REVIEW

Strength in numbers: preventing rereplication via multiple mechanisms in eukaryotic cells

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In eukaryotic cells, prereplication complexes (pre-RCs) are assembled on chromatin in the G1 phase, rendering origins of DNA replication competent to initiate DNA synthesis. When DNA replication commences in S phase, pre-RCs are disassembled, and multiple initiations from the same origin do not occur because new rounds of pre-RC assembly are inhibited. In most experimental organisms, multiple mechanisms that prevent pre-RC assembly have now been identified, and rereplication within the same cell cycle can be induced through defined perturbations of these mechanisms. This review summarizes the diverse array of inhibitory pathways used by different organisms to prevent pre-RC assembly, and focuses on the challenge of understanding how in any one cell type, various mechanisms cooperate to strictly enforce once per cell cycle regulation of DNA replication.

The ability of eukaryotic cells to duplicate vast amounts of genetic information quickly and accurately before each cell division is an awe-inspiring product of evolution. To achieve rapid replication, cells use a parallel processing approach in which multiple replisomes copy DNA simultaneously. A dramatic example of this strategy occurs during the early cleavage divisions of the frog *Xenopus laevis*, in which DNA replication initiates from ~300,000 sites, called origins, which are spaced ~10 kb apart (Blow 2001). As a result, 3 billion base pairs of DNA are duplicated within minutes. All eukaryotic organisms use a similar strategy, and the number of origins employed is generally correlated to the genome size and the length of the cell cycle. To ensure that DNA replication is not only fast, but accurate, it is essential that no segment of the chromosome be duplicated more than once. Indeed, reinitiation from even a single origin within the same cell cycle may cause genome instability. Thus, the ability of cells to restrict DNA replication to a single round per cell cycle is a fundamental requirement of cell proliferation and long-term survival.

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The two-state model for cell cycle regulation of DNA replication

Early insights into the regulation of eukaryotic DNA replication came from cell fusion experiments (Rao and Johnson 1970), which showed that union of an S-phase cell with a G1 cell accelerates the rate at which the latter enters S phase. In contrast, G2 cells are refractory to this stimulation. These results suggested that the initiation of DNA synthesis requires a positive, diffusible S-phase-promoting activity, and that G1 but not G2-phase cells are competent to respond to this signal. Building on these observations and on their own experiments in *Xenopus* egg extracts, Blow and Laskey (1988) developed the "licensing" model, in which a licensing factor that is required for replication initiation binds to chromatin in G1. At the G1/S transition, the factor is inactivated, and it cannot be replenished until cells pass through mitosis. These ideas were extended by in vivo footprinting experiments in yeast that showed that origins of replication alternate between two distinct states (Diffley et al. 1994). In G1, all origins exhibit a "prereplicative" pattern. In contrast, from the moment of initiation until passage through mitosis, the origin resides in a "postreplicative" state. Concurrently, experiments by Nurse and colleagues (Broek et al. 1991; Hayles et al. 1994) showed that cyclin-dependent kinases (CDKs) play a dual role in regulating DNA replication, being required not only to trigger replication initiation but also to limit DNA replication to a single round per cell cycle. When CDK activity is inhibited in G2 phase, origins of replication revert to the prereplicative state and reinitiation occurs (Dahmann et al. 1995; Piatti et al. 1996). Finally, the identification of the origin recognition complex (ORC), Cdc6, Cdt1, and the minichromosome maintenance (MCM) complex as licensing activities and/or key components of prereplication complexes (pre-RCs) gave molecular definition to the replication factors whose activity is controlled during the cell cycle (for review, see Bell and Dutta 2002).

These seminal experiments coalesced into an elegant model for the cell cycle regulation of DNA replication, the essence of which is that DNA replication occurs in two discrete steps, which are closely correlated with oscillations in CDK activity (see Fig. 1A; Diffley 1996). The first step occurs soon after M-CDK activity drops upon exit from mitosis, and it involves the assembly of a pre-RC at each origin via the ordered binding of at least four factors, ORC, Cdc6, Cdt1, and MCM2–7. The process of pre-RC assembly is often referred to as "licensing." The second step, replication initiation, is triggered by the increase in S-phase CDK activity (S-CDK), which occurs at the G1/S transition. When replication initiates, the pre-RC reverts to a post-replicative state due to loss of the MCM2–7 complex. Multiple initiations from the same origin do not occur because once cells pass through the G1/S transition, they cannot load new MCM2–7 complexes onto origins. In short, the cell cycle oscillates between two functional states. During the first state, which covers G1 phase and is characterized by low CDK activity, pre-RCs are assembled but cannot undergo initiation. In the second state, which spans S, G2, and M phases, high CDK activity allows replication to initiate, but it also prevents de novo pre-RC assembly. This separation of initiation into two distinct phases, only the first of which is blocked by CDK activity, and the disassembly of pre-RCs upon initiation, together ensure that no origin of DNA replication can initiate more than once per cell cycle. An important corollary to this model is that the inhibitory mechanisms, which prevent de novo MCM2–7 recruitment have no effect on the stability of existing pre-RCs. Thus, at origins that initiate DNA replication late in S phase, pre-RCs persist on chromatin for extended periods in an environment that is refractory to new pre-RC formation.

One important question that was left open by this model was whether CDK prevents rereplication by inhibiting the licensing reaction directly, or whether it functions indirectly, by specifying cell cycle position. As discussed in this review, detailed molecular mechanisms that prevent licensing have now been characterized in all major experimental systems. In yeast, this work has provided unequivocal evidence that CDKs directly inhibit origin licensing through direct phosphorylation of pre-RC components. In multicellular eukaryotes the evidence for direct CDK-mediated inhibition of pre-RC assembly is weaker, and, in contrast to yeast, the existence of CDK-independent mechanisms has been clearly established.

Step one: licensing origins of replication via pre-RC formation

All known mechanisms that restrict DNA replication to a single round per cell cycle inhibit the first phase of replication initiation, origin licensing (for review, see Bell and Dutta 2002; Blow and Dutta 2005). Licensing begins with DNA binding of the ORC, a six-subunit AAA$^+$ ATPase (Fig. 2). In budding and fission yeasts, ORC binds chromatin throughout the cell cycle, but in higher eukaryotes ORC phosphorylation during mitosis causes its transient release from chromatin (for review, see DePamphilis 2005). In *Saccharomyces cerevisiae*, ATP binding by ORC is required for the specific recognition of origin DNA sequences, and in multicellular eukaryotes, where ORC exhibits little or no sequence specificity, ATP binding is required for efficient interaction with any DNA sequence (for review, see Cvetic and Walter 2005). Chromatin-bound ORC recruits two additional proteins, Cdc6, another AAA$^+$ ATPase, and Cdt1, a coiled-coil domain protein. Together, ORC, Cdc6, and Cdt1 facilitate chromatin loading of the MCM2–7 com-

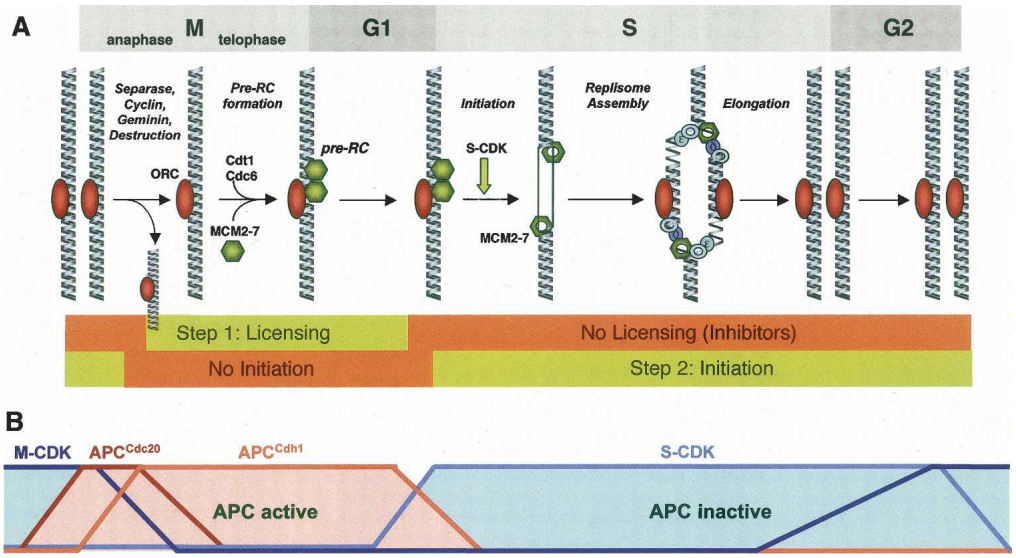


Figure 1. Two-step model for the cell cycle regulation of eukaryotic DNA replication. (*A*) The events that occur at origins of DNA replication at different stages of the cell cycle are shown. The green bars indicate when in the cell cycle licensing and initiation, respectively, are allowed. (*B*) Oscillations in APC and CDK activity during the cell cycle are indicated.

