluorescence microscopy (**C**) and (for YJL1935) immunoblot analysis of total Clb2 levels (**D**). Clb5$^{\Delta db}$ induction allowed YJL2964 and YJL2954 to pass START and bud.

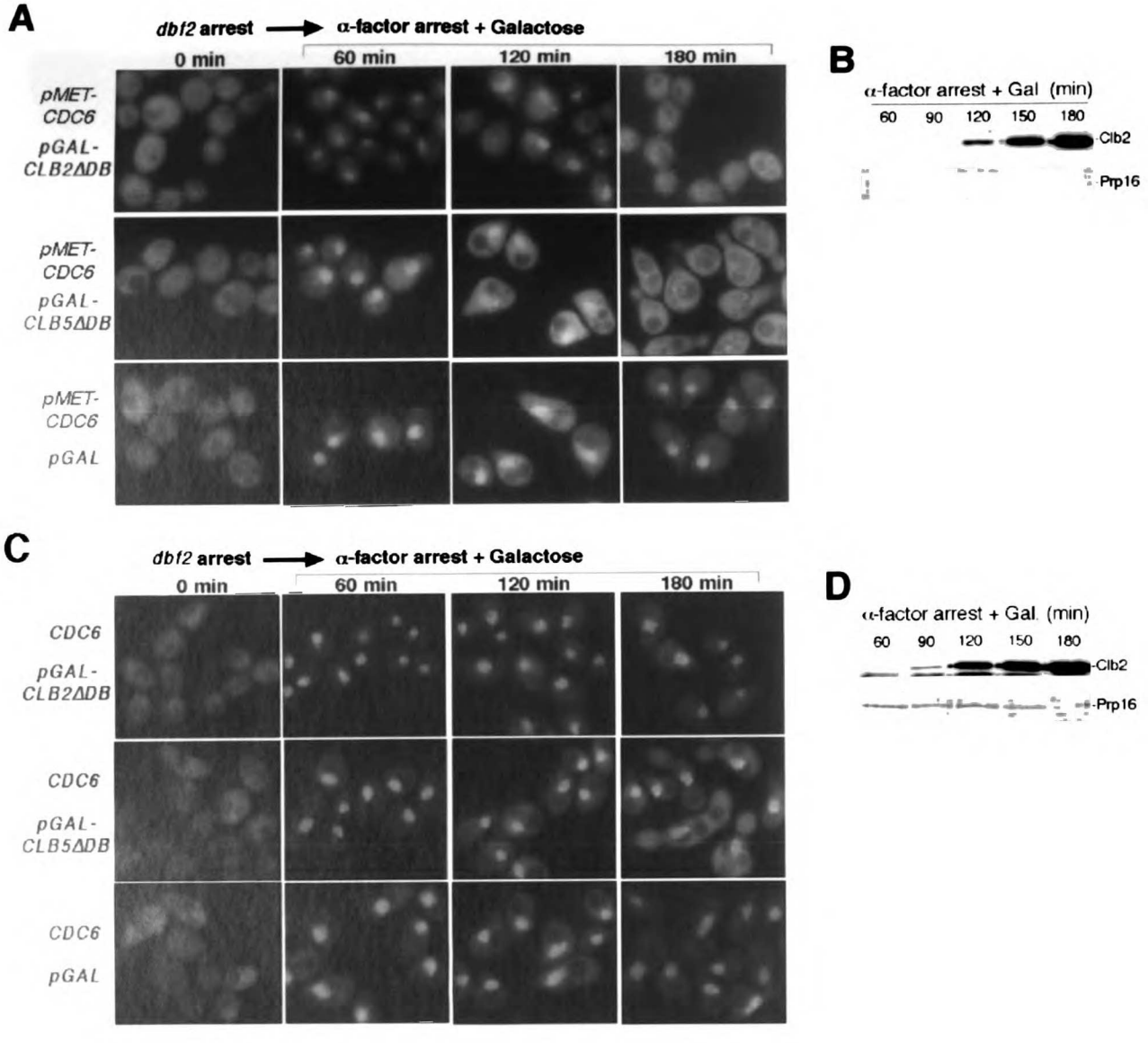
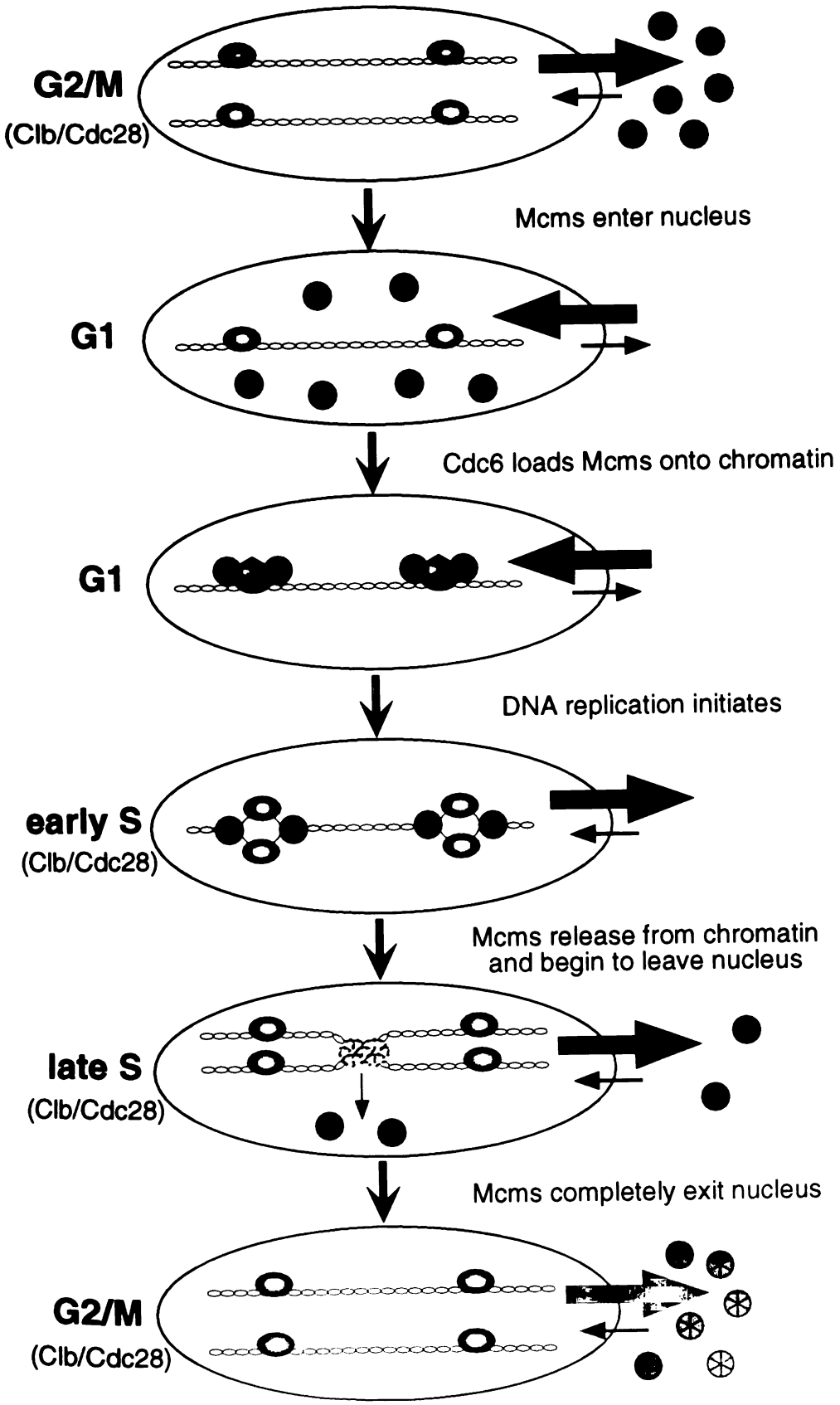

A
dbf2 arrest
α-factor arrest + Galactose
0 min
60 min
120 min
180 min
pMET-CDC6
pGAL-CLB2ΔDB
pMET-CDC6
pGAL-CLB5ΔDB
pMET-CDC6
pGAL
B
α-factor arrest + Gal (min)
60 90 120 150 180
Clb2
Prp16
C
dbf2 arrest
α-factor arrest + Galactose
0 min
60 min
120 min
180 min
CDC6
pGAL-CLB2ΔDB
CDC6
pGAL-CLB5ΔDB
CDC6
pGAL
D
α-factor arrest + Gal. (min)
60 90 120 150 180
Clb2
Prp16

Figure 7. Model for regulation of Mcm localization during the cell cycle. See Text for detailed explanation. In accordance with the work of Aparicio and Bell (Aparicio et al., 1997), we have depicted Mcm proteins as loading onto origins before initiation, shifting their association to replication forks after initiation, and dissociating from chromatin when forks disassemble. Although the model shows both import (leftward arrows) and export (rightward arrows) rates being regulated by Clb/Cdc28 kinases, a shift in net transport direction is possible with changes in only one of these rates.


G2/M
(Clb/Cdc28)
Mcms enter nucleus
G1
Cdc6 loads Mcms onto chromatin
G1
DNA replication initiates
early S
(Clb/Cdc28)
Mcms release from chromatin
and begin to leave nucleus
late S
(Clb/Cdc28)
Mcms completely exit nucleus
G2/M
(Clb/Cdc28)
:ORC
:Cdc6
:Mcm2-7

CHAPTER 3

Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms

The stable propagation of genetic information requires that the entire genome of an organism be faithfully replicated once and only once each cell cycle. In eukaryotes, this replication is initiated at hundreds to thousands of replication origins distributed over the genome, each of which must be prohibited from re-initiating DNA replication within every cell cycle. How cells prevent re-initiation has been a long-standing question in cell biology. In several eukaryotes, cyclin dependent kinases (CDKs) have been implicated in promoting the block to re-initiation (review in (Kelly and Brown, 2000)), but exactly how they perform this function is not clear. Here we show that B-type CDKs in *Saccharomyces cerevisiae* prevent re-initiation through multiple overlapping mechanisms, including phosphorylation of the origin recognition complex (ORC), down-regulation of Cdc6p activity, and nuclear exclusion of the Mcm2-7p complex. Only when all three inhibitory pathways are disrupted do origins re-initiate DNA replication in G2/M cells. These studies thus reveal that each of the three independent mechanisms of regulation is functionally important.

The mechanism of eukaryotic replication initiation and the role of CDKs in its regulation have been most extensively characterized in the budding yeast *Saccharomyces cerevisiae* (reviewed in (Kelly and Brown, 2000)). Initiation events at yeast origins can be divided into two fundamental stages: the assembly of pre-replicative complexes (pre-RCs) and the triggering of new DNA synthesis. The assembly of pre-RCs occurs shortly after mitosis and renders origins competent to initiate DNA synthesis. During this assembly, the origin recognition complex (ORC), which binds origins throughout the cell cycle, is joined by additional initiator proteins, including Cdc6p and the Mcm2-7p complex. Passage through the G1 commitment point (known as Start in yeast) then activates the kinases Cdc7p-Dbf4p and the B-type CDKs Clb-Cdc28p, which together trigger origin unwinding, assembly of the replication fork machinery, initiation of daughter strand synthesis, and pre-RC disassembly.

In addition to triggering initiation, Clb-Cdc28p is thought to prevent re-initiation, in part by blocking re-assembly of pre-RCs (Kelly and Brown, 2000). This block is maintained until the kinase is inactivated at the end of mitosis, thereby ensuring that origins initiate only once per cell cycle. CDKs have also been implicated in preventing re-replication in *Schizosaccharomyces pombe, Drosophila melanogaster,* and *Xenopus laevis* (Kelly and Brown, 2000). However, the mechanism by which CDKs prevent pre-RC re-assembly and the identity of their relevant inhibitory targets are not understood. Clb-Cdc28p is known to reduce Cdc6p levels in two ways: phosphorylation of Cdc6p promotes its ubiquitin-mediated degradation (Drury et al., 1997; Elsasser et al., 1999), and phosphorylation of Swi5p, a transcriptional activator of *CDC6*, prevents Swi5p from entering the nucleus and inducing Cdc6p expression (Moll et al., 1991). Clb-Cdc28p also promotes the net nuclear export of Mcm proteins, leading to their exclusion from the nucleus in G2 and M phases (Labib et al., 1999; Nguyen et al., 2000). The reduction in Cdc6p levels and the nuclear exclusion of Mcm2-7p, however, are not individually essential for the block to re-initiation, as constitutive expression of stabilized or nonphosphorylatable Cdc6p ((Drury et al., 1997) and data not shown) or constitutive nuclear localization of Mcm2-7p (Nguyen et al., 2000) do not induce re-replication within a cell cycle. Hence, it has not been possible to establish the functional importance of these mechanisms in the block to re-replication. Moreover, these results leave open the possibility that Clb-Cdc28p targets additional replication proteins to maintain this block.

Here we have examined the possible regulation of ORC by Clb-Cdc28p and its relevance to re-replication. Three of the six ORC proteins, Orc1p, Orc2p, and Orc6p, have consensus CDK phosphorylation sites ([S/T]-P-X-[K/R]), suggesting that they might be phosphorylated by the kinase *in vivo*. Consistent with this possibility, Orc6p and Orc2p each migrated on SDS-PAGE as a doublet, which was converted to the faster

migrating form upon phosphatase treatment (Fig. 1a, 1g). The presence of the slower migrating hyperphosphorylated form was dependent on both Cdc28p (Fig. 1b, 1h) and the CDK consensus phosphorylation sites (Fig. 1c, 1i) and was cell cycle regulated (Fig. 1d, 1j). Both Orc6p and Orc2p were hypophosphorylated in G1, became hyperphosphorylated after Start, and remained hyperphosphorylated until the next G1 phase. Incomplete conversion to the hypophosphorylated form in the second and third G1 phases was likely due to loss of cell synchrony, as both proteins displayed rapid and complete conversion to the hypophosphorylated form in cells synchronously released into G1 phase from a *dbf2* late mitotic arrest (Fig. 1e, 1k). These findings strongly suggest that Cdc28p phosphorylates Orc6p and Orc2p on at least some of their CDK consensus sites *in vivo*.

We determined more precisely the timing of this phosphorylation by examining Orc6p and Orc2p at different cell cycle arrests (Fig. 1f, 1l). Both proteins were hypophosphorylated after Start upon *cdc4* or *cdc34* arrest, when Cln-Cdc28p is active (Schwob et al., 1994), and only became hyperphosphorylated later in G1 at a *cdc7* arrest, when Clb-Cdc28p is active (Schwob et al., 1994). Orc2p and Orc6p remained hyperphosphorylated at all arrest points later in the cell cycle, paralleling the persistence of Clb-Cdc28p kinase activity through late anaphase. These data indicate that Clb-Cdc28p and not Cln-Cdc28p is responsible for the cell cycle-regulated hyperphosphorylation of Orc2p and Orc6p. Moreover, the results demonstrate that this phosphorylation is independent of Cdc7p-Dbf4p.

To determine the function of Orc2p and Orc6p phosphorylation, we constructed a strain in which the phospho-acceptor residues of all CDK consensus sites in these proteins were mutated to alanine. The strain was indistinguishable from its congenic wild-type parent in growth rate, plasmid loss rates (a measure of replication initiation

efficiency), and flow cytometry profile (data not shown). Similar results were obtained with a strain in which the last remaining CDK consensus site in ORC (on Orc1) was also mutated (data not shown). Thus, phosphorylation of ORC on its CDK consensus sites, like the reduction of Cdc6p levels and the nuclear exclusion of Mcm2-7p, is not essential for the block to re-initiation. These experiments also show that phosphorylation of ORC proteins on their CDK consensus sites is not required for the initiation of DNA replication.

To test whether Clb-Cdc28p employs multiple overlapping mechanisms as a safeguard against re-initiation, we constructed strains that combined various disruptions of ORC, Cdc6p and Mcm2-7p regulation. Phosphorylation of Orc2p and Orc6p on their consensus CDK sites was eliminated by mutating these sites as described above. Nuclear exclusion of Mcm2-7p by Clb-Cdc28p was disrupted by fusing two tandem copies of the SV40 nuclear localization signal (NLS) onto Mcm7p (Nguyen et al., 2000). In both cases, the wild-type genes were precisely replaced by their mutant counterpart. The restriction of Cdc6p expression to G1 phase was overridden by expressing a partially stabilized form of Cdc6p, ΔntCdc6p, under the control of the galactose-inducible *GAL1* promoter. ΔntCdc6p contains an N-terminal truncation of amino acids 2-46, which removes sequences that facilitate Cdc6p degradation[2] and are necessary for Clb-Cdc28p association (Elsasser et al., 1996). Despite these mutations, ΔntCdc6p can fully substitute for wild-type Cdc6p protein (as measured by plasmid loss rates) when expressed from the *CDC6* promoter (data not shown). *pGAL1-Δntcdc6* was introduced in addition to the endogenous *CDC6* gene.

We first constructed three congenic strains (YJL3239, YJL3242, YJL3244) containing all three possible pairwise combinations of regulatory perturbations described above and examined them for the ability to re-replicate their DNA at a G2/M phase arrest

(when replication is complete and Clb-Cdc28p kinase activity is high). To achieve a tight arrest, cells were both depleted of Cdc20p, which is required for the metaphase-to-anaphase transition (Visintin et al., 1997), and exposed to nocodazole, a microtubule-destabilizing agent that disrupts mitotic spindles. Only after cells were arrested was Δntdc6 induced by galactose. None of these strains increased their DNA content significantly beyond 2C (as measured by flow cytometry, Fig. 2a) or displayed any actively replicating chromosomes (Chapter 6, Fig. 1), which migrate with retarded mobility during pulsed-field gel electrophoresis (PFGE) (Hennessy et al., 1990). Moreover, *ARS305* (Fig. 2b) and *ARS1* (data not shown), which normally fire in early and mid-early S phase respectively (Brewer et al., 1993; Newlon et al., 1993), showed no signs of re-initiation or passive re-replication by neutral-neutral 2-D gel electrophoresis. We conclude that the block to re-initiation in G2/M phase remains largely intact despite simultaneous disruption of any two of the three regulatory mechanisms described above.

We next tested whether a strain containing disruptions in all three regulatory mechanisms (YJL3248) would undergo re-replication at a G2/M phase arrest. In contrast to the results above, galactose induction of ΔntCdc6p resulted in an increase in DNA content from 2C to ~3C (Fig. 2a) and induction of initiation bubbles at *ARS305*, *ARS121*, *ARS607* (Fig. 2b, Chapter 6, Fig. 2b, 2e, 2f), and *ARS1* (data not shown). The re-initiation at *ARS305* was dependent on its *ARS* consensus sequence and ORC binding site (Bell and Stillman, 1992) (Chapter 6, Fig. 2a), suggesting that re-initiation occurred through the same ORC-dependent mechanism as S phase initiation. Re-replication was also accompanied by re-association of Mcm2p with chromatin (Fig. 2c), suggesting that Mcm complexes reloaded onto origins to re-initiate replication. In addition, the mobility of all chromosomes was retarded during PFGE (Chapter 6, Fig. 1a), with southern analysis confirming that both large (chromosome 4 and 7, Chapter 6, Fig. 1b) and small (chromosome 3, data not shown) chromosomes experienced difficulty entering the gel.

Hence, all chromosomes appeared to participate in the re-replication. Together our results indicate that ORC, Cdc6p, and Mcm2-7p must be simultaneously deregulated in order to re-initiate DNA replication.

Given that re-initiation arose after induction of a partially stabilized form of Cdc6p from the strong *GAL1* promoter, we wished to confirm that ectopic expression of ΔntCdc6p and not massive overexpression (which might cause additional unknown perturbations) was responsible for the re-initiation. Western analysis indicated that the level of ΔntCdc6p induced in our re-replicating strain after two hours in galactose was less than two-fold higher than peak levels of endogenous Cdc6p expressed in early G1 phase (Fig. 3a). We also induced ΔntCdc6p synthesis for only one hour to mimic the normally transient G1 expression of Cdc6p. Considerable re-replication and re-initiation was still observed after this transient induction, and, in fact, some re-initiation was detected during the induction before ΔntCdc6p had fully accumulated (Fig. 3b). Finally, induction of the fully unstable wild-type Cdc6p in G2/M also triggered re-replication (Chapter 6, Fig. 3). Thus, ectopic expression of ΔntCdc6p without any significant overexpression was sufficient to induce re-replication in the triply de-regulated strain.

Despite re-initiating DNA replication, the triply deregulated strain did not completely duplicate its DNA, suggesting that we did not remove all restraints on re-replication. Although multiple origins re-initiated efficiently, several origins did not, including two early origins, *ARS306* and *ARS307* (Chapter 6, Fig. 2c and 2d), and two late origins *ARS501* and *ARS1413* (Chapter 6, Fig. 2h and 2i). We note that 2-D gel analysis of *ARS305*, *ARS306* and *ARS307* demonstrates that they all initiated efficiently during the S phase preceding the induced re-replication (data not shown). These observations suggest that additional mechanisms prevent re-initiation of some origins and raise the question of what distinguishes these origins from those that do re-initiate. Y

arcs were clearly induced at origins that failed to re-initiate indicating that these origins were still passively re-replicated, presumably by replication forks that had re-initiated from neighboring origins. The somewhat weaker intensity of these arcs, however, suggests that re-elongation may also have been partially inhibited. Forks originating from *ARS305* and *ARS607* appeared to have some difficulty re-replicating fragments only 30-35 kb away (*ARS306* and 607+30kb, Chapter 6, Fig. 2c and 2g), even though replication forks can travel at least 100-200 kb in S phase (Newlon et al., 1993). A more quantitative genome-wide analysis of re-replication will be needed to confirm and fully characterize any remaining inhibition of re-initation and/or re-elongation in our triply deregulated strain. Nonetheless, additional mechanisms besides those we specifically deregulated are likely to restrict re-replication within a single cell cycle. Some of these mechanisms may provide additional means of inhibiting ORC, Cdc6p, or Mcm2-7p, (just as Cdc18p in *S. pombe* is independently restrained by both decreased expression and phosphorylation[15]), whereas others may target additional replication proteins.

Unscheduled DNA replication has also been reported to arise from transient inactivation of CDK activity in G2 or G2/M-arrested cells (Broek et al., 1991; Dahmann et al., 1995; Noton and Diffley, 2000; Sauer et al., 1995). This CDK inactivation, however, resets the cell cycle to G1 phase in the absence of mitosis, thereby effectively inducing S phase in a new cell cycle and not re-initiation within G2 or G2/M phase of the original cell cycle. In *S. cerevisiae*, for example, transient inactivation of Clb-Cdc28p in G2/M-arrested cells by overexpression of the CDK inhibitor Sic1p induces transcription of G1-specific genes and a new round of budding, a G1-specific event (Dahmann et al., 1995). Consistent with the cell cycle being reset to G1 phase, the new round of replication that ensues upon release of Clb-Cdc28p inactivation can be blocked by the mating pheromone alpha-factor, which arrests cells in G1 (J. Diffley, personal communication). Thus, although this unscheduled DNA replication confirms that cell

cycle position is determined by CDK activity (Broek et al., 1991), it does not address how this CDK activity prevents re-replication within a cell cycle.

We therefore examined whether the re-replication in our triply deregulated strain was due to a similar resetting of the cell cycle by monitoring its cell cycle state while re-replication occurred. During this period, the strain did not rebud (Fig. 2a and 4a), maintained high mitotic levels of Clb2-Cdc28p kinase activity (Fig. 4b), and was able to re-replicate in the presence of alpha factor (Fig. 4c). Hence, these cells did not bypass mitosis and enter a G1-like state before they re-replicated. They also did not break through the mitotic arrest and enter S phase of the next cell cycle, as cells maintained a single undivided nucleus while they re-replicated (Fig. 4a). In addition, we note that, by inducing Δntcdc6p, we avoided Cdc6p association with and possible inhibition of Clb-Cdc28p, an interaction mediated by the N-terminus of Cdc6p (Elsasser et al., 1996). Thus, we conclude that the block to re-initiation was removed in the triply deregulated strain because we had simultaneously rendered ORC, Cdc6p, and Mcm2-7p refractory to the inhibitory action of Clb-Cdc28p, and not because we had inadvertently inactivated Clb-Cdc28p.

In summary, Clb-Cdc28p uses at least three overlapping inhibitory pathways involving phosphorylation of ORC, decreased expression of Cdc6p, and nuclear exclusion of Mcm2-7p to prohibit re-initiation of DNA replication in budding yeast (Fig. 5). Any one of these mechanisms is sufficient to block re-initiation in the absence of the others (Fig. 2 and 3, YJL3239, YJL3242, YJL3244), thus accounting for the observation that no single mechanism is individually essential for this block. We propose that budding yeast uses a combination of overlapping mechanisms targeting distinct initiation proteins to ensure that none of its hundreds of replication origins re-initiate within a cell cycle. Although no overt re-initiation was seen when any single mechanism was

disrupted, we suspect that each mechanism is important for preserving long-term genome stability by keeping the frequency of re-initiation events extremely low over the course of many cell divisions. Hence, we consider these overlapping mechanisms to be mutually reinforcing and not necessarily redundant.

Although multiple mechanisms targeting multiple proteins must be disrupted to induce re-initiation, given that these proteins work together in a complex, it is conceivable that mutation or pertubation of one of these proteins could override enough of these mechanisms to trigger re-initiation. Such an explanation could account for previous reports of re-replication arising from perturbation of just *CDC6* or its *S. pombe* ortholog *CDC18*. In one report, *cdc6-3*, a mutant severely defective for initiation, also accumulated a 2.5-3C DNA content in G2/M phase and exhibited both persistent initiation intermediates and persistent association of Mcm proteins with chromatin (Liang and Stillman, 1997). Although there was no direct demonstration that these persistent signs of replication arose from re-initiation, one could imagine that the *cdc6-3* mutation induced re-initiation by counteracting some of the mechanisms inhibiting ORC and Mcm proteins as well as those inhibiting Cdc6p. Similarly, massive overexpression of Cdc18p is sufficient to induce significant re-replication in *S. pombe* (Muzi-Falconi et al., 1996) (Nishitani and Nurse, 1995), but more modest constitutive expression does not do so unless a second initiation protein, Cdt1p, is simultaneously overexpressed (Nishitani et al., 2000). These results suggest that, in addition to overriding the G1-specific expression of Cdc18p, massive overexpression of Cdc18p may overwhelm other mechanisms that prevent re-replication in *S. pombe*.

We suggest that CDKs in other eukaryotes also depend on multiple downstream inhibitory targets to prevent re-replication within a single cell cycle. Such a model might explain why disruption of Cdc6p regulation in humans (Vas et al., 2001) and *Xenopus*

(Petersen et al., 2000) is not sufficient to induce re-replication. While basic mechanistic strategies used by *S. cerevisiae* to prevent re-replication, such as destruction, re-localization, or modification of replication proteins, are likely to be conserved, the precise implementation of those strategies may vary. For example, in most eukaryotes Mcm proteins are not excluded from the nucleus in S and G2 phase (Kelly and Brown, 2000), but in mammalian cells, at least, Cdc6p has been shown to be excluded after G1 phase (Fujita et al., 1999; Jiang et al., 1999; Pelizon et al., 2000; Petersen et al., 1999; Saha et al., 1998). The relative importance of various strategies may also differ in different organisms and at different times in the cell cycle. Nonetheless, by using multiple inhibitory mechanisms to target multiple replication proteins, eukaryotic cells can satisfy the need to maintain an exquisitely tight block to re-replication at hundreds to thousands of replication origins.

Methods

Details of plasmid/strain construction and experimental assays can be found in Supplementary Information. Yeast growth, galactose induction, methionine repression, and cell cycle arrest/release were performed as described (Nguyen et al., 2000).