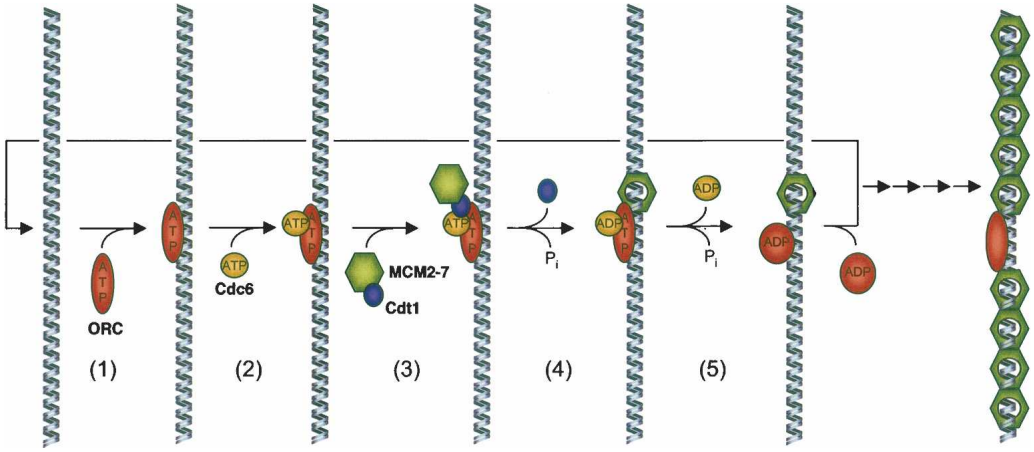


Figure 2. Model for the mechanism of pre-RC assembly in yeast. ORC:ATP binds to origin DNA (*1*) and recruits Cdc6:ATP (*2*). (*3*) ORC and Cdc6 then recruit a complex of MCM2–7 and Cdt1. (*4*) Upon ATP hydrolysis by Cdc6, MCM2–7 binds tightly to DNA, possibly by encircling the duplex. (*5*) ORC hyrdrolyzes ATP. The resulting conformational change releases MCM2–7 and presumably leads to release of the ORC complex from DNA. The cycle begins anew when a new ORC:ATP complex binds to the origin. Repetition of these five steps leads to the recruitment of multiple MCM2–7 complexes to a single origin of DNA replication.

plex, which almost certainly functions as the replicative DNA helicase (see below). Recent experiments in licensing-competent yeast extracts suggest distinct functions for the ATPase activities of ORC and Cdc6 in MCM2–7 recruitment (Bowers et al. 2004; Randell et al. 2006). Thus, when ATP hydrolysis by Cdc6 is blocked, MCM2–7 binds origin DNA but remains loosely associated. In contrast, in the absence of ATP hydrolysis by ORC, MCM2–7 is loaded and associates tightly with the origin, but the number of MCM2–7 complexes that bind to each origin is reduced. Interestingly, *S. cerevisiae* Cdt1 forms a complex with MCM2–7 in solution, but Cdt1 is only stably bound to the origin when ATP hydrolysis by Cdc6 is blocked (Tanaka and Diffley 2002; Randell et al. 2006). Together, these observations support a model in which Cdc6 associates with ORC, after which MCM2–7 is escorted to the origin via Cdt1 (Fig. 2). ATP hydrolysis by Cdc6 causes a conformational change in MCM2–7, which induces tight MCM2–7 binding to DNA and displacement of Cdt1. Finally, ATP hydrolysis by ORC inaugurates a new round of MCM2–7 loading. This cycle appears to be repeated multiple times, yielding pre-RCs containing many MCM2–7 complexes (see below). Unlike yeast Cdt1, metazoan Cdt1 does not form a stable complex with MCM2–7, but direct binding of Cdt1 to MCM2–7 is nevertheless thought to be essential for pre-RC assembly (Yanagi et al. 2002; Cook et al. 2004; Ferenbach et al. 2005).

In the last decade, a compelling body of evidence has accumulated that suggests that the MCM2–7 complex is the replicative DNA helicase. First, like most other replicative DNA helicases, the MCM2–7 complex consists of six AAA^+ ATPase subunits (Mcm2–Mcm7), which assemble into a ring-shaped hexamer. Second, recombinant Mcm4/Mcm6/Mcm7 subcomplexes from numerous eukaryotic organisms exhibit ATPase-dependent helicase activity, although they are not highly processive (for review, see Takahashi et al. 2005). Third, MCM2–7 travels with and is required for movement of the replisome (Aparicio et al. 1997; Labib et al. 2000; Pacek and Walter 2004). Fourth, ATP hydrolysis-deficient mutants of MCM2–7 assemble into pre-RCs but are specifically blocked at the origin-unwinding step (Ying and Gautier 2005). Fifth, inhibition of MCM2–7 blocks DNA unwinding at the replication fork (Pacek and Walter 2004; Shechter et al. 2004). Finally, MCM2–7 localizes to the site of unwinding under conditions when the replicative DNA helicase is uncoupled from the rest of the replisome (Pacek et al. 2006).

Interestingly, recent evidence suggests that MCM2–7 may not unwind DNA on its own. MCM2–7 has been purified in a ternary complex with two other replication initiation factors, Cdc45 and GINS (Kubota et al. 2003; Masuda et al. 2003; Gambus et al. 2006; Moyer et al. 2006), and highly purified preparations of this complex exhibit helicase activity (Moyer et al. 2006). Importantly, Cdc45 and GINS are associated with replication forks and are required for their progression (Aparicio et al. 1997; Tercero et al. 2000; Takayama et al. 2003; Pacek and Walter 2004; Pacek et al. 2006). Moreover, inhibition of Cdc45 blocks DNA unwinding at the replication fork in *Xenopus* egg extracts, and both Cdc45 and GINS associate with the uncoupled DNA helicase (Pacek and Walter 2004; Pacek et al. 2006). Because Cdc45 and GINS lack ATPase motifs, the data collectively suggest that MCM2–7 is the engine that stimulates DNA unwinding, while Cdc45 and GINS play auxiliary functions.

Considering that MCM2–7 likely functions as the replicative DNA helicase, it is puzzling that in most organisms, between five and 40 MCM2–7 complexes bind to each origin of DNA replication (Fig. 2; Randell et al. 2006; for review see Takahashi et al. 2005). In *Xenopus* egg extracts, these multiple MCM2–7 complexes are widely distributed on DNA, and they all appear to be functional (Edwards et al. 2002; Harvey and Newport 2003; Woodward et al. 2006). However, only a small sub-

set of the chromatin-bound complexes is normally required to support efficient DNA replication in this system (Mahbubani et al. 1997; Edwards et al. 2002; Woodward et al. 2006). In yeast, mutations in ORC that limit the number of MCM2–7 complexes loaded onto each origin of replication are lethal (Randell et al. 2006), but the reason underlying lethality is unknown. Presently, it is unclear why so many MCM2–7 complexes are loaded onto origins, although several models have been proposed that suggest that the "latent" MCM2–7 complexes are activated during replicative crises (Edwards et al. 2002; Hyrien et al. 2003; Woodward et al. 2006). Whatever their function, all the MCM2–7 complexes associated with chromatin must be removed during the first round of DNA replication to prevent reinitiation. Most likely, passage of the DNA replication fork displaces latent MCM2–7 complexes (Brewer and Fangman 1993; Santocanale et al. 1999).

The dynamics of pre-RCs are highly relevant for the cell cycle regulation of DNA replication. MCM2–7 complexes bind very stably to DNA, being resistant to extraction by high salt (Donovan et al. 1997; Rowles et al. 1999; Edwards et al. 2002; Bowers et al. 2004), consistent with the observation that MCM2–7 complexes persist at origins for extended periods of the cell cycle, especially at late origins. The molecular basis for MCM2–7's tight grip on DNA is not understood, although it has been proposed that MCM2–7 may encircle double-stranded DNA within pre-RCs (Mendez and Stillman 2003; Takahashi et al. 2005). This idea is based in part on the apparent similarity between pre-RC assembly and the deposition of polymerase processivity factors onto DNA by clamp loaders (Perkins and Diffley 1998; Randell et al. 2006). Importantly, after MCM2–7 is loaded, ORC, Cdc6, and Cdt1 are no longer required for initiation of DNA replication, indicating that their primary function in DNA replication is to deliver MCM2–7 to origins (Muzi Falconi et al. 1996; Hua and Newport 1998; Duncker et al. 1999; Rowles et al. 1999; Maiorano et al. 2000; Shimada et al. 2002). This feature of pre-RCs is crucial, because it means that ORC, Cdc6, and Cdt1 can be inactivated at the G1/S transition to prevent de novo pre-RC assembly without affecting subsequent initiation. Interestingly, it appears that binding of the MCM2–7 complex to origins of replication stimulates the dissociation of ORC, Cdc6, and Cdt1 (Rowles and Blow 1997; Harvey and Newport 2003; Randell et al. 2006). Dissociation from pre-RCs should liberate these factors for assembly of new pre-RCs elsewhere, while perhaps also rendering them more accessible for inactivation by proteolysis and other mechanisms when cells enter S phase (see below).

Step two: initiation of DNA replication and origin inactivation

Origins that are licensed during the G1 phase do not initiate DNA synthesis until they are acted on by a plethora of other initiation factors in S phase (for recent reviews, see Bell and Dutta 2002; Takeda and Dutta 2005; Walter and Araki 2006). The key event in the initiation of DNA replication is thought to be activation of MCM2–7 helicase activity, which requires assembly of the Cdc45–GINS–MCM2–7 complex. Briefly, two protein kinases, CDK and DDK (Dbf4 and Drf1-dependent kinase) cooperate with many other factors, including Mcm10 and Dpb11, to deposit Cdc45 and GINS onto the MCM2–7 complex (Sld2 and Sld3, two proteins implicated in this process in yeast, remain to be clearly identified in metazoans). Recent evidence supports the longstanding idea that DDK functions by directly phosphorylating the MCM2–7 complex, an event believed to facilitate its interaction with Cdc45 (Masai et al. 2006; Sheu and Stillman 2006; Tsuji et al. 2006). While two CDK substrates, Sld2 and Sld3, have been identified in yeast (Masumoto et al. 2002; Tanaka et al. 2007; Zegerman and Diffley 2007), the targets of this protein kinase are still unknown in metazoans. Once the origin is unwound, DNA polymerase α/primase is recruited, synthesizing an ~10 nucleotide RNA primer, which it further extends with ~30 deoxynucleotides. The RNA–DNA primer is recognized by replication factor C (RFC), which displaces DNA polymerase α/primase and in turn recruits PCNA, a ring-shaped trimer that encircles DNA and functions as the processivity factor for DNA polymerase δ (Maga and Hubscher 2003).