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Figure 1 Clb-Cdc28p phosphorylation of Orc6p and Orc2p *in vivo*. **a-f**, Immunoblot of Orc6p using anti-HA detection of Orc6p-HA3 (**a-d, f**) or anti-Orc6p (**e**). **g-l**, Immunoblot of Orc2p using anti-HA detection of Orc2p-HA3 (**g**) or anti-Orc2p (**h-l**). Extracts used in **h, j, k, l** were identical to those used in **b, d, e, f**, respectively. **a, g**, Immunoprecipitates from YJL921 (*ORC6-HA3*) (**a**) or YJL963 (*ORC2-HA3*) (**g**) treated with λ phosphatase with or without phosphatase inhibitors. **b, h**, YJL934 (*cdc28-4 ORC6-HA3*) or YJL865 (*CDC28 ORC6-HA3*) grown at 23° C were arrested in early S with hydroxyurea (after a pre-arrest in G1 with α-factor) then shifted to either 38° C or kept at 23° C for an additional 3 hr. **c**, Log phase YJL865 (*ORC6-HA3*) and YJL1394 (*orc6-4A-HA3*). **i**, Log phase YJL3155 (*ORC2*) and YJL1737 (*orc2-6A*). **d, j**, YJL865 (*ORC6-HA3*) cells were released at time 0 from an α-factor arrest in G1 and samples taken every 10 min for analysis by immunoblot, FACS (to determine time of S phase) , and budding index with DAPI staining (to determine time of Start and Mitosis). 80 min time point in **j** is absent. **e, k**, YJL1937 (*dbf2-2 ORC6*) cells were grown at 37° C for 150 min to arrest them in late mitosis, released from the arrest at time 0 by shifting them to 23° C, and sampled every 10 min for immunoblot analysis. **f, l**, immunoblot of *ORC6-HA3 cdc* strains arrested by growth at 37° C for 2-3 hr (till >95% have appropriate bud morphology); drug arrests were performed on YJL864 (*ORC6-HA3*) using α-factor, hydroxyurea (HU), or nocodazole (NOC).

a
+Phosphatase
+Phosphatase
+Inhibitor
Orc6p-HA3
b
HU arrest
cdc28 CDC28
23°C
38°C
23°C
38°C
c
Log phase
ORC6
orc6-4A
Orc6p-HA3
d
Start
S phase
Mitosis
e
dbf2 release
Time (min)
Orc6p-HA3
Loading control
f
Log
cdc25
cdc28
α factor
cdc4
cdc34
cdc7
HU
cdc17
cdc9
NOC
cdc14
cdc15
Orc6p-HA3
g
+Phosphatase
+Phosphatase
+Inhibitor
Orc2p-HA3
h
HU arrest
cdc28 CDC28
23°C
38°C
23°C
38°C
i
Log phase
ORC2
orc2-6A
Orc2p
j
Start
S phase
Mitosis
k
dbf2 release
Time (min)
Orc2p
Loading control
l
Log
cdc25
cdc28
α factor
cdc4
cdc34
cdc7
HU
cdc17
cdc9
NOC
cdc14
cdc15
Orc2p

Figure 2 Induction of re-replication and re-initiation in G2/M by deregulation of ORC, Mcm2-7p, and Cdc6p. ORC phosphorylation, Mcm2-7p localization, and Cdc6p expression were deregulated in YJL3239 (*ORC2 ORC6 MCM7-2NLS CDC6 pGAL1-Δntcdc6 pMET3-CDC20*), YJL3242 (*orc2-6A orc6-4A MCM7-2nls3A CDC6 pGAL1-Δntcdc6 pMET3-CDC20*), YJL3244 (*orc2-6A orc6-4A MCM7-2NLS CDC6 pGAL1 pMET3-CDC20*), and YJL3248 (*orc2-6A orc6-4A MCM7-2NLS CDC6 pGAL1-Δntcdc6 pMET3-CDC20*) as described in the text and summarized at the top of the figure; - deregulated, + regulated. Deregulation of Cdc6p was conditional and dependent on galactose induction of *pGAL1-Δntcdc6*. Cells were initially grown in medium lacking methionine and containing raffinose to prevent expression of ΔntCdc6p. They were arrested in G2/M by addition of 2mM methionine (which induced depletion of Cdc20p) followed 2.5 hr later by 15 μg/ml of nocodazole. After a further 30 min, galactose was added to induce ΔntCdc6p at time 0 hr. **a**, Budding index and flow cytometry. **b**, Strains were sampled at 0 hr or between 1-2 hr (sampled every 2 min and pooled) for analysis of DNA replication intermediates at *ARS305* by neutral-neutral 2D gel electrophoresis[30]. Similar 2-D gel results obtained from cells sampled between 0-1 hr, 1-2 hr, and 2-3 hr were seen with congenic strains that were wild-type for *CDC20* and arrested in G2/M solely with nocodazole (data not shown). No replication intermediates were induced in any strain if dextrose, which represses the *GAL1* promoter, was added to the medium instead of galactose. **c**, Mcm2 reassociates with chromatin during re-replication. Re-replication was induced in YJL3244 and YJL3248 as described above and chromatin-enriched fractions[20] analyzed at the indicated time points (lanes 3-6, 9-12) by immunoblotting with anti-Mcm2p and anti-Orc3p antibodies. Log phase (lane 1, 7) and alpha factor (lane 2, 8) arrested cells were examined in parallel.

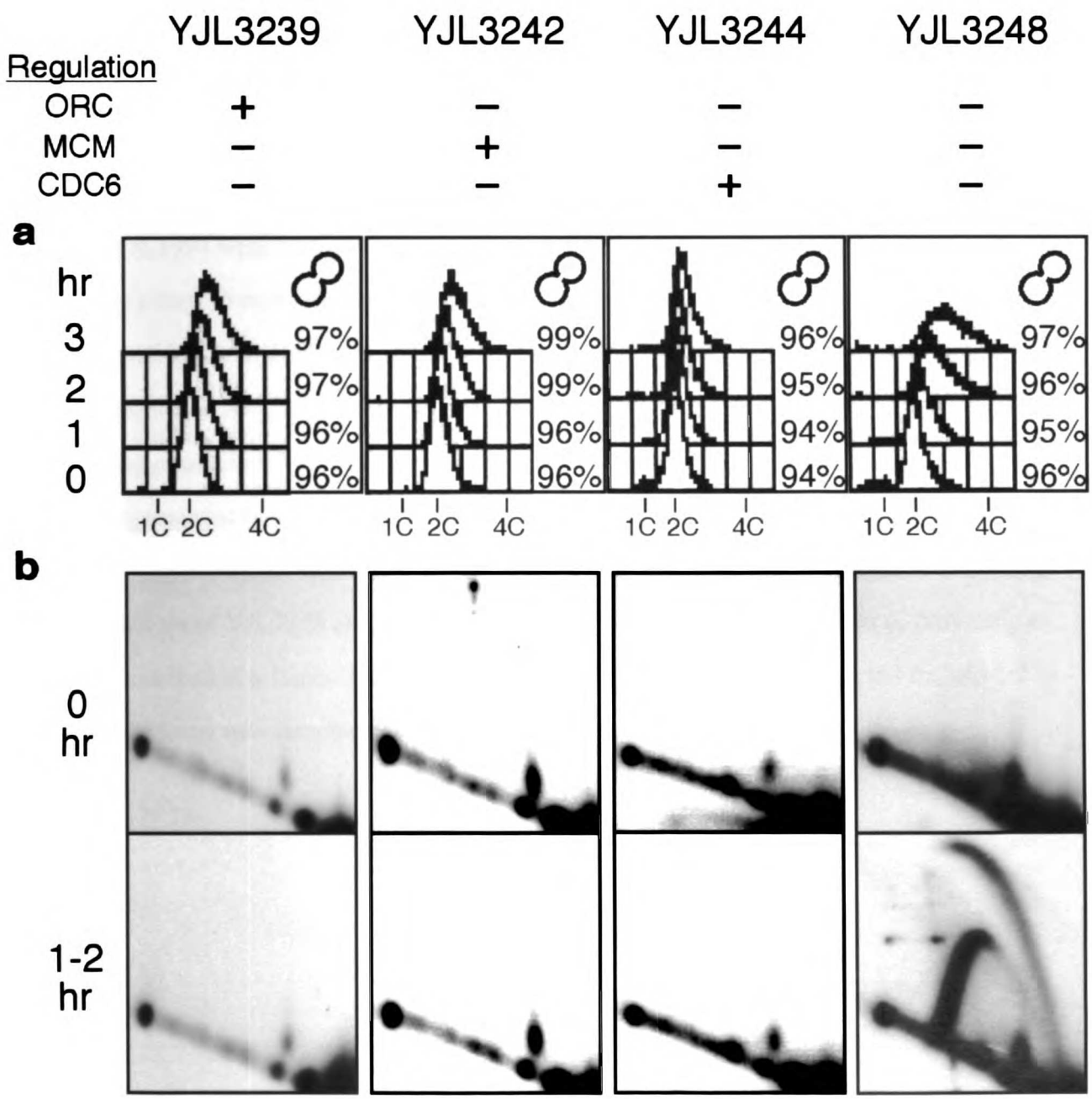

YJL3239
YJL3242
YJL3244
YJL3248
Regulation
ORC + − − −
MCM − + − −
CDC6 − − + −
a
hr
3
2
1
0
97%
97%
96%
96%
99%
99%
96%
96%
96%
95%
94%
94%
97%
96%
95%
96%
1C 2C 4C
b
0 hr
1-2 hr
c
YJL3244
YJL3248
pGAL1
pGAL1-Δntcdc6
Log
α f arrest
0 hr 1 hr 2 hr 3 hr
Mcm2
Orc3
1 2 3 4 5 6 7 8 9 10 11 12

Figure 3 Re-replication and re-initiation are induced with physiological levels of ΔntCdc6p. **a**, Immunoblot with anti-Cdc6 antibodies of re-replicating strain YJL3248 (lanes 1-4, 9-13) and *dbf2-2* strain YJL1937 (lanes 5-8). ΔntCdc6p was induced in YJL3248 as described in Fig. 2 (lanes 1-4) or as in Fig. 2 with the addition of dextrose after 60 min to repress further induction (lanes 9-13). Endogenous levels of Cdc6p in YJL1937 were monitored every 10 min following release from a *dbf2-2* late mitotic arrest or every 15 min following release from an alpha factor arrest. Time points containing the peak levels immediately after *dbf2* release (lane 5, G1) or in the first (lane 6, 1st G1) or second (lane 7, 2nd G1) G1 phases after alpha factor release are shown and compared to a log phase population (lane 8). Peak G1 levels in cycling cells appear to be best represented by peak levels following *dbf2* release. Band intensities quantified by densitometry are expressed in arbitrary units (amount). **b**, FACS analysis and budding indices of YJL3248 at indicated times during a transient 1 hr induction of ΔntCdc6p as described in **a** (lanes 9-13); 2D gel analysis[30] of *ARS607* taken at 0hr, 0-1 hr, and 1-2 hr (the latter two sampled every two min and pooled).

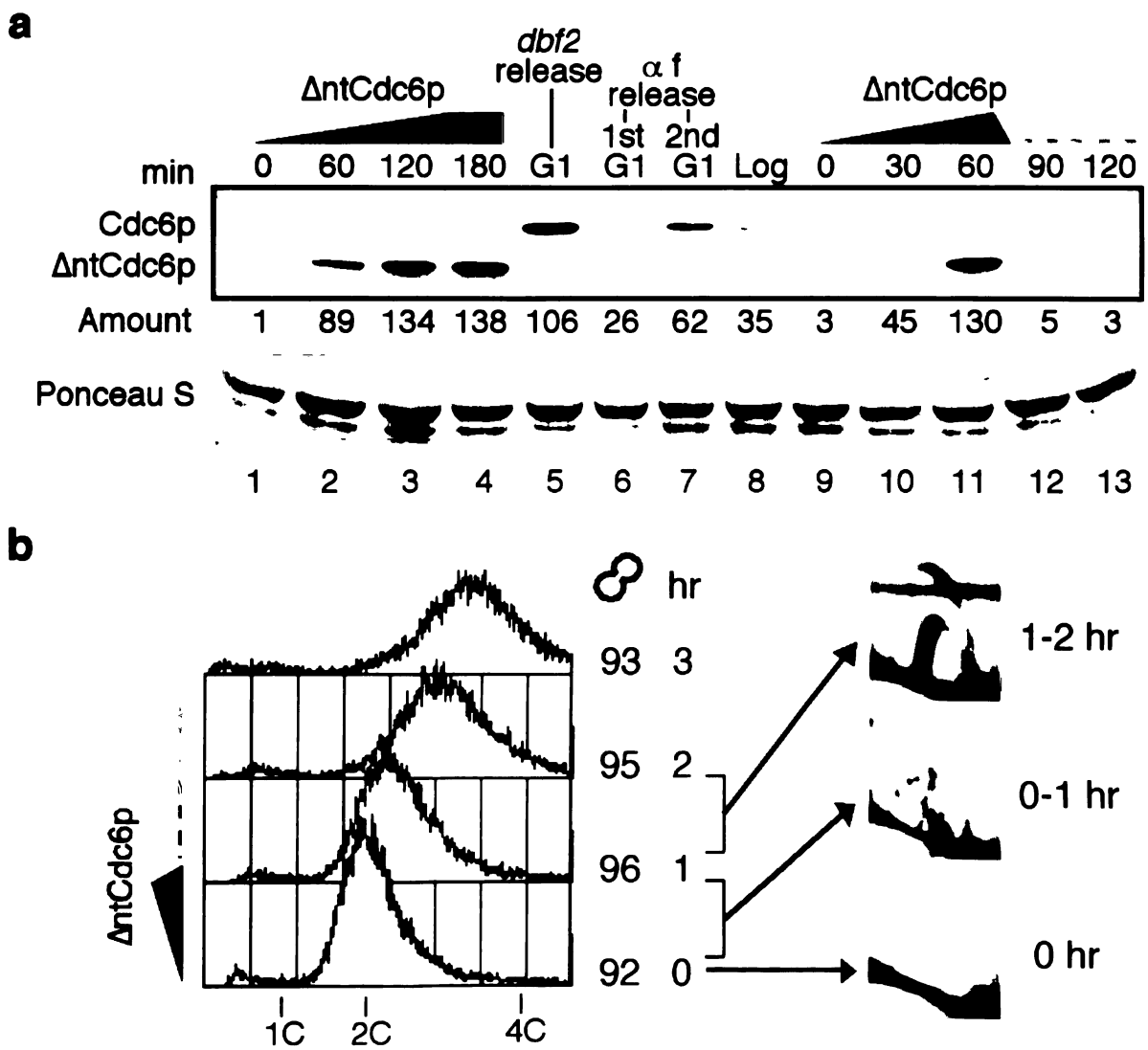

a
dbf2 release
α f release
1st G1
2nd G1
ΔntCdc6p
ΔntCdc6p
min
0 60 120 180 G1 G1 G1 Log 0 30 60 90 120
Cdc6p
ΔntCdc6p
Amount
1 89 134 138 106 26 62 35 3 45 130 5 3
Ponceau S
1 2 3 4 5 6 7 8 9 10 11 12 13
b
hr
93 3
95 2
96 1
92 0
1-2 hr
0-1 hr
0 hr
ΔntCdc6p
1C 2C 4C

Figure 4 Re-replicating cells remain in G2/M phase. YJL3244 and YJL3248 are a control and re-replicating strain, respectively, induced to re-replicate as described in Fig. 2. **a**, Percent of cells that are large budded with a single nucleus. Pictures show DAPI fluorescence overlaying bright field microscopy of cells at 0 and 3 hr time points. **b**, H1 kinase assays performed on anti-Clb2p immunoprecipitates taken every 0.5 hr following galactose induction of re-replication. Measurements were performed in the linear range of the assay. **c**, Galactose induction of re-replication was performed in the presence or absence of α-factor (to arrest any cells entering G1 phase) and samples were taken every hour for flow cytometry.

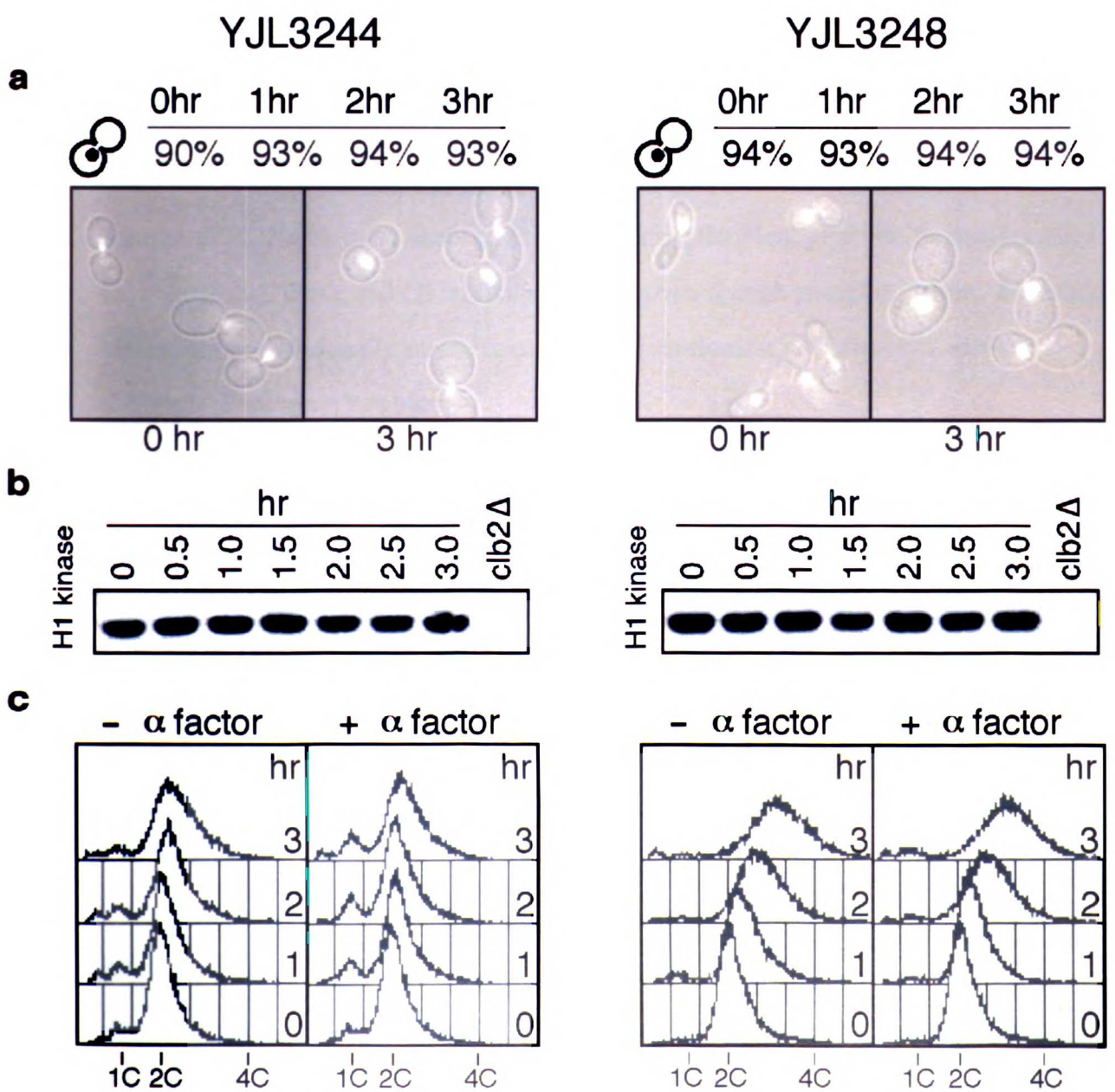
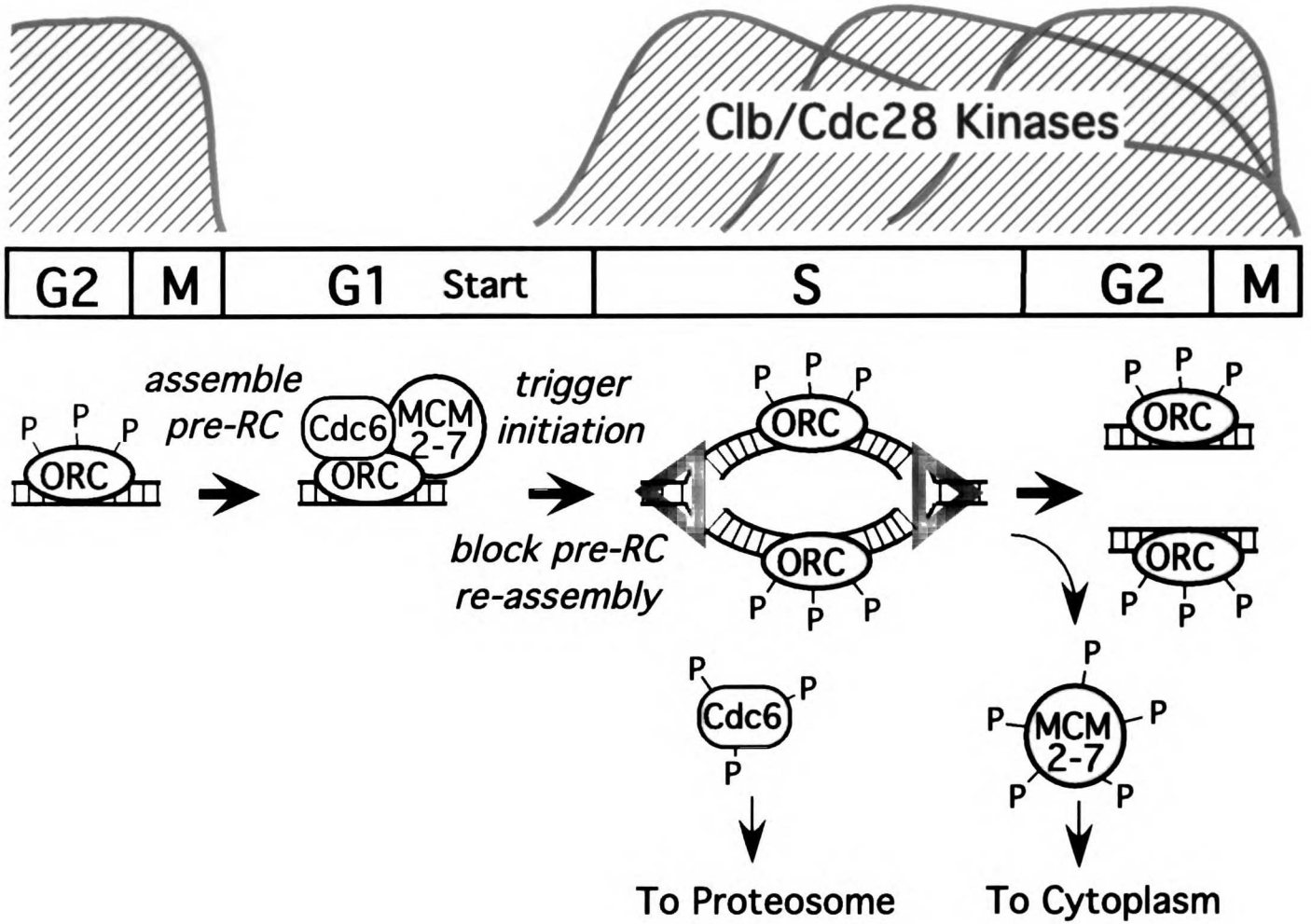

YJL3244
YJL3248
a
0hr 1hr 2hr 3hr
90% 93% 94% 93%
94% 93% 94% 94%
0 hr
3 hr
b
hr
0 0.5 1.0 1.5 2.0 2.5 3.0 clb2Δ
H1 kinase
c
- α factor
+ α factor
hr
3
2
1
0
1C 2C 4C

Figure 5 Model for Clb-Cdc28p inhibition of re-replication through multiple overlapping mechanisms. Mcm2-7p and Cdc6p join ORC at the origin to form the pre-replicative complex (pre-RC) in G1 phase when Cdc28p kinase activity is low. Induction of Clb-Cdc28p after Start helps to trigger initiation, resulting in assembly of the replication fork machinery (triangles) and disassembly of the pre-RC. The kinase simultaneously prevent re-initiation by at least three overlapping mechanisms: (1) phosphorylating Cdc6p and facilitating its polyubiquitination and degradation (Drury et al., 1997; Elsasser et al., 1996); (2) promoting nuclear exclusion of Mcm proteins (Labib et al., 1999; Nguyen et al., 2000), most likely by phosphorylating the Mcm proteins (A. Rosales and J. Li, unpublished data); and (3) inhibiting ORC activity though phosphorylation. No single mechanism is individually essential to prevent re-replication in G2/M cells, as each is sufficient to maintain this block.


Clb/Cdc28 Kinases
G2
M
G1
Start
S
G2
M
assemble
pre-RC
Cdc6
MCM
2-7
ORC
trigger
initiation
block pre-RC
re-assembly
ORC
ORC
ORC
ORC
Cdc6
MCM
2-7
To Proteosome
To Cytoplasm

CHAPTER 4

Conclusion

In order to maintain a stable genome over many rounds of cell division cycle, eukaryotic cells must replicate their DNA precisely once and only once per cell cycle and coordinate it with other events in the cell cycle. In eukaryotes, DNA replication initiates at multiple replication origins scattered throughout the genome. Re-initiation within a cell cycle must be prevented at each of these origins. When I began my thesis project, it was unclear exactly how initiation is controlled with such precision. What was known was that CDKs have a central role in preventing re-initiation by inhibiting the re-assembly of pre-RCs (Detweiler and Li, 1998; Hua et al., 1997). The presence of active CDKs from START until the end of mitosis ensures that re-initiation is prevented for the remainder of the cell cycle. Moreover, destruction of CDKs at the end of mitosis allows cells to re-initiate DNA replication in G1 phase of the next cell cycle.

When I started to work in the Li lab, the molecular targets of the CDKs that mediate inhibition of re-initiation were not known. My thesis project was to identify the critical targets of these kinases and to determine how phosphorylation of these targets inhibits re-initiation. My basic strategy was to identify initiation proteins that are regulated by CDKs and to determine whether disrupting this regulation would lead to re-initiation. The known pre-RC components at that time, ORC, Cdc6, and Mcm were the most obvious candidates for inhibitory targets of the CDKs and the ones that I initially studied. Below I will summarize my contributions toward understanding how CDKs prevent re-initiation of DNA replication within a single cell cycle at the molecular level.

CDKs promote the nuclear export of Mcm2-7 after they dissociate from chromatin during replication

Indirect immunofluorescence and cell fractionation studies in the budding yeast *S. cerevisiae* demonstrate that the nuclear localization of the Mcm2-7 proteins is regulated during the cell cycle (Dalton and Whitbread, 1995; Hennessy et al., 1990; Yan et al., 1993). The Mcm2-7 proteins accumulate in the nucleus in G1 phase, disappear from the nucleus during S phase, and remain in the cytoplasm in G2/M phase. This reduction of Mcm protein levels in the nucleus offers a potential mechanism to prevent re-initiation of DNA replication. However, the role of this regulation in preventing re-initiation had not been directly tested, and furthermore the basic observation about this regulation had been called into question by report based on cell fractionation studies indicating that Mcm proteins are constitutively nuclear in budding yeast (Young and Tye, 1997).

To resolve this issue and to examine the mechanism of Mcm regulation in budding yeast in more detail, I fused the green fluorescent protein (GFP) to five of the six Mcm proteins and investigated their localization in living cells. In addition to confirming that Mcm localization is regulated during the cell cycle, I showed that Mcm proteins are actually excluded from the nucleus by net nuclear export. Moreover, I demonstrated that Mcm2-7 proteins co-localize as a complex, and CDKs promote the exclusion of Mcm2-7 proteins from the nucleus in G2/M phase. These results raise the possibility that this nuclear exclusion might be one of the mechanisms that CDKs use to prevent re-replication of DNA within a single cell cycle (Chapter 2) (Labib et al., 1999). Interestingly, I showed that constitutive nuclear localization of the Mcm proteins is not sufficient to lead to re-initiation of DNA replication in G2/M cells (Chapter 2).

Therefore, either regulation of Mcm2-7 nuclear localization was not important for the block to re-initiation or was one of several overlapping mechanisms preventing re-initiation. The latter case turned out to be true necessitating that I identify and disrupt all the overlapping mechanisms before I could establish the significance of any of them.

In contrast to the budding yeast Mcm2-7 proteins, fission yeast and mammalian Mcm proteins are constitutively nuclear throughout the cell cycle (Kearsey and Labib, 1998; Pasion and Forsburg, 1999), but their chromatin association is regulated during the cell cycle. The Mcm proteins associate with chromatin in G1 phase, gradually dissociate from the chromatin in S phase, and remained dissociated until the end of mitosis. Extraction study of human cells suggests that Mcm proteins associate with chromatin in G1 phase and dissociate from the chromatin as cells enter S phase, when CDKs are active (Todorov et al., 1995). The correlation between the dissociation of Mcm from chromatin and the high CDK activity suggests that CDK activity might regulate the association of Mcm with chromatin. Studies in *Xenopus* egg extracts suggest that high level of CDKs inhibit the pre-RC formation by preventing the association of Mcm3 to chromatin (Hua et al., 1997). ChIP experiment in *S. cerevisiae* indicated that high CDK activity in G2/M phase prevents the association of Mcm7 with chromosomal origins (Tanaka et al., 1997). Studies in *Xenopus* and human demonstrated that CDKs phosphorylate Mcm2 and Mcm4 *in vitro* and *in vivo*, and this phosphorylation prevents the association of Mcm with chromatin (Fujita et al., 1998; Hendrickson et al., 1996; Pereverzeva et al., 2000). This result suggests that in higher eukaryotes CDKs inhibits re-initiation and re-replication by likely phosphorylating Mcm proteins and preventing them from associating with chromatin during S, G2, and M phase.

CDKs use multiple overlapping mechanisms to prevent re-initiation of DNA replication

Although there were several potential mechanisms to prevent re-replication, the fact that none were required suggested that these mechanisms could act in concert to provide a very tight block to re-initiation. A potential complication in these studies results from the possibility that CDKs use multiple overlapping mechanisms to block re-initiation. Given such overlapping mechanisms that cells use, one would predict that preventing CDKs from acting on any single target would have no effect on the block to re-initiation. This prediction proves to be true for Mcm (as mentioned above) because constitutive nuclear localization of Mcm2-7 has no effect on the block to re-initiation.