The two-step, cell cycle regulation of DNA replication can now be described from the perspective of the replicative DNA helicase. In the G1 phase, origins of DNA replication are rendered competent when the core of the helicase, the MCM2–7 complex, is assembled into pre-RCs. In S phase, helicase assembly is completed with the recruitment of Cdc45 and GINS. During initiation, the pre-RC is dismantled when the helicase moves away from the origin. Critically, new MCM2–7 complexes cannot be recruited once cells enter S phase, so helicase reassembly, which would be required for reinitiation, is not possible. In contrast, the subsequent steps, Cdc45 and GINS recruitment, are promoted in S phase. Indeed, transient destruction of MCM2–7 in mid-S phase causes permanent cell cycle arrest, whereas after transient elimination of Cdc45, DNA replication resumes (Labib et al. 2000; Tercero et al. 2000). By targeting the replicative DNA helicase, cells block rereplication at the earliest enzymatic step of chromosome duplication, thereby minimizing the possibility of genomic instability.

Anaphase-promoting complex (APC): the master regulator of licensing

The cell cycle regulation of DNA replication is inextricably connected to the cell cycle engine, whose major feature is the periodic oscillation of CDK activity (Fig. 1B; for review, see Morgan 2007). CDKs consist of a catalytic subunit that is activated upon binding to a cyclin subunit, which also confers substrate specificity. Cyclins that control cell proliferation can be grouped into four categories: the G1 cyclins, the G1/S cyclins, the S-phase cyclins, and the M-phase cyclins. With few ex-

ceptions, only the S- and M-phase CDKs inhibit pre-RC formation, so it is important to consider how their abundance is regulated. The exit from mitosis, which is marked by destruction of M-CDK activity, is stimulated by a multisubunit E3 ubiquitin ligase called the APC. M-CDKs promote their own destruction by phosphorylating a version of the APC that contains the activator protein Cdc20 (APC^{Cdc20}). APC^{Cdc20} then targets Cyclins A and B for destruction. Importantly, most eukaryotic cells also express, APC^{Cdh1}, whose activator subunit Cdh1 is inhibited by M-CDK activity. Thus, when M-phase cyclins are destroyed, and APC^{Cdc20} becomes inactive, APC^{Cdh1} steps in. Like APC^{Cdc20}, APC^{Cdh1} destroys M- and S-phase-specific cyclins, and stabilizes CDK inhibitors (CKIs). APC^{Cdh1} thereby perpetuates a G1 state that is characterized by low S- and M-CDK activity. The G1 phase ends when growth signals activate expression of G1/S cyclins. G1/S-CDKs, which are not substrates of APC^{Cdh1}, phosphorylate and switch off APC^{Cdh1}. APC^{Cdh1} activity is also blocked in S and G2 phase by Emi1. Subsequently, S- and M-phase cyclins can reaccumulate and CKIs are destroyed. In yeast, activation of APC at the end of mitosis sets the stage for origin licensing by establishing a window of low S/M-CDK activity. In higher eukaryotes, the APC additionally targets the licensing inhibitor Geminin (see below). In summary, the APC is a master regulator whose activity determines whether cells are in a state that is permissive or restrictive for pre-RC formation.

Rereplication, failed mitosis, endoreduplication, and gene amplification

The ploidy of eukaryotic cells can change for a variety of reasons. First, during rereplication, replication origins initiate DNA synthesis more than once but there is no coordination between reinitiation events, and thus, the resulting increase in ploidy is usually partial. Second, if mitosis fails due to a defect in cytokinesis, cyclins are still destroyed by the APC, allowing pre-RC reassembly and progression into a new cell cycle. Mitotic failure causes a doubling of DNA content. Rereplication and mitotic failure are generally not programmed events, but rather result spontaneously from defects in the cell cycle machinery. Third, cells can undergo endoreduplication, which usually involves consecutive and complete S phases that are not separated by mitosis. Endocycles occur in many metazoans during normal development. Fourth, during gene amplification, specific segments of the chromosome undergo repeated initiation events, leading to increased copy number of particular loci. We collectively refer to these four phenomena as "overreplication." This review focuses primarily on the mechanisms that prevent rereplication.

Redundancy and the regulation of origin firing

It is generally assumed (but not proven) that reinitiation from even a single origin of DNA replication is undesirable and possibly fatal. Considering that some eukaryotic cells initiate DNA replication at up to 300,000 sites during a single S phase, it follows that, in some cases, cells must reduce the incidence of origin refiring to <0.0003%. This requirement for virtually absolute repression of rereplication is probably why cells have developed multiple mechanisms to inhibit pre-RC assembly. The presence of multiple inhibitory mechanisms raises conceptual questions concerning redundancy that are intertwined with the details of experimental methods. Thus, if at least two separate mechanisms must be disrupted to detect rereplication in a particular system, and the limit of detection is 1% of the genome rereplicated, then it follows that each individual mechanism is by itself >99% efficient. When acting together, these two mechanisms would achieve at least 99.99% repression. In this scenario, the mechanisms are overlapping, and they should not be considered functionally redundant unless each alone is sufficient to maintain genome stability over many generations. In some instances, inhibitory mechanisms have been characterized that are not essential for viability. Such mechanisms are considered dispensable. As discussed below, cells probably achieve the desired level of origin repression by employing multiple overlapping inhibitory mechanisms whose combined, multiplicative effect is adequate to support viability.

Strategies to prevent rereplication in different eukaryotes

Using defined manipulations, rereplication can now be induced in most major systems used to study eukaryotic DNA replication. As described in the sections below, these experiments indicate that all eukaryotes employ multiple mechanisms to prevent pre-RC formation, but the number, nature, and interplay of these mechanisms varies substantially between organisms.

S. cerevisiae

Budding yeast initiates DNA replication at some 400 origins of DNA replication in every S phase. This organism is unique in that every known inhibitory mechanism is dependent on CDK activity (see Fig. 3). These mechanisms target each of the pre-RC components, sometimes in multiple ways. Cdc6 is inhibited at three levels. First, phosphorylation of Cdc6 marks it for ubiquitylation by the E3 ligase SCF^{Cdc4}, leading to proteasome-mediated destruction (Drury et al. 1997). Second, CDK inhibits Cdc6 transcription by blocking the nuclear import of the transcription factor, Swi5 (Moll et al. 1991). Finally, phosphorylation of Cdc6 at N-terminal CDK sites induces stable association with the mitotic CDK, Clb2–Cdc28, which blocks the licensing activity of Cdc6 (Mimura et al. 2004). The MCM2–7 complex is exported from the nucleus under the control of CDK phosphorylation (Labib et al. 1999; Nguyen et al. 2000; Liku et al. 2005), which also localizes Cdt1 to the cytoplasm during