I next examined the regulation of Cdc6 by CDKs. Our lab and others had already shown that Cdc6 protein is periodically expressed during the cell cycle in G1 phase (Detweiler and Li, 1997; Nishitani and Nurse, 1995; Piatti et al., 1995). Its absence at other points in the cell cycle was shown by others to be due to repression of *CDC6* transcription (Moll et al., 1991) and promotion of Cdc6 degradation by CDKs (Drury et al., 1997). Deregulation of Cdc6 by constitutive overexpression of the protein, however, was not sufficient to trigger re-initiation. I demonstrated that constitutive nuclear localization of the Mcm proteins is not sufficient to lead to re-initiation of DNA replication in G2/M cells, even if Cdc6 is ectopically expressed at the same time. Therefore, as with the regulation of Mcm, I needed to investigate other potentially overlapping regulatory mechanisms before I could establish the significance of CDK regulation of Mcm localization in the block to re-initiation (Chapter 3).

In contrast to budding yeast *S. cerevisiae*, early studies in fission yeast showed that overexpression of Cdc6 homolog, Cdc18, which normally disappears after G1 phase, leads to re-replication, suggesting that the block to re-initiation in fission yeast occurs primarily by limiting Cdc18 levels (Nishitani and Nurse, 1995). Recent experiment showed that overexpression of a short non-functional N-terminal fragment of human Orc1 can also lead to re-replication in fission yeast *S. pombe* (Wolf et al., 1996). Since it has been shown that the N-terminus of Cdc6 binds CDKs (Brown et al., 1997; Elsasser et al., 1996), it is reasonably thought that overexpression of Cdc18 can bind and inhibit CDK activity, thus promoting re-replication just as CDK activity is directly inhibited.

I then examined the regulation of ORC during the cell cycle. With the technical assistance of Carl Co, I showed that ORC is phosphorylated in a cell cycle dependent manner by CDKs, but that disruption of this phosphorylation does not lead to re-initiation. Only when I simultaneously disrupted the regulation of ORC, Cdc6, and Mcm did I observe re-initiation and re-replication (Chapter 3). This result demonstrates that each of these mechanisms is sufficient to block re-initiation and that CDKs use multiple overlapping mechanisms, including phosphorylation of ORC, nuclear exclusion of Mcm2-7, and down-regulation of Cdc6 levels, to ensure that the block is strictly maintained at every origin in the budding yeast genome.

Like budding yeast, fission yeast Orc2 and Orc6 are phosphorylated in a cell cycle dependent manner by CDKs (Vas et al., 2001). In contrast to budding yeast, disruption of fission yeast Orc2 phosphorylation by CDKs leads to DNA re-replication when Cdc6 is deregulated (Vas et al., 2001). These results suggest that phosphorylation of ORC by

CDKs negatively regulates replication. Therefore, like the budding yeast, fission yeast CDKs also use multiple overlapping mechanisms to prevent re-replication.

Limitation of re-replication

Although I clearly saw re-initiation at several origins during the induction of re-replication, I also observed several origins that did not re-initiate. Even more surprising was the observation that replication forks from re-initiating origins appear to have difficulty moving down the chromosome. These results suggest that additional mechanisms inhibiting re-elongation as well as re-initiation remain to be uncovered. *S. cerevisiae* Cdt1 was recently identified (Tanaka and Diffley, 2002). Like budding yeast Mcm2-7, nuclear localization of Cdt1 is regulated during the cell cycle and the entire complex of Cdt1-Mcm2-7 is required for nuclear accumulation. This regulation is also dependent on CDK activity. Even though regulation of Cdt1, Cdc6, ORC, and Mcm2-7 is simultaneously disrupted, re-replication is still incomplete in G2/M (Morreale R., personal communication), strongly suggesting that these may not be the only mechanisms to inhibit re-replication.

Moreover, budding yeast Mcm3 contains five full consensus CDK sites and two degenerate sites and has been shown to be phosphorylated by CDKs. But in the re-replication cells Mcm3 is still wild type. If all the CDK sites on Mcm3 are mutated in combination with the other mutants in the re-replication strain, the re-replication phenotype might be more dramatic. As mention in Chapter 1, Sld2 levels seem to drop at the end of S phase and remain low until late G1 of the next cell cycle. The disappearance of Sld2 could contribute to the block to re-initiation. Furthermore, DNA polymerase α has been shown to associate with chromatin in G1 phase and release from chromatin at

the end of S phase (see Chapter 1). The regulation of DNA polymerase α association with chromatin could contribute to the block to re-initiation. The regulation of both Sld2 and DNA polymerase α is still intact in the re-replication. If both proteins are deregulated in the re-replication strain, the re-replication phenotype might be more dramatic. Thus, the regulation of these proteins might be additional mechanisms to prevent re-initiation and re-replication.

Relevance to other organisms

CDKs are always thought to be required for triggering initiation of DNA replication and at the same time play a central role in preventing the assembly of new pre-RC (Diffley, 1996). Recent study in *Xenopus* showed that geminin is an inhibitor of pre-RC formation (McGarry and Kirschner, 1998). This result suggests that CDK is not the only player to prevent the assembly of the pre-RCs. Geminin inhibits DNA replication by preventing the loading of Mcm onto chromatin. Studies in both human and *Xenopus* demonstrated that Cdt1 is negatively regulated by an interaction with geminin (Tada et al., 2001; Wohlschlegel et al., 2000). It has been showed that inhibition of CDKs in metaphase in combination with the depletion of geminin stimulates the pre-RC formation. Thus as in yeast, CDKs also use redundant ways to block the assembly of the pre-RC in *Xenopus* egg extracts. CDKs inactivate APC in G1 allowing the accumulation of geminin which prevent the loading of Mcm onto chromatin. They also phosphorylate Cdc6 to promote its nuclear export (Petersen et al., 1999).

Thus, the idea that CDKs use multiple overlapping mechanisms to prevent re-initiation and re-replication is still hold true for all eukaryotes. The exact mechanisms that budding yeast uses may not be conserved in other eukaryotes, but the target proteins

and the use of multiple mechanisms is probably conserved. For example, in budding and fission yeast Cdc6 protein level is regulated in a CDK-dependent manner. In *Xenopus* and human, Cdc6 remains stable during the cell cycle but phosphorylation of Cdc6 by CDKs promotes its nuclear export as cells enter S phase (Petersen et al., 1999; Saha et al., 1998). In human, Orc1 accumulates during G1, and is reduced as cells enter S phase. Orc1 is phosphorylated by CDKs and targeted for ubiquitin-mediated degradation (Natale et al., 2000).

Conclusion

In summary, the results discussed in this thesis show that CDKs use multiple overlapping mechanisms to prevent re-initiation and re-replication within a single cell cycle. It is extremely important for cells to have multiple safety nets to prevent DNA re-replication because such failure will lead to genomic instability. These results also provide one of the few examples where we have a mechanistic understanding of how CDKs regulate a key cell cycle event. In order to understand how eukaryotes regulate initiation of DNA replication so that it occurs once and only once per cell cycle at the molecular level, the future of the field lies in the development of in vitro replication system. Recent in vitro studies of the pre-RC formation at S. cerevisiae origin provides powerful tools to dissect the discrete steps of the pre-RC assembly (Seki and Diffley, 2000). Such studies have potential to identify additional components of the pre-RC. Moreover, this cell-free system should be useful for understanding how pre-RC is regulated during the cell cycle. Future studies include trying to obtain clues to identify of these additional mechanisms by examining the regulation of other replication proteins during the cell cycle. Moreover, it is interesting to investigate whether and exactly how

re-replication might lead to genomic instability. These studies will hopefully give us a better understanding of why the cell goes to such great lengths to prevent the re-replication of its DNA.

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CHAPTER 5

Supplementary data for Chapter 2

MATERIALS AND METHOD

Plasmids and Strains

Integrating replacement plasmids (Table 1) were constructed in three stages: (1) *MCM2-7* genomic fragments were subcloned into pRS306; (2) an 18 bp cassette containing the *Not*I and *Sgr*AI restriction sites was inserted just downstream of either the first or last codon of each *MCM* ORF; (3) one or more cloning cassettes encoding GFP, $SVNLS_2$ (tandem copies of the SV40 NLS), or $svnls3A_2$ (tandem copies of a mutant SV40 NLS) were inserted into the NotI or SgrAI sites. Integrating tagging plasmids (Table 2) were derived from the integrating replacement plasmids by deletion. Whenever site-directed mutagenesis, oligonucleotide ligations, or PCR were used in cloning steps, sequences of the resulting clones were verified by Sanger sequencing. Nucleotide numbering is relative to the A of the start codon (+1) on the sense strand; upstream is negative (starting with –1) and downstream is positive.

The genomic and vector fragments that make up each *MCM* subclone are as follows: pJL973, *Sac*II-*Asp*718 *MCM2* and*Sac*II-*Asp*718 pRS306; pJL1034, *Pvu*II-*Pvu*I* *MCM3* and *Xho*I*-*Not*I* pRS306; pNL975, *Sac*I-*Hind*III *MCM4* and *Sac*I-*Hind*III pRS306 (minus Cla I site); pJL1035, *Xho*I-*Age*I* *MCM5* and *Xho*I-*Not*I* pRS306; pKI (*Xho*I:-971)-*Pst*I* *MCM6* and *Xho*I-*Not*I* pRS306; pJL1033, *Pst*I*-*Sna*BI *MCM7* and *Xho*I*-*Sac*I* pRS306. An asterisk indicates the restriction end was blunted by Klenow (if a 5' overhang) or T4 DNA polymerase (if a 3' overhang). The *MCM6* genomic fragment had a *Xho*I site added to the end of nt –971 and an internal *Sgr*AI site destroyed by pt mutation (–47T>a). The sequence 5'-GGCGGCCGCGCACCGGTG-3' (encoding GGRGPV) was inserted immediately downstream of the last codon of the *MCM2*, *MCM4* , *MCM5*, and *MCM7* ORFs to generate, respectively, the *MCM-NotI-SgrAI* constructs pKI1145, pKI1147, pKI1148, and pVN003. The sequence 5'-GCACCGGTGGGCGGCCGC-3' (encoding GPVGGR) was inserted immediately

downstream of the first codon for the *MCM3* and *MCM6* ORFs to generate, respectively, the *SgrAI-NotI-MCM* constructs pKI1253 and pKI1250.

Three GFP cassettes were generated by PCR to be in frame with the *MCM* ORFs when inserted into the NotI restriction sites discussed above: (1) a *NotI-Gly6-GFP-NotI* cassette encoding a Gly6 linker attached to an enhanced GFP mutant (S65T and V163A); (2) a *NotI-GFP-Gly6-NotI* cassette identical to (1) but with the Gly6 and GFP in reverse order; and (3) a *NotI-GFP-NotI* cassette encoding an enhanced GFP mutant (F64L and S65T). All are simply referred to as GFP in Tables 1-3, but cassette 1 was used for *MCM-NotI-SgrAI* constructs, cassette 2 was used for *SgrAI-NotI-MCM* constructs, and cassette 3 was used for one*MCM7-GFP* construct (pVN018). The following two tags were generated by oligonucleotide synthesis for in frame insertion into the *Sgr*AI sites discussed above: (1) $SVNLS_2$ encodes for RSG-PPKKKRKVE-GGSG-PPKKKRKVE-GGSR, which contains two copies of the SV40 NLS; (2) $svnls3A_2$ encodes for RSG-PPAKAAKVE-GGSG-PPAKAAKVE-GGSR, which contains two copies of a mutant SV40 NLS. The exact nucleotide sequence of these tags can be obtained from the authors. The integrating replacement plasmids resulting from insertion of these tags into the *MCM-NotI-SgrAI* and *SgrAI-NotI-MCM* constructs are listed in Table 1. Plasmids listed in Table 1 were used to replace the endogenous *MCM* gene with a tagged version by two-step gene replacement (see strains listed in Table 3).

The integrating tagging plasmids listed in Table 2 are related to those in Table 1 by deletions between the following restriction sites: (1) *MCM2* series, *Eco*RI*-*Ecl136*III; (2) *MCM3* series, *Cla*I*-*Ecl136*III; (3) *MCM4* series, *BstAP*I*-*Ecl136*III*; (4) *MCM5* series, *SnaB*I-*Asp718**; (5) *MCM6* series, *Sac*I-*Sac*I; (6) *MCM7* series, *Pac*I*-*Xho*I*. One-step integrative recombination of these plasmids simultaneously disrupted the endogenous MCM gene and introduced a tagged version under the control of the endogenous promoter (see strains listed in Table 3). *S. cerevisiae* strains used in these

constructions were derivatives of YJL310 (R. Deshaies, California Institute of Technology, Pasadena, California).

pMET-CDC6 in YJL1607, 1613, 1939, 1945, 2334, 2335, 2336, and 2340 was originally derived by two-step gene replacement of the wild-type *CDC6* gene using pCD29 (Detweiler and Li, 1998). *pGAL-CLB2ΔDB* in YJL1607, 1935, and 1939 was originally introduced by integration of pCD25 (Detweiler and Li, 1998) at the *TRP1* locus. *pGAL* in YJL2951 and 2962 was introduced by integration of pJL806 at *URA3* locus. *pGAL-CLB5ΔDB* in YJL2954 and 2964 was introduced by integration of *GAL1-CLB5db*^{HA} (Cross et al., 1999) at the *ARS1* locus. *pGAL-sic1-9A* in YJL2959 and 2971 was introduced for this study by integration of pVN179 at the *URA3* locus. pVN179 was constructed by subcloning an EcoRI-HindIII fragment of AH971216B1 (gift of M. Mendenhall, University of Kentucky) containing sic1-9A (with the following amino acid substitutions: 2A,5A,33V,45A,69A,76A,80A,173A,191A) under the control of the *GAL1* promoter into pRS306. *ts-degron-cdc45* in YJL2340 and 2342 was introduced by integrative gene replacement using pubi-ts-CDC45 (Hopwood and Dalton, 1996).

TABLES

Table 1. Integrating Replacement Plasmids

	GFP	*GFP-SVNLS*$_2$	*GFP-svnls3A*$_2$
MCM2	pKI1151	pKI1165	pKI1241
MCM3	pKI1296	pKI1309	pKI1310
MCM4	pKI1157	pKI1174	pKI1175
MCM5	ND	pKI1178	ND
MCM6	pKI1298	pKI1313	pKI1314
MCM7	pVN018*/pVN142	pVN151	pVN148

* Uses *GFP* encoding the F64L, S65T enhanced GFP mutant;

all other *GFP*s encode S65T, V163A enhanced GFP mutant

Table 2. Integrating Tagging Plasmids

	GFP	$SVNLS_2$	$svnls3A_2$
MCM2	pKI1183	pKI1188	pKI1263
MCM3	pKI1299	pKI1323	pKI1324
MCM4	pKI1212	pKI1218	pKI1267
MCM5	ND	pKI1226	pKI1270
MCM6	pKI1302	pKI1341	pKI1342
MCM7	pKI1198	pJL1208	pKI1273

Table 3. Yeast Strains

Strain	Genotype	Source
YJL310*	*MAT***a**	(Detweiler and Li, 1998)
YJL345	*MAT***a** *cdc6-1 ura3-52 leu2-3,112*	(Detweiler and Li, 1998)
YJL1389	*MAT*α *dbf2-2 ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 psi+*	K. Nasmyth
YJL1607*	*MAT***a** *MCM7-GFP[l] METpCDC6 trp1-289::{pGAL-CLB2ΔDB,TRP1}*	YJL1167 (Detweiler and Li, 1998)Tfn[a]
YJL1613*	*MAT***a** *MCM7-GFP[l] METpCDC6 trp1-289::(TRP)*	YJL1169 (Detweiler and Li, 1998)Tfn[a]
YJL1647*	*MAT*α *MCM7-GFP[l]*	This study[d]

YJL1925 *MATa MCM7-GFP[l] cdc6-1 ura3-52 trp1-289 bar1::LEU2* YJL345x1647,2-3D

YJL1935 *MATa MCM7-GFP[l] trp1-289::{pGAL-Clb2ΔDB,TRP1} dbf2- his3-11,15 ura3 bar1::LEU2 leu2-3, 1122* YJL1607x1389 2-14C

YJL1937 *MATa MCM7-GFP[l] dbf2-2 trp1-289 ura3-52 bar1:: LEU2 leu2-3,112* YJL1607x1389,3-6D

YJL1939 *MATa MCM7-GFP[l] trp1-289::{pGAL-Clb2ΔDB, TRP1} dbf2 -2 his3-11,15 ura3-52 bar1::LEU2 leu2-3,112 pMET-CDC6* YJL1607x1389,3-17A

YJL1945 *MATa MCM7-GFP[l] pMET-CDC6 dbf2-2 bar1:: LEU2 ura3-52 trp1-289 his3-11,15 leu2-3,112* YJL1607x1389,3-12A

YJL1969* *MATa MCM2-GFP* YJL310 Tfn[a]

YJL1973* *MATa MCM4-GFP* YJL310 Tfn[a]

YJL1977* *MATa MCM7-GFP* YJL310 Tfn[a]

YJL1981* *MATa MCM7-GFP-2NLS* YJL310 Tfn[a]

YJL1985* *MATa MCM7-GFP-2nls3A* YJL310 Tfn[a]

YJL1991* *MATa MCM5-GFP-2NLS* YJL310 Tfn[a]

YJL2039* *MATa mcm4::{MCM4-GFP-2NLS, URA3}* YJL310 Tfn[b]

YJL2043* *MATa MCM2-GFP mcm5::{MCM5-2NLS, URA3}* YJL1969 Tfn[b]

YJL2045* *MATa MCM4-GFP mcm2::{MCM2-2NLS, URA3}* YJL1973 Tfn[b]

YJL2047* *MATa MCM7-GFP mcm2::{MCM2-2NLS, URA3}* YJL1977 Tfn[b]

YJL2050* *MATa MCM7-GFP mcm5::{MCM5-2NLS, URA3}* YJL1977 Tfn[b]

YJL2151* *MATa MCM2-GFP-2NLS* YJL310 Tfn[a]

YJL2155* *MATa MCM4-GFP-2NLS* YJL310 Tfn[a]

YJL2160* *MATa GFP-MCM3* YJL310 Tfn[a]

YJL2163* *MATa GFP-MCM6* YJL310 Tfn[a]

YJL2170* *MATa MCM2-GFP mcm4::{MCM4-2NLS, URA3}* YJL1969 Tfn[b]

YJL2172* *MATa MCM2-GFP mcm4::{MCM4-2nls3A, URA3}*	YJL1969 Tfn[b]
YJL2188* *MATa MCM4-GFP mcm7::{MCM7-2NLS, URA3}*	YJL1973 Tfn[b]
YJL2211* *MATa MCM4-GFP mcm5::{MCM5-2NLS, URA3}*	YJL1973 Tfn[b]
YJL2221* *MATa mcm4::{MCM4-GFP-2nls3A, URA3}*	YJL310 Tfn[b]
YJL2227* *MATa mcm3::{2NLS-GFP-MCM3 ,URA3}*	YJL310 Tfn[b]
YJL2229* *MATa mcm6::{2NLS-GFP-MCM6,URA3}*	YJL310 Tfn[b]
YJL2231* *MATa MCM2-GFP mcm3::{2NLS-MCM3, URA3}*	YJL1969 Tfn[b]
YJL2237* *MATa MCM2-GFP mcm6::{2NLS-MCM6, URA3}*	YJL1969 Tfn[b]
YJL2241* *MATa GFP-MCM3 mcm2::{2NLS-MCM2, URA3}*	YJL2159 Tfn[b]
YJL2245* *MATa GFP-MCM3 mcm4::{2NLS-MCM4, URA3}*	YJL2159 Tfn[b]
YJL2249* *MATa GFP-MCM3 mcm5::{MCM5-2NLS, URA3}*	YJL2159 Tfn[b]
YJL2253* *MATa GFP-MCM3 mcm6::{2NLS-MCM6, URA3}*	YJL2159 Tfn[b]
YJL2257* *MATa MCM4-GFP mcm3::{2NLS-MCM3, URA3}*	YJL1973 Tfn[b]
YJL2263* *MATa MCM4-GFP mcm6::{2NLS-MCM6, URA3}*	YJL1973 Tfn[b]
YJL2267* *MATa GFP-MCM6 mcm2::{MCM2-2NLS, URA3}*	YJL2163 Tfn[b]
YJL2271* *MATa GFP-MCM6 mcm3::{2NLS-MCM3, URA3}*	YJL2163 Tfn[b]
YJL2273* *MATa GFP-MCM6 mcm4::{MCM4-2NLS, URA3}*	YJL2163 Tfn[b]
YJL2277* *MATa GFP-MCM6 mcm5::{MCM5-2NLS, URA3}*	YJL2163 Tfn[b]
YJL2281* *MATa MCM7-GFP mcm3::{2NLS-MCM3, URA3}*	YJL1977 Tfn[b]
YJL2289* *MATa MCM7-GFP mcm6::{2NLS-MCM6, URA3}*	YJL1977 Tfn[b]
YJL2331* *MATa mcm7::{MCM7-GFP,URA3}*	YJL310 Tfn[b]
YJL2334* *MATa pMET-CDC6 mcm7::{MCM7-GFP,URA3}*	YJL1169 Tfn[b]
YJL2336* *MATa MCM7-GFP pMET-CDC6 cdc7-4*	This study[d]
YJL2338* *MATa MCM7-GFP cdc7-4*	This study[d]
YJL2340* *MATa MCM7-GFP pMET-CDC6 cdc45::{ts-degron-cdc45,URA3} trp1-289::(TRP1)*	YJL1613 Tfn[c]
YJL2342* *MATa MCM7-GFP cdc45::{ts-degron-cdc45,URA3}*	YJL1977 Tfn[c]

YJL2756	*MATa/MATa mcm3::{GFP-MCM3, URA3}/mcm3::{GFP-MCM3,URA3} bar1::LEU2/bar1::LEU2 ura3-52/ura3-52 leu2-3,112/leu2-3,112 trp1-289/trp1-289*	This study[d]
YJL2951	*MATa dbf2-2 MCM7-GFP ura3-52::{pGAL, URA3}*	YJL1937 Tfn[c]
YJL2954	*MATa dbf2-2 MCM7-GFP ARS1::{pGAL-CLB5ΔDB, URA3, ARS1}*	YJL1937 Tfn[c]
YJL2959	*MATa MCM7-GFP ura3::{pGAL-sic1-9A,URA3} dbf2-2*	YJL1937 Tfn[c]
YJL2962	*MATa dbf2-2 MCM7-GFP pMET-CDC6 ura3-52::{pGAL, URA3 }*	YJL1945 Tfn[c]
YJL2964	*MATa dbf2-2 MCM7-GFP pMET-CDC6 ARS1::{pGAL-CLB5ΔDB , URA3, ARS1}*	YJL1945 Tfn[c]
YJL2971	*MATa MCM7-GFP pMET-CDC6 ura3::{pGAL-sic1-9A,URA3} dbf2-2*	YJL1945 Tfn[c]

(*) These strains are congenic to YJL310 whose full genotype is *MATaura3-52 leu2-3,112 trp1-289 bar1::LEU2.*

1. These*GFP*s encode the F64L, S65T enhanced GFP mutant; all other *GFP*s encode S65T, V163A enhanced GFP mutant.

(a) Strain derived by two-step gene replacement using plasmids from Table 1

(b) Strain derived by one-step integrative gene replacement using plasmids from Table 2

(c) Strain derived by one-step integration as described in text

(d) See Plasmids and Strains

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Hopwood, B., and Dalton, S. (1996). Cdc45p assembles into a complex with Cdc46p/Mcm5p, is required for minichromosome maintenance, and is essential for chromosomal DNA replication. Proc Natl Acad Sci U S A *93*, 12309-14.

Figure 1. Nuclear accumulation of Mcm7-GFP in G1 phase is independent of Cdc45. *CDC45* (YJL1977, *MAT*a *CDC45 CDC6 MCM7-GFP*) and *cdc45*^{ts} (YJL2342, *MAT*a *ts-degron-cdc45 MCM7-GFP*) cells growing exponentially in YEPD at 23° C were arrested in G2/M phase by addition of nocodazole for 3 hr then shifted to 37° C to inactivate the ts mutants. After 30 min at 37° C, cells were released from the G2/M phase arrest by filtration and resuspended in prewarmed 37° C YEPD medium containing α factor. Cells were examined by fluorescence microscopy just before release from the G2/M arrest (NOC) and 90 min later when they had fully arrested in G1 phase (αF arrest).

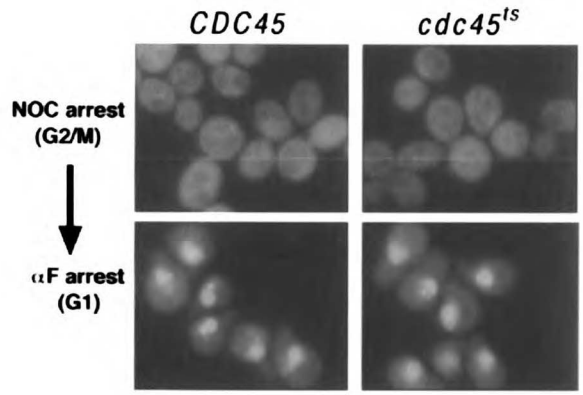
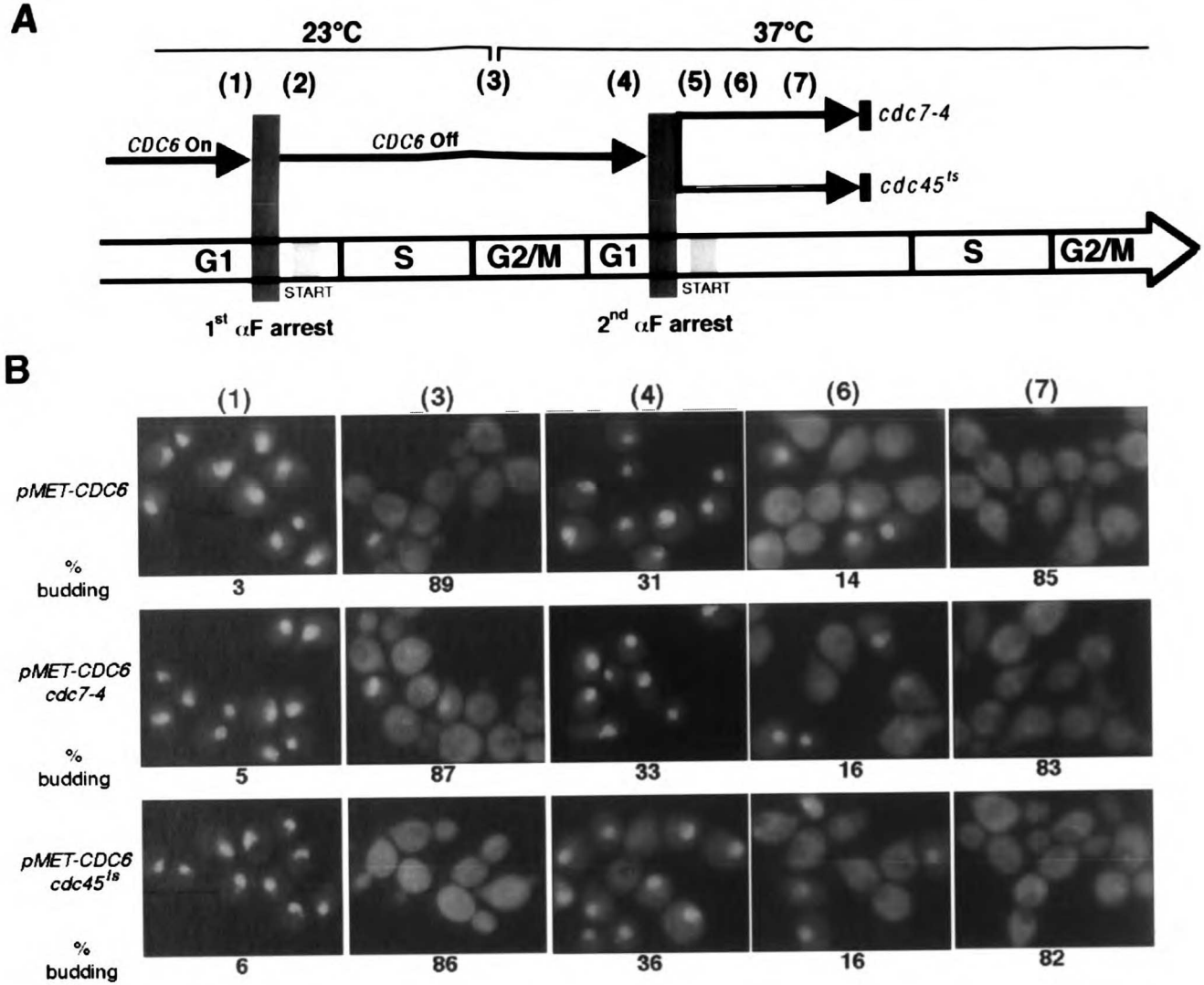

CDC45
cdc45ts
NOC arrest
(G2/M)
αF arrest
(G1)

Figure 2. Cdc7 and Cdc45 are not necessary for Mcm7-GFP export in Cdc6-depleted cells. (**A**) Experimental protocol. Cells growing in SCD-Met at 23° C were arrested in α-factor for 2.5 hr (1), then synchronously released into YEPD (2) containing additional methionine to represses *CDC6* transcription. After 2 hr the cells had reached G2 phase (3). and they were then shifted to the restrictive temperature of 37° C in the presence of α-factor to rearrest them in G1 phase of next cell cycle. After 80 min, when the cells were rearrested (4), pronase (and nocodazole) was added (5) to release them from this second α factor arrest, and samples were examined 20 min (6) and 40 min (7) later. (**B**) YJL1613 (*MAT***a** *pMET-CDC6 MCM7-GFP*), YJL2336 (*MAT***a** *cdc7-4 pMET-CDC6 MCM7-GFP*), and YJL2340 (*MAT***a** *ts-degron-cdc45 pMET-CDC6 MCM7-GFP*) were treated as in (A) and examined by fluorescence microscopy and % budding at the indicated points in the protocol. *CDC6* strains congenic to YJL1613, YJL2336, and YJL2340 (YJL1977, YJL2338, and YJL2342, respectively) were treated in parallel and analyzed by flow cytometry to monitor DNA replication after the last cell cycle release. YJL2338 and YJL2342 maintained a 1C DNA content (data not shown), consistent with inactivation of Cdc7 and Cdc45 in these strains.