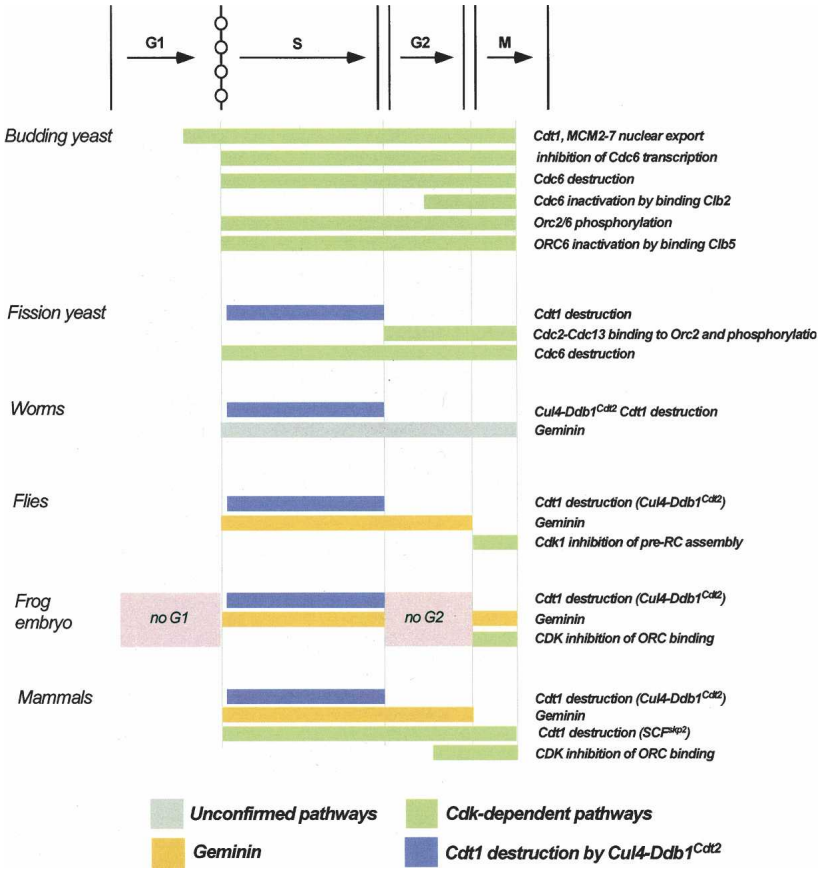


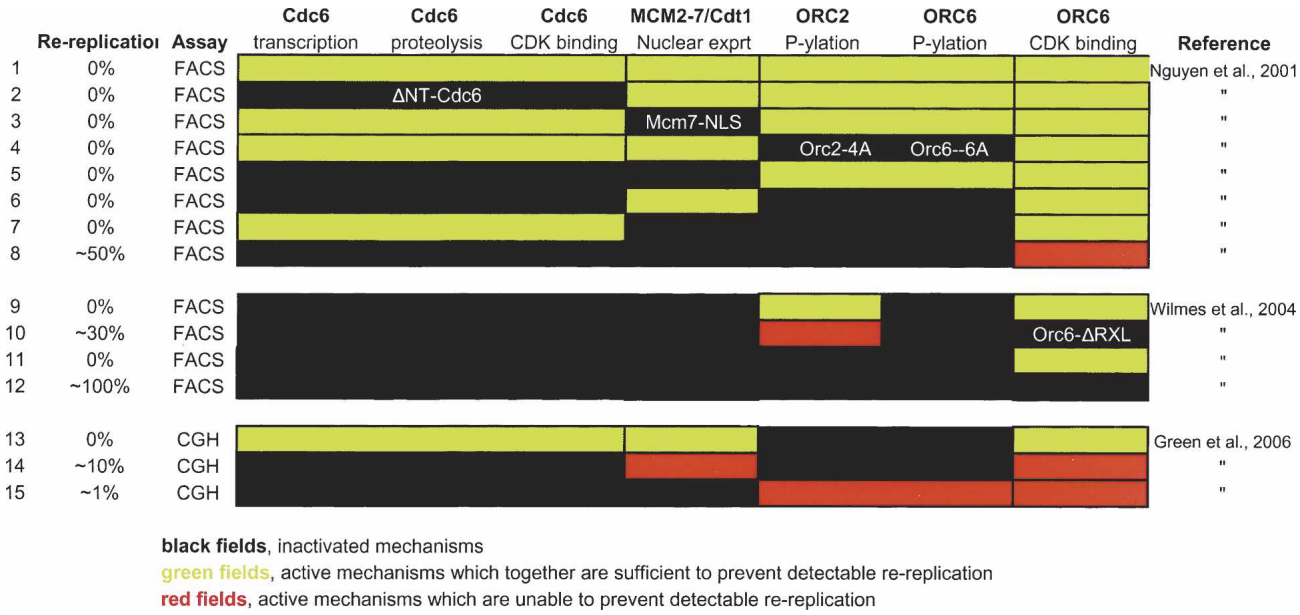
Figure 3. Species-specific pathways that prevent rereplication. The *top* of the figure shows the different stages of the eukaryotic cell cycle. For each species, each regulatory mechanism and the time in the cell cycle when it is active is represented by a horizontal bar. (Gray) Unconfirmed pathways; (green) pathways that are directly regulated by CDK activity; (blue) PCNA-dependent Cdt1 proteolysis pathways; (yellow) Geminin.

S phase, due to its association with MCM2–7 (Tanaka and Diffley 2002). ORC is inhibited by CDK phosphorylation of Orc2 and Orc6 (Nguyen et al. 2001). In addition, the S-phase cyclin Clb5 binds directly to an RXL motif in Orc6, but only after origins have fired. This interaction helps to inhibit the ability of ORC to assemble new pre-RCs (Weinberg et al. 1990; Wilmes et al. 2004). Given that Orc6–RXL mutations further increase rereplication in strains containing unphosphorylatable Orc2 and Orc6, it seems that binding of Clb5 to Orc6 is itself inhibitory to ORC activity, perhaps by creating a steric barrier that blocks binding of Cdc6 and/or Cdt1, or by locally increasing CDK activity. In summary, budding yeast cells appear to contain at least six independent, CDK-based strategies to prevent pre-RC formation during the S, G2, and M phases.

In a seminal paper, different combinations of these inhibitory mechanisms were inhibited to explore the effects on DNA rereplication (Table 1; Nguyen et al. 2001). An N-terminal deletion of Cdc6 was used to eliminate the three known mechanisms that inactivate Cdc6. Fusion of nuclear localization signals onto MCM2–7 short-circuited export of the MCM2–7/Cdt1 complex from the nucleus. Finally, mutation of CDK phosphorylation sites in Orc2 and Orc6 was used to attenuate negative regulation of ORC. Fluorescence-activated cell sorting (FACS) showed that simultaneous deregulation of ORC, Cdc6, and MCM2–7/Cdt1 was needed to induce significant rereplication (see Table 1, rows 1–8; Nguyen et al. 2001; Vas et al. 2001). In other words, any single mechanism was sufficient to block detectable rereplication. The paper concluded that rereplication is prevented by overlapping mechanisms, but has often also been interpreted as evidence that three redundant mechanisms block rereplication. In a later study, a mutation in the RXL motif in Orc6 was combined with the other three mutations, and was found to further sensitize cells to rereplication, supporting an important role for Clb5 binding to Orc6 (Table 1, rows 10 and 12; Wilmes et al. 2004).

More recently, microarray comparative genomic hybridization (CGH) has been used to measure rereplication in these strains (Green et al. 2006; Tanny et al. 2006). Using this more sensitive assay, deregulation of only two pre-RC components (ORC and Cdc6) was now sufficient to cause rereplication on most chromosomes (Table 1, row 14). Thus, the remaining inhibitory mechanisms, MCM2–7/Cdt1 export and the binding of Clb5 to Orc6, are in fact not sufficient to prevent rereplication. Analogously, when MCM2–7/Cdt1 and Cdc6 were deregulated, rereplication occurred, but it was only detectable at a single origin of replication (Table 1, row 15). While this latter result shows that the combined mechanisms targeting ORC are quite potent in preventing the vast majority of rereplication, they are not sufficient for complete inhibition. The CGH data amend the earlier FACS results to show that no single inhibitory mecha-

Table 1. *Conditions that induce rereplication in* S. cerevisiae



nism is by itself sufficient to block rereplication, suggesting that the control of rereplication is not redundant (Green et al. 2006).

The most difficult remaining question is what contribution any individual inhibitory mechanism makes in the context of the other mechanisms. Can some mechanisms be eliminated without any consequences to the cell? Although the CGH assay detects no rereplication in cells in which only the ORC complex is deregulated (Table 1, column 13), this assay is still not very sensitive, since a positive result requires rereplication at the same locus in a significant percentage of cells. Moreover, although these cells show normal viability, standard assays cannot detect small numbers of dying cells. Thus, only when rereplication is detectable in individual cells will it become possible to address this issue. If elimination of individual inhibitory mechanisms has detectable effects in single-cell assays, it will clearly demonstrate that each inhibitory mechanism makes measurable contributions toward the prevention of rereplication, even when many other mechanisms are operative. Such a scenario would explain how multiple inhibitory mechanisms are maintained during evolution.

Insulating licensing and initiation in S. cerevisiae

With respect to regulating DNA replication, the most challenging periods of the cell cycle occur at the transition points between low and high CDK activity, when origin firing and pre-RC formation could potentially overlap (for review, see Diffley 2004). Thus, at the M/G1 transition, when CDK activity drops, it is crucial to insure that conditions that allow origin firing decline before pre-RCs are allowed to reassemble (Fig. 1A). This "insulation" is achieved in yeast through a temporally ordered cascade of protein degradation. At the metaphase-to-anaphase transition, APC^{Cdc20} ubiquitylates Clb5 and presumably Dbf4, eliminating the ability of cells to trigger replication initiation (Oshiro et al. 1999; Shirayama et al. 1999). Under these conditions, Clb2–Cdc28, which is poor at stimulating initiation, is still present and prevents pre-RC formation (Donaldson 2000). Subsequently, APC^{Cdh1} triggers Clb2 destruction, allowing pre-RC formation. Thus, initiation becomes unfavorable before pre-RC assembly begins. Conversely, at the G1/S transition, it is crucial that the period of origin licensing ends before CDK activity rises, lest some origins initiate twice. To achieve this, G1 CDKs, which are not able to promote replication initiation, inhibit pre-RC formation via at least two mechanisms: Cdc6 proteolysis and MCM2–7 nuclear export (Labib et al. 1999; Drury et al. 2000; Perkins et al. 2001). Other organisms presumably also insulate the initiation and licensing periods from one another, and some details of how this might occur are discussed below.

In summary, all known mechanisms in budding yeast that prevent rereplication are CDK-dependent, and these various mechanisms are thought to combine multiplicatively to create a virtually insurmountable barrier to rereplication (Fig. 3).

Schizosaccharoymces pombe

Like budding yeast, fission yeast replicates its genome from several hundred origins of replication spaced tens of kilobase pairs apart. *S. pombe* contains a single Cdk, called Cdc2. Cig1, Cig2, and Puc1 are S-phase cyclins, whereas Cdc13 is the mitotic cyclin. Rum1 inhibits the

activity of Cdc2–Cdc13 (Correa-Bordes and Nurse 1995). As discussed above, the supreme role of CDKs in preventing rereplication was first discovered in fission yeast, where mutations in Cdc13, transient inhibition of Cdc2, or overexpression of Rum1 caused endocycles: multiple, discrete rounds of DNA replication with periodic origin licensing in the absence of mitosis. These observations suggest that in the absence of Cdc2–Cdc13, another Cdc2-dependent kinase stimulates DNA replication, and that this CDK undergoes periodic oscillations to allow repeated rounds of licensing and DNA replication. Cdc2–Cig2 is a good candidate because Cig2 overexpression in cells lacking Cdc13 prevents endoreduplication (Lopez Girona et al. 1998).

Early efforts to identify the targets of CDK inhibition focused on Cdc18, the fission yeast ortholog of Cdc6. As seen in budding yeast, Cdc18 is phosphorylated by Cdc2 in S phase, triggering ubiquitylation by the SCF^{Pop1} ligase and destruction by the proteasome (Jallepalli et al. 1997; Kominami and Toda 1997). Initially, it was thought that Cdc18 might be the only target of CDK-dependent inhibition because high level Cdc18 overexpression, in particular alleles containing mutated CDK phosphorylation sites, causes rereplication (Nishitani and Nurse 1995; Jallepalli et al. 1997). However, a physiological level of nondegradable Cdc18 expression has no detectable effects on DNA replication in FACS assays, suggesting that other inhibitory mechanisms to prevent rereplication do exist (Nishitani and Nurse 1995; Muzi Falconi et al. 1996).

Like Cdc18, *S. pombe* Cdt1 is also regulated by proteolysis in the S and G2 phases (Nishitani et al. 2000; Gopalakrishnan et al. 2001). Cdt1 proteolysis requires a novel E3 ubiquitin ligase called $Cul4–Ddb1^{Cdt2}$ (Hu and Xiong 2006; Ralph et al. 2006). Interestingly, there is so far no evidence that this pathway is directly regulated by CDK, and experiments in other organisms show it is coupled to DNA replication (see below). Constitutive expression of Cdt1 alone is not sufficient to induce detectable rereplication, but when Cdt1 overexpression is combined with expression of nonphosphorylatable Cdc18, rereplication occurs, as seen by a gradual increase in ploidy (Nishitani et al. 2000; Gopalakrishnan et al. 2001; Yanow et al. 2001). Therefore, destruction of Cdt1 and Cdc18 represent overlapping mechanisms to prevent rereplication.

In addition to promoting Cdc18 destruction, Cdc2–Cdc13 inhibits pre-RC assembly by binding to the fission yeast Orc2 (called Orp2), at origins of replication (Wuarin et al. 2002). This mechanism is thought to locally antagonize pre-RC assembly either via Cdc18 and/or Orp2 phosphorylation (Vas et al. 2001) or by creating a steric barrier to pre-RC assembly. In either case, localization of Cdc2–Cdc13 to origins is specific to G2 and M phase, and origin localization of Cdc2 is not conferred by S-phase cyclins. Interestingly, ablation of Cdc2–Cdc13 binding to Orp2 does not cause rereplication, but *endoreduplication* (Wuarin et al. 2002). Thus, association of Cdc13–Cdc2 with Orp2 is not essential to prevent origin refiring within S phase. Rather, this mechanism ensures that within G2 and M phase, further rounds of pre-RC assembly are blocked until the cell has entered anaphase.

In summary, *S. pombe* uses at least two strategies to prevent replication within S phase (Cdt1 and Cdc18 proteolysis), as well as a third mechanism to prevent relicensing in G2 and M (ORC inactivation by Cdc2–Cdc13) (Fig. 3). Since MCM2–7 is constitutively nuclear, *S. pombe* may not inhibit this pre-RC component.

X. laevis

The early embryonic cells cycles of the frog *X. laevis* take ~30 min and consist of alternating S and M phases without intervening gap phases (for review, see Blow 2001). DNA replication initiates in a sequence-independent fashion at ~300,000 origins spaced ~10 kb apart. Replication initiation is dependent on Cdk2–Cyclin E, mitotic entry is driven by Cdk1–Cyclin B, and mitotic exit is promoted by APC^{Cdc20}. Two unique features of these cell cycles are noteworthy. First, these cells do not express APC^{Cdh1}, explaining the absence of a G1 period (Lorca et al. 1998). Second, the activity of key cell cycle regulators fluctuates largely as a result of subcellular localization rather than expression level. Thus, Cdk2–Cyclin E levels are constant throughout the cell cycle (Hua et al. 1997). However, in mitosis, when there is no nuclear envelope, the Cdk2–Cyclin E concentration surrounding chromatin is low. Starting at telophase, Cdk2–Cyclin E is imported into nuclei, causing a dramatic rise in its nuclear concentration that triggers initiation. Importantly, extracts prepared from unfertilized *Xenopus* eggs undergo precisely one round of DNA replication per in vitro cell cycle, making this a powerful tool to understand the regulation of DNA replication.

Geminin

Geminin was initially discovered in *Xenopus* egg extracts in a screen for APC^{Cdc20} substrates, and it was immediately recognized as an inhibitor of MCM2–7 loading (McGarry and Kirschner 1998). Present in all metazoans but apparently not in yeast, Geminin inhibits pre-RC assembly by sequestering Cdt1 in an inactive complex that is unable to interact with or recruit MCM2–7 (Wohlschlegel et al. 2000; Tada et al. 2001; Cook et al. 2004; Lee et al. 2004; Ferenbach et al. 2005; Lutzmann et al. 2006). Interestingly, Geminin does not inhibit the interaction of Cdt1 with origins of DNA replication (Gillespie et al. 2001). Crystal structures and mutational analyses reveal that Geminin assembles into a homodimer with a central coiled-coil domain that interacts extensively with Cdt1, and thereby probably creates a steric barrier that prevents association of Cdt1 with MCM2–7 (Lee et al. 2004; Saxena et al. 2004).

In *Xenopus* egg extracts, Geminin is inactivated by APC^{Cdc20} at the metaphase–anaphase transition (McGarry and Kirschner 1998). Together with the destruction of mitotic cyclins, this event inaugurates the licensing period. Interestingly, only about half the endogenous

Geminin is degraded in anaphase; the remainder is inactivated via an unknown mechanism that involves Geminin ubiquitylation (Li and Blow 2004). Geminin is reactivated when licensing ends in telophase, indicating that the licensing period during these cell cycles lasts only a few minutes. The mechanism of reactivation is not understood, but it is dependent on nuclear import of Geminin, after which the binding of Geminin to Cdt1 increases dramatically (Hodgson et al. 2002; Arias and Walter 2005; Lutzmann et al. 2006).

The properties of the Geminin that persists during the licensing period are controversial. The conventional view is that inactivated Geminin is unable to bind Cdt1 and therefore does not prevent licensing (Hodgson et al. 2002). However, a recent study suggests that Geminin and Cdt1 form a complex that is competent for licensing (Lutzmann et al. 2006). In this view, Geminin only inhibits Cdt1 activity when a critical Geminin:Cdt1 ratio is achieved, which occurs after Geminin is reactivated in telophase. The fact that *Xenopus* Geminin is regulated post-translationally is probably crucial to allow it to switch rapidly between functional states, thereby accommodating the rapid embryonic cell cycles. It is unclear whether somatic cells contain similar populations of Geminin that are unable to inhibit the function of Cdt1.

The ability of Geminin to inhibit Cdt1 is apparently independent of CDK status (Ballabeni et al. 2004; Sugimoto et al. 2004; Li and Blow 2005). As such, the inhibition of Cdt1 by Geminin represents the first known CDK-independent means to prevent pre-RC assembly. Notably, immunodepletion of Geminin from *Xenopus* egg extracts was reported to induce no detectable rereplication (McGarry and Kirschner 1998), suggesting the existence of additional mechanisms to prevent rereplication (see next section).

PCNA-dependent and Cul4–Ddb1^{Cdt2}-dependent Cdt1 proteolysis

Cdt1 is destroyed in the S phase of all metazoan cells (Nishitani et al. 2001; Zhong et al. 2003; Thomer et al. 2004; Arias and Walter 2005), and it has recently emerged that its proteolysis is intimately linked to DNA replication (Fig. 4A). Thus, in *Xenopus* egg extracts, it was first discovered that Cdt1 is ubiquitylated on chromatin in a manner that depends on the initiation of DNA replication (Arias and Walter 2005). Subsequent analysis showed that Cdt1 destruction depends on its binding to PCNA (Arias and Walter 2006). *Xenopus* Cdt1 contains a PCNA-interacting protein (PIP) box at its N terminus (shown as a red box in Fig. 4A). Mutation of the PIP box abolishes Cdt1 binding to PCNA, its ubiquitylation on chromatin, and its destruction in S phase. Interestingly, PCNA-dependent Cdt1 ubiquitylation occurs exclusively on chromatin. Therefore, it appears that Cdt1 can only interact with chromatin-bound PCNA, a feature of the proteolysis mechanism that prevents Cdt1 destruction in G1 phase. Because it participates in the synthesis of every Okazaki fragment, PCNA accumulates to high levels on chromatin during S phase (Arias and Walter 2006), making it a well-suited trigger for rapid Cdt1 destruction. Importantly, all metazoan organisms contain a PIP box at the extreme N terminus of Cdt1 (Arias and Walter 2006; Senga et al. 2006), and current evidence indicates that PCNA-dependent Cdt1 destruction is highly conserved among these organisms (see below).

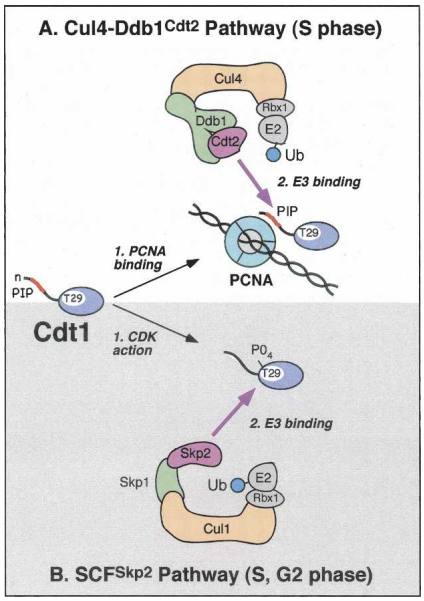


Figure 4. Cdt1 proteolysis pathways. The two major pathways that target Cdt1 for destruction are shown. The PIP box of Cdt1 is indicated in red. (*A*) Cul4–Ddb1^{Cdt2}-dependent Cdt1 destruction occurs in two steps. First, Cdt1 docks onto PCNA at DNA replication forks or sites of DNA damage. Second, Cdt2 interacts with the complex of Cdt1 and PCNA and ubiquitylates Cdt1. (*B*) SCF^{Skp2}-dependent Cdt1 destruction also occurs in two steps. First, CDK phosphorylates Thr 29 (in humans). Second SCF^{Skp2} binds to the phoshporylated T29 via Skp2 and ubiquitin tranfer occurs.

The E3 ubiquitin ligase that stimulates replication-dependent Cdt1 destruction in *Xenopus* egg extracts is a canonical cullin-based complex, Cul4–Ddb1^{Cdt2} (Fig. 4A; Arias and Walter 2006; Jin et al. 2006). Cul4 functions as a scaffold whose N terminus is bound by the adaptor Ddb1 and whose C terminus associates with an E2 ubiquitin-conjugating enzyme. The Ddb1 adaptor, in turn, interacts with the WD40 repeat protein, Cdt2, which probably binds directly to Cdt1. As expected from the ubiquitylation of Cdt1 on chromatin, Cul4–Ddb1^{Cdt2} is also found on chromatin in S phase. Its binding to chromatin is dependent on initiation of DNA replication, and it requires the interaction of Cdt1 with PCNA. Thus, one model for PCNA-dependent Cdt1 destruction envisions that Cdt1 docks onto PCNA via its PIP box, thereby creating a binding site for Cul4–Ddb1^{Cdt2} (Fig. 4A). It has also been reported that Cdt1 is destroyed upon mitotic exit by the APC, but the function of this pathway in regulating replication is presently unclear (Li and Blow 2005).