A
23°C
37°C
(1) (2) (3) (4) (5) (6) (7)
CDC6 On
CDC6 Off
cdc7-4
cdc45ts
G1
S
G2/M
G1
S
G2/M
START
START
1st αF arrest
2nd αF arrest
B
(1) (3) (4) (6) (7)
pMET-CDC6
% budding
3 89 31 14 85
pMET-CDC6 cdc7-4
% budding
5 87 33 16 83
pMET-CDC6 cdc45ts
% budding
6 86 36 16 82

CHAPTER 6

Supplemental data for Chapter 3

Plasmid construction. Plasmid pJL737 (*ORC6*) contains nucleotides –665 to +1856 (relative to the first coding nucleotide) of ORC6 inserted in the polylinker of pRS306 (Sikorski and Hieter, 1989) flanked by a cut and filled in BamHI site (-665) and an intact SalI site (+1856). Plasmid pJL921 (*ORC6-HA3*) is identical except (1) the polylinker sites SpeI and NotI were destroyed by religating the cut and filled-in sites to each other, (2) a new NotI site was introduced at the 3' end of the *ORC6* ORF by inserting 5'-AGCGGCCGC-3' just before the stop codon, and (3) a triple hemagglutinin epitope HA3[2] was cloned into the NotI site. The resulting construct encodes Orc6 protein fused at its C-terminus to HA3. Plasmid pJL1544 was derived from pJL921 by excising the 5' portion of *ORC6* with BsrG1 and NgoMV, filling-in the two ends, and ligating them together. Loop-in integration of pJL1544 (linearized with BstXI) at the *ORC6* locus simultaneously disrupts the endogenous *ORC6* gene and introduces *ORC6-HA*.

Plasmids pMP933 and pMP934 contains *ORC2* from the first SacI site 5' of the ORF to the first SalI site 3' of the ORF cloned into SacI and SalI of pRS306. In pMP933 (*ORC2*), SgrAI and NotI sites were introduced at the 5' end of the ORF by inserting 5' – ATGGCACCGGTGGGCGGCCGC – 3' just before the ATG. In pMP934, SgrAI and NotI sites were introduced at the 3' end of the ORF by inserting 5' – GGCGGCCGCGCACCGGTG – 3' just before the stop codon. Insertion of HA3 into the NotI site of pMP934 generated pMP944 (*ORC2-HA3*), which encodes Orc2 fused at its C-terminus to HA3.

Plasmid pTW000 (*orc6-4A-HA3*) was derived from pJL921 by introducing mutations that change serine/threonine to alanine in the CDK consensus phosphorylation

sites at residues 106, 116, 123, and 146; excision of HA3 from pTW000 generated pJL1096 (*orc6-4A*). Plasmid pJL1095 (*orc2-6A*) was generated by similar mutations in *ORC2* of pMP934 that change residues 16, 24, 70, 174, 188, and 206 in its consensus CDK phosphorylation sites.

Plasmid pJL806 (*pGAL1*) contains the *GAL1* promoter from –663 to –5 (relative to the first nucleotide of the *GAL1* ORF) inserted in the polylinker of pRS306 (Sikorski and Hieter, 1989) flanked by a cut and filled in SpeI site (-663) and an intact BamHI site (-5). Plasmid pJL1489 (*pGAL1-Δntcdc6*) contains the sequence 5' – TATGAGCGGCCGC – 3' followed by *CDC6* from +139 to +1983 inserted in the SmaI site of pJL806 downstream of the *GAL1* promoter; it expresses a truncated Cdc6p with amino acids 2-47 replaced by amino acids S-G-R. Plasmid pJL1490 (*pGAL1-CDC6*) contains the sequence 5' – TATGAGCGGCCGC – 3' followed by *CDC6* from +1 to +1983 inserted in the same SmaI site; it expresses full length Cdc6p with the amino acids M-S-G-R appended to the N-terminus.

pJL1206 (*MCM7-NLS*), which encodes Mcm7p fused at its C-terminus to two tandem copies of the SV40 NLS, was derived by NotI excision of GFP from pVN151 (*MCM7-GFP-NLS*) (Nguyen et al., 2000). pKI1260 (*MCM7-nls3A*), which encodes Mcm7p fused at its C-terminus to two mutated and nonfunctional copies of the SV40 NLS, was derived by NotI excision of GFP from pVN148 (*MCM7-GFP-nls3A*) (Nguyen et al., 2000).

Strain construction. pJL921 was used to construct YJL865 (*CDC-wt ORC6-HA3*) by a loop-in/loop-out gene replacement in YJL310 (Detweiler and Li, 1997). YJL865 was

crossed to *cdc* mutants (previously backcrossed three to four times against YJL310) to generate the following *ORC6-HA3* strains used in Fig. 1: YJL929 (*cdc25-5*), YJL934 (*cdc28-4*), YJL905 (*cdc4-1*), YJL926 (*cdc34-2*), YJL907 (*cdc7-4*), YJL903 (*cdc17-1*), YJL922 (*cdc9-1*). Loop-in integration of pJL1544 (*orc6-HA3*) into *cdc15* and *cdc14* generated *ORC6-HA3* strains YJL3099 (*cdc15-1*), and YJL3101 (*cdc14-1*), respectively. Only YJL1937[3] (*dbf2-2*) contains untagged *ORC6*. All the strains described above were *MATa ORC2 bar1::LEU2 ura3-52 leu2-3,112 trp1-289* in the A364a background. pMP944 was used to construct YJL963 (*ORC2-HA3*) by loop-in/loop-out gene replacement in YJL310. YJL963 was later found to have diploidized (as a *MATa/a* diploid) during its construction. pTWOOO was used to construct YJL1394 (*orc6-4A-HA3*) by loop-in/loop-out gene replacement in YJL312, which is a *pep4::TRP1* strain congenic to YJL310. Sequential loop-in/loop-out gene replacements with pJL1096 and pJL1095 in the A364a background generated YJL1737 (*MATa orc2-6A orc6-4A leu2 ura3-52 trp1-289 ade2 ade3 bar1::LEU2*).

Strains in Fig. 2-4 were derived from YJL1737 using various combinations of the following plasmids: pMP933 (*ORC2*), pJL737 (*ORC6*), pJL1206 (*mcm7-NLS*), pKI1260 (*MCM7-nls3A*), pJL1489 (*pGAL1-Δntcdc6*), pJL806 (*pGAL1*) and pMET-CDC20 (Uhlmann et al., 2000) which replaces the *CDC20* promoter with the *MET3* promoter. pMP933, pJL737, pJL1206, and pKI1260 were used in loop-in/loop-out gene replacements at their respective chromosomal loci, pMET-CDC20 was used in one-step gene replacements at the *CDC20* locus, and pJL1489 and pJL806 were inserted at the *URA3* locus by loop-in integration. These genetic manipulations generated YJL3239 (*ORC2 ORC6 MCM7-NLS CDC6 ura3-52::{pGAL1-Δntcdc6, URA3} cdc20Δ::{pMET3-*

CDC20, TRP1}); YJL3242 (*orc2-6A orc6-4A MCM7-nls3A CDC6 ura3-52::{pGAL1-Δntcdc6, URA3} cdc20Δ::{pMET3-CDC20, TRP1}*;, YJL3244 (*orc2-6A orc6-4A MCM7-NLS CDC6 ura3-52::{pGAL1, URA3} cdc20Δ::{pMET3-CDC20, TRP1}*); and YJL3248 (*orc2-6A orc6-4A MCM7-NLS CDC6 ura3-52::{pGAL1-Δntcdc6, URA3} cdc20Δ::{pMET3-CDC20, TRP1}*). All strains are *MATa leu2 ura3-52 trp1-289 ade2 ade3 bar1::LEU2.*

YJL3122 and YJL3510 are both congenic to the re-replicating strain YJL3248. YJL3122 contains *pGAL1-CDC6* instead of *pGAL1-Δntcdc6* integrated at the *ura3-52* locus. YJL3510 contains an 8 bp linker substitution in the ARS consensus sequence and ORC binding site (Bell and Stillman, 1992) of *ARS305* that was introduced by loop-in/loop-out replacement using plasmid pDK-ARS305Lin1 (Huang and Kowalski, 1996).

Protein Analysis. Samples were prepared for immunoblot analysis as described (Owens et al., 1997). The two forms of Orc2p were separated by electrophoresis on a 6-12% SDS-PAGE gradient gel. Primary antibodies used in this paper were: 12CA5 anti-HA mouse monoclonal ascites (1:2000); affinity purified anti-Orc6 rabbit polyclonal antibodies (1:2000); SB67 (Liang and Stillman, 1997) anti-Orc2 mouse monoclonal cultured supernatant (1:100); SB3 (Liang and Stillman, 1997) anti-Orc3 mouse monoclonal ascites (1:10000); #28 (Liang and Stillman, 1997) anti-Mcm2 mouse monoclonal ascites (1:3000); 9H8/5 (Donovan et al., 1997) anti-Cdc6 mouse monoclonal ascites (1:200). Secondary antibodies used were anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (1:5000) (Biorad or ProMega). Chemiluminescent detection was performed using Supersignal reagents from Pierce. To prepare the anti-

Orc6 antibodies, anti-GST-Orc6 antiserum from rabbit was depleted of anti-GST antibodies by passage over a GST column then purified by elution from a GST-Orc6 column as described (Asubel et al., 2000).

For phosphatase analysis of *ORC6-HA3* or *ORC2-HA3*, cells were lysed by bead beating in SDS Lysis Buffer (10 mM Tris-Cl pH7.5, 50 mM NaCl, 5 mM EDTA, 1% (w/v) SDS, 1 mM DTT, 1mM Na_3VO_4, 50 mM NaF, 50 mM _-glycerophosphate, 0.1 mM PMSF, 1 mM benzamidine, and 1 μg/ml each of leupeptin, pepstatin A, and chymostatin). Lysates were converted to IP Buffer conditions by dilution in four volumes of Triton Dilution Buffer (SDS Lysis Buffer with SDS replaced by 1 % (v/v) Triton X-100 and NaCl at 150 mM). The HA-tagged Orc proteins were immunoprecipitated from 200 μg of diluted lysate with 12CA5 anti-HA monoclonal ascites and protein A sepharose as described (Harlow and Lane, 1988) and immunoprecipitates washed three times in IP Buffer. Phosphatase treatment of the immunoprecipitates with or without phosphatase inhibitors was performed as described (Jaspersen and Morgan, 2000).

H1 Kinase and Replication Assays. H1 kinase assays were performed as described (Rudner et al., 2000), except reactions were performed in 20 μl of 50 mM HEPES pH7.4, 2 mM $MgCl_2$, 0.1 mM ATP, 1 mM DTT, containing 5 μg Histone H1 (Upstate Biological) and 5 μCi [_-^{32}P] ATP (NEN, BLU502Z). For flow cytometry, cells were fixed and stained with 1 μM Sytox Green (Molecular Probes) as described (Haase and Lew, 1997). For pulsed-field gel electrophoresis (PFGE), cells were prepared in agarose plugs as described (Asubel et al., 2000) and elecrophoresis was carried out in 1% LE

agarose (Seakem) in a CHEF DRII apparatus from Bio-Rad using the following conditions: 14° C, 0.5X TBE, 200 V, 120° field angle, 36 hour run, switch time ramped from 40 to 70 sec. For neutral-neutral 2-D gel electrophoresis, DNA was prepared as described (Huberman et al., 1987) and 3 μg digested with EcoRV (for *ARS305*, *ARS307*, *ARS607*+30kb), NciI (for *ARS306*), EcoRI and BamHI (for *ARS121*), SacI and ApaLI (for *ARS607*), XbaI (for *ARS501* and *ARS1413*), or NcoI (for *ARS1*). The digested DNA was subjected to electrophoresis as described (Friedman and Brewer, 1995).

Southern analysis of both PFGE and 2-D gels were performed as described (Asubel et al., 2000). For the PFGE southern analysis the *ARS1* probe detected both chromosome 4 (*ARS1* locus) and 7 (*MET3-CDC20* locus marked with *TRP1-ARS1*). Probes were PCR amplified from yeast genomic DNA with the following primers: for *ARS305*, OJL1028 5'-ATTCGCCTTCTGACAGGACG-3' and OJL1029 5'-ATAACGGAGACTGGCGAACC-3'; for *ARS306*, OJL1033 5'-TGGTTTGGACGACGGATTGG-3' and OJL1034 5'-TATGGGATGCTGTTGCGAGC-3'; for *ARS307*, OJL1037 5'- TGTGTTCCACTCAATCTGCGG-3' and OJL1038 5'-GGGTTCTTGGTCAATGCCTG-3'; for *ARS121*, OJL1088 5'-AAACCATTCCTGCCTCTGTG-3' and OJL1089 5'- GAAGCCCTTTGTTGAGAACC-3'; for *ARS607*, OJL1024 5'- GTCCCAATAGTGGCTCTGTG-3' and OJL1025 5'-GCTTTCTAGTACCTACTGTGC-3'; for *ARS501*, OJL1031 5'-TAAGACAGCGTGTGTACTCC-3' and OJL1032 5'-AATTGAGCCCGATGACTACG-3'; for *ARS1413*, OJL1039 5'-ATTTCTGAAGTCGTTCCCAGCC-3' and OJL1040 5'-TCTGTCGCCAAGAGCAATCTAC-3'; for *ARS1*, OJL1026 5'-TTCCGATGCTGACTTGCTGG-3' and OJL1027 5'-GACGACTTGAGGCTGATGGT-

3'; for 30kb distal to ARS607, OJL1090 5'-GCCGTACAAATTCTTCCTCTAG-3' and OJL1091 5'-TCTGCTGTTCGCTACATTCC-3'.