What is the division of labor between Geminin and Cdt1 destruction in preventing rereplication? Blocking proteolysis of Cdt1 by itself yields no detectable rereplication (Arias and Walter 2005, 2006; Li and Blow 2005; Maiorano et al. 2005; Yoshida et al. 2005). Similarly, in the absence of Geminin, rereplication is not detectable (McGarry 2002; Arias and Walter 2005) or very inefficient (Li and Blow 2005; Yoshida et al. 2005; Kerns et al. 2006). However, when both mechanisms are neutralized, substantial rereplication occurs (Li and Blow 2005; Maiorano et al. 2005; Yoshida et al. 2005; Arias and Walter 2006). Therefore, it appears that Geminin and Cul4–Ddb1^{Cdt2}-dependent Cdt1 destruction are each sufficient to prevent the large majority of rereplication in interphase egg extracts.

CDKs and rereplication in Xenopus

Geminin and replication-dependent Cdt1 destruction both appear to be CDK-independent mechanisms to prevent rereplication, raising the question of whether CDK phosphorylation plays any role in blocking rereplication in *Xenopus* egg extracts. It was reported that recombinant human Cdk2–Cyclin E inhibits pre-RC formation when added at high concentrations to *Xenopus* egg extracts (Hua et al. 1997). However, simultaneous removal of Geminin and stabilization of Cdt1 induces rereplication in the presence of high nuclear concentrations of endogenous Cdk2–Cyclin E (see above), calling into question an inhibitory role for this kinase. The situation for Cdk1 is different. In metaphase-arrested *Xenopus* egg extracts, Cdk1 is nearly as potent as Geminin in preventing licensing (Tada et al. 2001). It appears to exert this effect by inhibiting the binding of the ORC complex to DNA (Mahbubani et al. 1997; Findeisen et al. 1999).

Insulating the licensing and initiation periods in Xenopus *egg extracts*

To prevent reinitiation during mitotic exit, cells must curtail initiation *before* enabling licensing. Importantly, new initiations cease well before mitotic exit, as soon as the nuclear envelope breaks down, because the concentration of replication factors such as Cdk2–Cyclin E around chromatin collapses (Arias and Walter 2004). Therefore, when the licensing period is subsequently inaugurated by APC^{Cdc20} in anaphase, there is no danger of reinitiation. Conversely, at the onset of DNA replication, which occurs after telophase, licensing must be terminated before initiation commences. We speculate that Geminin is imported and reactivated faster than Cdk2–Cyclin E and other initiation factors. Importantly, the only other known inhibitory mechanism that operates in S phase, Cdt1 destruction, commences only after replication has initiated, and therefore cannot contribute to insulation. The low amount of rereplication sometimes observed in the absence of Geminin (Li and Blow 2005; Yoshida et al. 2005; Kerns et al. 2006) may reflect a low level of reinitiation events that occur in early S phase before all the Cdt1 has been destroyed.

In summary, early frog embryos use Geminin, Cdt1 destruction, and Cdk1 activity to prevent rereplication (Fig. 3). Unlike Geminin, which is active from the start of S phase until anaphase, Cdt1 destruction can only begin after replication forks have been established, and it may cease well before anaphase, if PCNA is unloaded rapidly upon completion of DNA replication. Finally, Cdk1-mediated inhibition is expected to commence sometime in G2, perhaps before Cdt1 destruction subsides. In this view, there is one window of time, very early in S phase, in which only one mechanism (Geminin) is known to operate. Perhaps additional, unknown mechanisms exist that help add an extra layer of control at this apparently vulnerable time in the *Xenopus* cell cycle.

Mammals

In mammalian cells, DNA replication initiates from some 25,000 origins spaced ~100 kb apart. Replication initiation is promoted by Cdk2–Cyclin A, whereas mitosis depends on Cdk1–Cyclin A and Cdk1–Cyclin B. As seen in the early embryonic cell cycles of *Xenopus*, mammalian cells employ Geminin, Cul4–Ddb1^{Cdt2}-dependent Cdt1 destruction, and Cdk1-mediated inhibition of ORC loading to prevent rereplication (Fig. 3). However, they also engage a second Cdt1 proteolysis pathway that requires SCF^{Skp2}. In addition, several inhibitory mechanisms involving Cdc6 and Orc1 have been postulated.

Cdt1 destruction in S phase

Before the PCNA-dependent Cdt1 destruction pathway was discovered, there was evidence in mammalian cells that Cdk2 targets Cdt1 for destruction via the SCF^{Skp2} E3 ubiquitin ligase. Thus, Cdt1 was found to coimmunoprecipitate with Cdk2–Cyclin A, and phosphorylation of Cdt1 on Thr 29 was shown to be essential for its binding to the F-box protein, Skp2, which serves as the substrate receptor for SCF^{Skp2} (see Fig. 4B; Li et al. 2003; Liu et al. 2004; Sugimoto et al. 2004; Takeda et al. 2005). At the time, it was surprising that mutations in human Cdt1 that abolish its interaction with Skp2 had little or no effect on Cdt1 stability in S phase (Takeda et al. 2005; Nishitani et al. 2006; Senga et al. 2006), and that *Skp2*$^{-/-}$ mouse embryonic fibroblasts show no increase in Cdt1 levels (Nishitani et al. 2006). This paradox was resolved when it was shown that human Cdt1 is also destroyed by the PCNA-dependent pathway (Nishitani et al. 2006; Senga et al. 2006). Thus, when the SCF^{Skp2} pathway is neutralized, mutation of Cdt1's PIP box or siRNA against PCNA, Cul4, or Ddb1 all stabilize Cdt1 in S phase. In addition, indirect evidence now indicates that Cdt2 is also required for Cdt1 destruction in S phase (Jin et al. 2006; Sansam et al. 2006).

What is the relative importance of the SCF^{Skp2} and Cul4–Ddb1^{Cdt2} Cdt1 destruction pathways? In mammals, the two may overlap in S phase, since both path-

ways must be neutralized to detect maximum Cdt1 accumulation (Nishitani et al. 2006; Senga et al. 2006). However, other results show that silencing of Cdt2 or Ddb1 is sufficient to induce Cdt1-dependent rereplication, whereas silencing of Skp2 has little or no effect (Jin et al. 2006; Lovejoy et al. 2006; Sansam et al. 2006), suggesting that the Cul4–Ddb1^{Cdt2} pathway may predominate in S phase (see also below). In G2, the converse appears to be true: The SCF^{Skp2} pathway remains active due to the continued presence of CDK activity, whereas the PCNA pathway subsides, presumably because PCNA has been unloaded from chromatin by this time (Nishitani et al. 2006). In other metazoans, the Cul4–Ddb1^{Cdt2}-dependent Cdt1 destruction pathway appears to predominate. Thus, mutation of the PIP box in *Xenopus* Cdt1, inhibition of DNA replication in flies, elimination of Cul4 in worms, and mutation of Cdt2 in zebrafish are all sufficient to stabilize Cdt1 and/or to induce rereplication (Zhong et al. 2003; Arias and Walter 2005, 2006; May et al. 2005; Sansam et al. 2006).

Cdt1 destruction after DNA damage

Experiments in human and *Drosophila* cells first showed that Cdt1 is destroyed not only in S phase, but also within minutes of exposure to DNA damage (Higa et al. 2003; Hu et al. 2004), a response that has been confirmed in fission yeast and frogs (Hu and Xiong 2006; Jin et al. 2006; Ralph et al. 2006). Like S phase Cdt1 destruction, DNA damage-induced Cdt1 destruction requires Cul4, Ddb1, Cdt2, Cdt1's PIP box, and PCNA (Higa et al. 2003, 2006; Hu et al. 2004; Hu and Xiong 2006; Jin et al. 2006; Nishitani et al. 2006; Ralph et al. 2006; Sansam et al. 2006; Senga et al. 2006), but it does not require checkpoint kinases (Higa et al. 2003; Jin et al. 2006; Ralph et al. 2006). Therefore, the requirements for Cdt1 destruction in S phase and after DNA damage are indistinguishable, and both mechanisms likely involve docking of Cdt1 and the Cul4–Ddb1^{Cdt2} ubiquitin ligase onto chromatin-bound PCNA (Fig. 4A).

It has been proposed that destruction of Cdt1 is important to delay replication when DNA damage is incurred during G1 phase (Higa et al. 2003). However, considering that Cdt1 is dispensable for replication once MCM2–7 has been loaded (Maiorano et al. 2000, 2004), which normally occurs in telophase (Mendez and Stillman 2000; Okuno et al. 2001), this appears unlikely. As an alternative explanation, we propose that damage-induced destruction of Cdt1 may be important during G2 and M phase to prevent a spurious round of rereplication after checkpoint activation. DNA damage in G2 phase triggers checkpoint pathways that block entry into mitosis via inhibition of Cdk1 activity (Bartek et al. 2004). Because mitotic CDK activity is essential to prevent pre-RC assembly during M phase (see below), down-regulation of Cdk1 by the DNA damage checkpoint could result in a new round of origin licensing. Upon checkpoint silencing, the reaccumulation of M-phase CDK activity would be predicted to trigger an additional round of origin firing (Bates et al. 1998; Prokhorova et al. 2003). Thus, damage-induced destruction of Cdt1 may be critical to prevent a second round of replication indirectly brought on by checkpoint signaling.

It is interesting to revisit the mechanism of Cdt1 destruction in *S. pombe*. Because DNA damage in *S. pombe* also stimulates Cdt1 turnover, and this process requires PCNA, Ddb1, and Cdt2 (Hu and Xiong 2006; Ralph et al. 2006), it appears that Cdt1 docking onto chromatin-bound PCNA and subsequent ubiquitylation by Cul4–Ddb1^{Cdt2} is conserved in this yeast. Although fission yeast Cdt1 does not contain a canonical PIP box [Q-x-x-(L/V/I/M)-x-x-(F/Y)-(F/Y)], its extreme N terminus contains a partial PIP box (Q-T-K-L), a motif recently shown to mediate binding of proteins to PCNA in budding yeast (Moldovan et al. 2006). A key question is now whether PCNA-dependent Cdt1 destruction contributes to the periodic expression of Cdt1 in unperturbed fission yeast cell cycles. In support of this idea, Cdt1 levels normally drop after the G1/S transition (Nishitani et al. 2000; Gopalakrishnan et al. 2001), and mutations in *S. pombe* Ddb1 or Cdt2 lead to massive Cdt1 stabilization in unperturbed cells (Hu and Xiong 2006; Ralph et al. 2006). So far, however, PCNA has not been implicated in Cdt1 turnover in unstressed cells (Hu and Xiong 2006), and this issue requires further investigation.

Geminin

Human Geminin is expressed during S, G2, and M phase, the periods of the cell cycle when licensing is prohibited (McGarry and Kirschner 1998; Wohlschlegel et al. 2000). In support of Geminin being an inhibitor of licensing in human cells, it binds to Cdt1 (Wohlschlegel et al. 2000), and its elimination in several cell lines causes substantial rereplication (Melixetian et al. 2004; Zhu et al. 2004). Geminin is a substrate of APC^{Cdc20} and APC^{Cdh1} (McGarry and Kirschner 1998; Rape et al. 2006). It is therefore destroyed together with mitotic cyclins in early anaphase, and it does not reaccumulate until late G1 when APC^{Cdh1} is inactivated. Ideally, Geminin levels should exceed Cdt1 levels in late G1, so that the licensing period terminates before replication initiates. This insulation may be achieved by SCF^{Skp2}-dependent Cdt1 destruction.

Geminin can also play a positive role in origin licensing by preventing Cdt1 proteolysis during mitosis (Ballabeni et al. 2004). Silencing of Geminin during G2/M phase causes a drastic decrease in Cdt1 levels, resulting in reduced MCM2–7 chromatin loading in the following G1 phase. The binding of Geminin to Cdt1 probably protects the latter from destruction by the SCF^{Skp2} pathway in M phase, which is consistent with the fact that in the presence of roscovotine, a Cdk1 inhibitor that should block the SCF pathway, Geminin depletion has little effect on Cdt1 stability. The destruction of Geminin in anaphase presumably liberates Cdt1, insuring that cells exit mitosis with a functional licensing apparatus (Fig. 5). This model does not, however, explain why Cdt1 is destroyed in S phase despite the presence of Geminin

(Nishitani et al. 2006; Senga et al. 2006). One possibility, illustrated in Figure 5, is that SCF^{Skp2} only targets free Cdt1, in contrast to Cul4–$Ddb1^{Cdt2}$, which eliminates free Cdt1 as well as the Geminin-bound form (Arias and Walter 2005). Thus, in S phase, Cdt1 is completely destroyed because all forms of Cdt1 are targeted. In G2, when the Cul4–$Ddb1^{Cdt2}$ pathway is switched off, Cdt1 bound to Geminin begins to accumulate, and this population peaks in mitosis, before its liberation from Geminin in anaphase. Protection of mammalian Cdt1 by Geminin is functionally analagous to Clb2–Cdc28 binding to Cdc6 in budding yeast (Mimura et al. 2004), in that both mechanisms repress pre-RC assembly in M phase yet also ensure that pre-RC components are available at the start of the next cell cycle, when the inhibitor is destroyed by the APC.

CDK

While neutralization of Cdt1 is essential to prevent rereplication during S phase, accumulating evidence indicates that the block to relicensing during G2 and M phase in mammals is critically dependent on Cdk1 activity. First, conditional inactivation of Cdk1 in cycling cells is sufficient to induce endoreduplication (Itzhaki et al. 1997). Second, and similarly, transient overexpression of the CKI p21 during G2 phase induces a complete round of rereplication (Bates et al. 1998), reminiscent of genetic experiments in budding and fission yeast (Broek et al. 1991; Dahmann et al. 1995). Third, human and murine cells arrested in G2 or mitosis recruit ORC and MCM2–7 to chromatin if CDK activity is inhibited (Coverley et al. 1996, 1998; Fujita et al. 1998; Ballabeni et al. 2004; Li et al. 2004; Sugimoto et al. 2004). Strictly speaking, these experiments do not prove a direct role for CDK in preventing pre-RC formation, since in each experiment the cell cycle may have effectively been reset to a G1-like state. Another potential caveat is that the elimination of Cdk1 activity causes activation of APC^{Cdh1} and destruction of Geminin. However, in at least one study, chemical inactivation of Cdk1 induced pre-RC formation in mitosis in the continued presence of Geminin (Ballabeni et al. 2004). It has been proposed that in hamster cells, phosphorylation of Orc1 by Cdk1/Cyclin A prevents its association with chromatin (Li et al. 2004). Moreover, mouse Cdt1 has DNA-binding activity that is blocked by CDK, but the physiological role of the DNA binding and its regulation have not been established (Yanagi et al. 2002; Sugimoto et al. 2004). To test whether Cdk1 directly regulates these pre-RC components, it will be essential to eliminate their CDK phosphorylation sites and ask whether they can bind to chromatin in mitosis.

The role of Cdk2 in preventing rereplication in mammals is controversial. On the one hand, deregulation of Cdt1 during S phase by different means is sufficient to induce rereplication (Vaziri et al. 2003; Melixetian et al. 2004; Zhu et al. 2004; Jin et al. 2006; Lovejoy et al. 2006; Sansam et al. 2006), and there is no reason to believe that Cdk2 activity was attenuated in these experiments, at least not initially, before rereplication induces DNA damage and checkpoint signaling (see below). Thus, any Cdk2-dependent inhibitory mechanisms, including SCF^{Skp2}-dependent Cdt1 destruction, are apparently not sufficient to prevent rereplication. On the contrary, expression of Cyclin A enhanced rereplication in mammalian tissue culture cells (Vaziri et al. 2003). The lack of an essential role for Cdk2 in regulating DNA replication is dramatically underscored by the observation that Cdk2 and Cyclin E knockout mice are viable (Berthet et al. 2003; Geng et al. 2003). On the other hand, a recent report suggests that Cdk1 and Cdk2 play redundant roles in preventing rereplication in Hela cells (Machida and Dutta 2007). As described above, Cdk2 is proposed to promote Cdt1 destruction via SCF^{Skp2}, although there is so far no evidence that elimination of this pathway potentiates rereplication. Finally, expression of Cyclin E in human cells results in a shortened G1 phase and a decrease in MCM2–7 bound to chromatin (Ekholm-Reed et al. 2004). However, the mechanism underlying this overexpression phenotype remains unknown. In summary, more work is needed to determine what role, if any, Cdk2 kinase plays in regulating DNA replication during the mitotic cell cycle.

Negative regulation of ORC and Cdc6

In mammals, considerable attention has focused on Orc1, the largest ORC subunit, as a target of negative regulation during the cell cycle (for review, see DePamphilis 2005). While there is good evidence that ORC is inhibited by Cdk1 activity in mitosis (Li et al. 2004), its fate during S and G2 is less clear. Some studies indicated that in S phase, Orc1 is partially destroyed via proteoly-

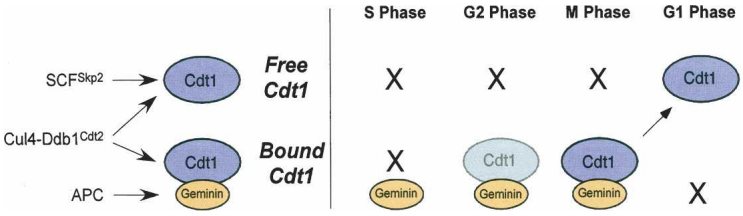


Figure 5. Model for cell cycle regulation of Cdt1 levels by Geminin and proteolysis. The *left* side of the figure shows that SCF^{Skp2} targets only free Cdt1, whereas Cul4–$Ddb1^{Cdt2}$ can destroy free Cdt1 and Geminin-bound Cdt1. The *right* side of the figure shows the abundance of Geminin and Cdt1 at different cell cycle stages. In cases where a factor is absent, it is replaced by an X. Thus, in S, G2, and M, there is no free Cdt1 due to the combined actions of SCF^{Skp2} and Cul4–$Ddb1^{Cdt2}$. In G2 and M phase, Cdt1 bound to Geminin accumulates because only Cul4–$Ddb1^{Cdt2}$ can target this form of Cdt1, and this proteolysis pathway is turned off after S phase. Upon mitotic exit, APC destroys Geminin, thereby creating a free pool of Cdt1 in G1.

sis (Mendez et al. 2002; Tatsumi et al. 2003), and that it is no longer detected at certain origins of replication (Ladenburger et al. 2002; Abdurashidova et al. 2003). Moreover, it has been reported that the interaction of Orc1 with chromatin is less tight in S and G2 than in G1 (Li and DePamphilis 2002). However, other studies conclude that the level of Orc1 and its interaction with chromatin do not fluctuate during this period (Saha et al. 1998; Okuno et al. 2001; McNairn and Gilbert 2005; McNairn et al. 2005). The reason for these differences is unclear. It is important to point out that mammalian cells, especially when transformed, contain a large functional excess of ORC (Dhar et al. 2001; McNairn and Gilbert 2005). Therefore, any mechanism of inhibition would have to eliminate the vast majority of cellular ORC to be effective. This is apparently not always the case, since mammalian G2 nuclei can replicate in ORC-depleted *Xenopus* egg extracts (Romanowski et al. 1996). A future challenge is to define and specifically disrupt mechanisms that regulate ORC function, and to determine whether cells are sensitized to rereplicate.

The role of mammalian Cdc6 in preventing rereplication is also uncertain. Initial studies in vertebrates showed that transfected Cdc6 is exported from nuclei under the control of CDK activity (Saha et al. 1998; Jiang et al. 1999; Petersen et al. 1999). However, subsequent work found that endogenous Cdc6 was bound to chromatin in S and G2 (Coverley et al. 2000; Mendez and Stillman 2000; Oehlmann et al. 2004), and that only the soluble pool of Cdc6 is exported (Alexandrow and Hamlin 2004). The observation that Cdc6 is chromatin-bound throughout S phase may also cast doubt on the functional relevance of ORC inactivation. Curiously, human Cdc6 is ubiquitylated by the APC during early G1 phase and gradually reaccumulates during S, G2, and M phases (Mendez and Stillman 2000; Petersen et al. 2000). It is unclear whether this destruction plays any role in preventing rereplication, as overexpression of nondegradable Cdc6 is not sufficient to induce rereplication (Petersen et al. 2000). Instead, APC-mediated destruction of Cdc6 may be important for maintaining low levels of Cdc6 during quiescence. Upon withdrawal from the cell cycle, mammalian cells arrest with an active APC that degrades Cdc6. When they are stimulated to re-enter the cell cycle, human Cdc6 reaccumulates despite APC activity because phosphorylation of Cdc6 by Cdk2/Cyclin E prevents its proteolysis (Mailand and Diffley 2005). Interestingly, no evidence for Cdk2/Cyclin E-dependent stabilization of Cdc6 has so far been observed in the mouse. When murine Cyclin E knockout cells are stimulated to re-enter the cell cycle from a quiescent state, Cdc6 levels accumulate with wild-type kinetics, despite the absence of protective Cyclin E phosphorylation (Geng et al. 2003). In summary, Cdc6 does not appear to be a major target of negative regulation during mitotic cell cycles, whereas it is down-regulated during quiescence.