Chromatin Association Assay. Chromatin association assays were performed as described (Liang and Stillman, 1997) but with several modifications. Log, alpha factor, and 0 hr samples were spheroplasted in 30 µl of 2 mg/ml Oxalyticase (Enzogenetics, Corvallis, OR); 1, 2, and 3 hr samples were spheroplasted in 40, 45, and 50 µl of 2 mg/ml Oxalyticase, respectively. Lysis was performed in 400 µl extraction buffer and the extracts were underlaid with 200 µl of a 30% sucrose cushion before pelleting the chromatin enriched fraction. The pellets were washed with the same volume of extraction buffer and spun through the same volume of sucrose cushion. The washed pellets were resuspended in 100 µl BE10 (20 mM Hepes pH 7.9, 1.5 mM Magnesium Acetate, 50 mM Potassium Acetate, 10% Glycerol, 0.5 mM DTT, 1 mM PMSF, 0.1 mM Sodium Vanadate, 1 mM Benzamidin, and 1 µg/ml each of Chymostatin, Pepstatin A, and Leupeptin) supplemented with 2 µl (220 U) DNase I (Sigma, D7291) and 150 mM NaCl and incubated at 25° C for 10 min to solubilize chromatin bound proteins. After pelleting insoluble material, the supernatant was collected and immunoblotted as described above.

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Figure 1 All chromosomes experience gel retardation as a consequence of re-replication. ORC phosphorylation, Mcm2-7p localization, and Cdc6p expression were deregulated in YJL3239 (*ORC2 ORC6 MCM7-2NLS CDC6 pGAL1-Δntcdc6 pMET3-CDC20*), YJL3242 (*orc2-6A orc6-4A MCM7-2nls3A CDC6 pGAL1-Δntcdc6 pMET3-CDC20*), YJL3244 (*orc2-6A orc6-4A MCM7-2NLS CDC6 pGAL1 pMET3-CDC20*), and YJL3248 (*orc2-6A orc6-4A MCM7-2NLS CDC6 pGAL1-Δntcdc6 pMET3-CDC20*) as described in the text and summarized at the top of the figure: minus, deregulated; plus, regulated. Deregulation of Cdc6p was conditional and dependent on galactose induction of *pGAL1-Δntcdc6*. Cells were initially grown in medium lacking methionine and containing raffinose to prevent expression of ΔntCdc6p. They were arrested in G2/M by addition of 2mM methionine (which induced depletion of Cdc6p) followed 2.5 hr later by 15 μg/ml of nocodazole. After another 30 min, galactose was added to induce ΔntCdc6p at time 0 hr. **a**, Chromosome migration after pulsed-field gel electrophoresis (PFGE) and ethidium bromide staining. **b**, Southern analysis of PFGE probed for chromosome 4 and 7. Similar results were seen with congenic strains that were wild-type for *CDC20* and arrested in G2/M solely with nocodazole (data not shown).

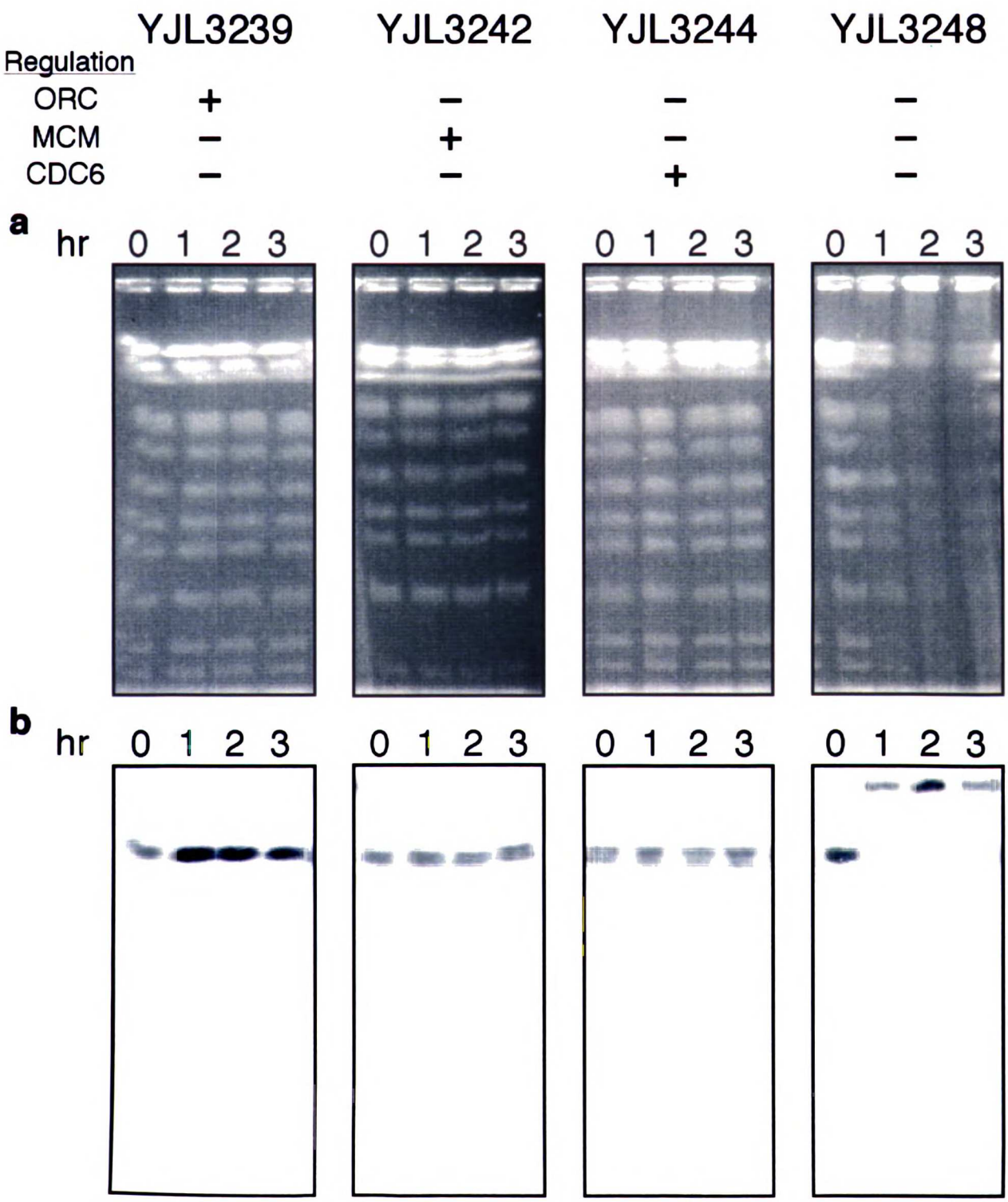

YJL3239
YJL3242
YJL3244
YJL3248
Regulation
ORC + − − −
MCM − + − −
CDC6 − − + −
a
hr 0 1 2 3
b
hr 0 1 2 3

Figure 2 Re-initiation occurs at some but not all origins. Induction of re-replication and analysis of replication intermediates by neutral-neutral 2D gel electrophoresis was performed as described in Fig. 2 on congenic re-replicating strains YJL3510 (*ars305-lin1*) (**a**) and YJL3248 (*ARS305*) (**b-i**). The nonfunctional *ars305-lin1* has an eight bp linker substitution disrupting the ORC binding site for *ARS305*[6,7]. The origins that were probed for each pair of panels (0 hr and 1-2 hr) are indicated in the figure. 607 + 30kb is a locus 30kb distal to *ARS607* that is replicated in S phase by a fork initiated from *ARS607* (Friedman et al., 1997; Yamashita et al., 1997). Bottom right schematic shows expected positions of bubble arcs (indicating initiation within fragment), Y arcs (indicating passive replication), and non-replicating DNA. Arc intensities were normalized by selecting exposures that displayed equivalent signal intensities at the position of the non-replicating DNA (as extrapolated from signal intensities observed in very light exposures).

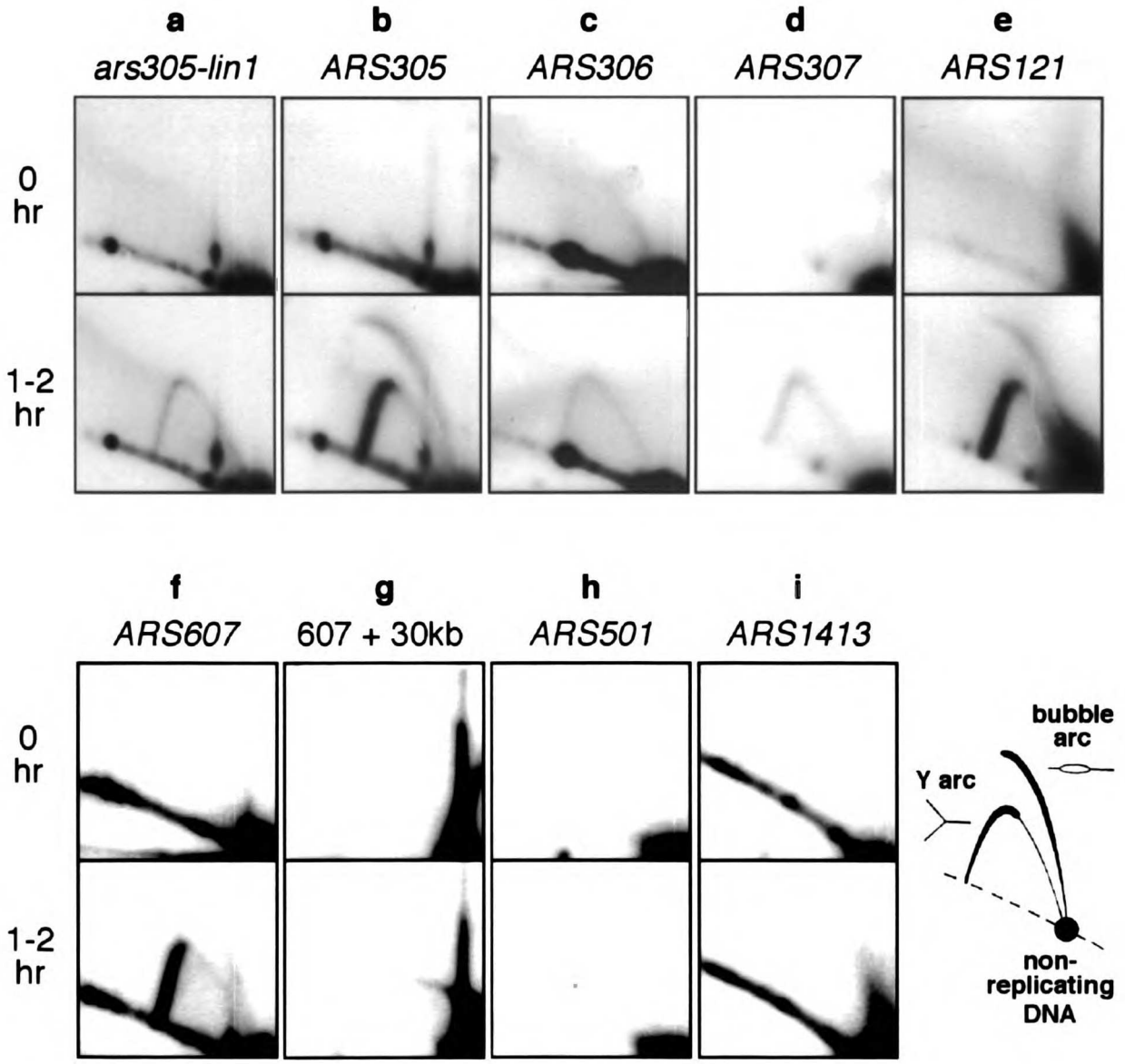
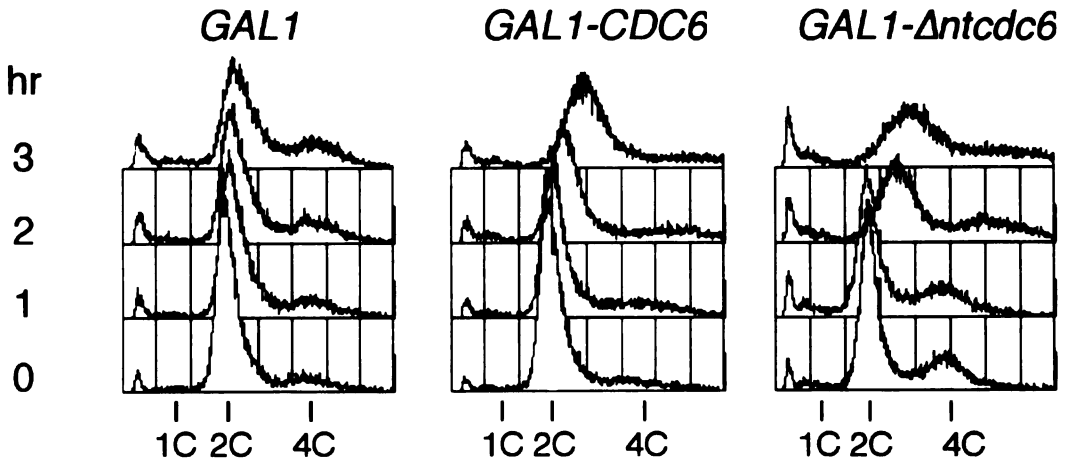

a
ars305-lin1
b
ARS305
c
ARS306
d
ARS307
e
ARS121
0 hr
1-2 hr
f
ARS607
g
607 + 30kb
h
ARS501
i
ARS1413
0 hr
1-2 hr
bubble arc
Y arc
non-replicating DNA

Figure 3 Expression of Cdc6p from the *GAL1* promoter can induce re-replication. YJL3038 (*pGAL1 orc2-6A orc6-4A MCM7-2NLS CDC6*), YJL3122 (*pGAL1-CDC6 orc2-6A orc6-4A MCM7-2NLS CDC6*), YJL2099 (*pGAL1-Δntcdc6 orc2-6A orc6-4A MCM7-2NLS CDC6*) growing in rich medium containing raffinose were arrested at G2/M phase with nocodazole for 3 hr. Galactose was added (0 hr) to induce expression from the *GAL1* promoter and FACS samples were processed every hour.


GAL1
GAL1-CDC6
GAL1-Δntcdc6
hr
3
2
1
0
1C 2C 4C
1C 2C 4C
1C 2C 4C