There is currently no evidence to suggest that MCM2–7 is subject to direct negative regulation in mammals or any other metazoan. Unlike in budding yeast, MCM2–7 remains nuclear throughout S and G2 phases (Madine et al. 1995a,b; Todorov et al. 1995; Mendez and Stillman 2000). Given that Cdt1 deregulation is sufficient to promote rereplication in all metazoans examined, functional MCM2–7 complexes are probably present in the nucleus throughout the cell cycle.

Interplay between mammalian inhibitors of pre-RC assembly

As discussed above, *Xenopus* egg extracts contain several overlapping, if not redundant, mechanisms that prevent rereplication, and elimination of more than one mechanism is required to detect substantial rereplication. The situation in mammalian cells is less straightforward, since these cells contain the same mechanisms (and perhaps others), yet elimination of a single pathway is often sufficient to promote extensive rereplication.

In HeLa and MCF10A cells, Geminin siRNA does not induce detectable rereplication (Kulartz and Knippers 2004; Nishitani et al. 2004; Machida and Dutta 2007), demonstrating that Geminin is not essential to prevent the vast majority of rereplication in these cells. This implies that other mechanisms, such as Cdt1 proteolysis in S phase (by SCF^{Skp2} and $Cul4–Ddb1^{Cdt2}$) and G2 (by SCF^{Skp2}), and CDK-mediated inhibition of Cdt1 in mitosis, are highly active. Consistent with this idea, cosilencing of Geminin and Cyclin A leads to twice as much rereplication than when Cyclin A alone is inhibited, which also demonstrates that Geminin is active in these cells (Machida and Dutta 2007). A similar effect is achieved via silencing of Emi1, which leads to ectopic activation of APC^{Cdh1} and concomitant destruction of Geminin and Cyclin A (Machida and Dutta 2007). In contrast to the situation for Geminin, siRNA knockdown of Cdt2 or Ddb1 in HeLa cells is sufficient to cause significant rereplication, presumably due to relicensing in S phase (Jin et al. 2006; Lovejoy et al. 2006). In this situation, why are the SCF^{Skp2} and Geminin pathways insufficient to prevent relicensing? One possibility is that the SCF^{Skp2} pathway is, in fact, not very active in these cells; as a result, Cdt1 accumulates in S phase and eventually exceeds Geminin, allowing relicensing. A related possibility is that local accumulation of small amounts of Cdt1 induces limited rereplication, triggering DNA damage, followed by down-regulation of Cdk2, inhibition of the SCF^{Skp2} pathway, and further Cdt1 accumulation. In such an amplification loop, cells could eventually undergo massive rereplication. In summary, in Hela cells, Geminin is not essential to prevent detectable rereplication, in contrast to $Cul4–Ddb1^{Cdt2}$ and Cyclin A.

In other cell types (e.g., H1299 or HCT116 cancer cells), Geminin siRNA causes significant rereplication (Melixetian et al. 2004; Zhu et al. 2004). It may be that in these cells, the SCF^{Skp2} pathway is inactive. In this case, some origins might refire at the beginning of S phase before Cdt1 has been destroyed via the PCNA-dependent pathway. Another explanation is that in these cell types, one or more Cdt1 proteolysis pathways could depend on

Geminin. Although experiments in *Xenopus* egg extracts and HeLa cells argue against this notion (Nishitani et al. 2004; Arias and Walter 2005; Kerns et al. 2006), it is possible that cooperation between inhibitory pathways exists in some settings. Importantly, rereplication in the absence of Geminin also occurs in primary cells (Melixetian et al. 2004) and probably in mouse embryos (Gonzalez et al. 2006), indicating that this result is not an artifact of examining transformed cells.

Caenorhabditis elegans

In worms, the study of DNA replication is in its infancy, but this highly tractable system has already begun to make valuable contributions. For example, the observation that siRNA knockdown of Cul4 causes stabilization of Cdt1 and massive rereplication in the S phase of a wide range of embryo blast cells, provided the first clue that a Cul4-based E3 ubiquitin ligase participates in the negative regulation of DNA replication (Zhong et al. 2003). Given that worms contain Cdt2 and that the PIP box of *Ce*Cdt1 is required for CeCdt1 destruction (Kim and Kipreos 2006), it appears that replication-dependent Cdt1 destruction is an essential defense against rereplication in these cells. Worms contain a putative geminin ortholog (GMN-1), but attempts to knock down its expression resulted in no clear DNA replication defects (Yanagi et al. 2005). The SCF^{Skp2}-dependent Cdt1 destruction pathway may not operate in worms, because *skp2*-mutant larvae are viable and exhibit no defects in Cdt1 destruction or DNA replication, nor does eliminating Skp2 enhance the replication defects seen when Cul4–Ddb1 function is compromised (Kim and Kipreos 2006). In summary, worms represent another example where experimental targeting of a single inhibitory pathway causes massive rereplication, and more work is needed to understand why this is the case.

Flies

Work in the flies *Drosophila melanogaster* and *Sciara coprophila* have illuminated the regulation of DNA replication during mitotic cell cycles and how this regulation is circumvented during endoreduplication and gene amplification. Flies are somewhat unique in that Cyclin E acts as the primary S-phase cyclin, both during mitotic and endocycles, whereas Cyclins A and B function as the mitotic cyclins (for review, see Lee and Orr-Weaver 2003). As seen in frogs and mammals, Cdt1 (called Double-parked, or Dup, in flies) is the major target of mechanisms that prevent rereplication. Thus, *Drosophila* Geminin binds Cdt1/Dup, and elimination of Geminin causes rereplication in *Drosophila* tissue culture cells and embryos (Quinn et al. 2001; Mihaylov et al. 2002; Higa et al. 2003). Cdt1/Dup is also degraded in the S phase of different cell types in *Drosophila* embryos (Whittaker et al. 2000; Thomer et al. 2004). Mutations in DNA replication proteins, including PCNA, stabilize Cdt1/Dup in S phase, as does removal of the Cdt1/Dup N terminus, which contains the PIP box motif, suggesting that the Cul4–$Ddb1^{Cdt2}$ pathway is active (Thomer et al. 2004; May et al. 2005). Cdt1/Dup destruction may also be controlled by CDK, since Cdt1/Dup is phosphorylated by CDK in vivo and because mutation of CDK consensus phosphorylation sites appears to enhance the rereplication activity of Cdt1/Dup under some conditions. Consistent with Cdt1/Dup proteolysis playing a role in regulating S phase, Cdt1 overexpression induces rereplication (Thomer et al. 2004). *Drosophila* Orc1 is also regulated by proteolysis, but it is ubiquitylated during the early G1 phase by APC^{Cdh1}, a time when pre-RCs are normally formed, and the function of its destruction is not understood (Araki et al. 2003, 2005).

CDKs also play a critical role in regulating DNA replication in *Drosophila*. As seen in *S. pombe*, mutations in mitotic cyclins disrupt the dependency of successive S phases on an intervening mitosis. Thus, disruption of Cyclin A in embryos or tissue culture cells induces a switch from a mitotic cell cycle to an endocycle (Sauer et al. 1995; Mihaylov et al. 2002). However, the targets of negative regulation by Cdk1–Cyclin A are not known.

Endocycles

We now turn to endoreduplication, a special case of cell cycle-regulated DNA replication in which DNA synthesis is uncoupled from cell cycle progression. During metazoan development, certain cell types exit from the mitotic cell cycle and enter an endocycle, in which successive rounds of S phase are separated by a Gap phase, in the absence of mitosis (Edgar and Orr-Weaver 2001). During *Drosophila* endocycles, Cdk2/Cyclin E activity oscillates between high levels in S phase and low levels in Gap phases (Lilly and Spradling 1996; MacAuley et al. 1998; Hattori et al. 2000). This cyclical expression is essential because continuous Cyclin E activity blocks endoreduplication (Calvi et al. 1998; Follette et al. 1998; Weiss et al. 1998). These results suggest that while Cdk2/Cyclin E is essential to promote origin firing in the endocycle, the cell must pass through a window of low Cdk2/Cyclin E activity, presumably to allow origin licensing. Endoreduplication therefore represents the clearest example in which Cdk2 can inhibit licensing.

The relevant target that is "reset" by low Cdk2 activity during the endocycle is unknown. However, it is not likely to be MCM2–7, because transient expression of Cyclin E in endocycling cells actually *promotes* rapid loading of MCM2–7 onto chromatin (Su and O'Farrell 1998). Thus, it appears that a low CDK environment is necessary for loading of an earlier licensing component (i.e., ORC, Cdc6, and/or Cdt1) and Cdt1 is a particularly attractive candidate. Both Cdt1 and Geminin are present in endocycling cells and during gene amplification cycles in developing nurse cells (Whittaker et al. 2000; Quinn et al. 2001). Moreover, Cdt1/Dup levels drop as endoreduplicating cells enter S phase, and Cdt1/Dup is rate-limiting during the amplification cycles (Thomer et al. 2004). Therefore, an attractive idea is that Cdk2–Cyclin E must be switched off in order to stop SCF^{Skp2}-depen-

dent Cdt1 turnover. Although regulation of Geminin activity within the endocycle has not been analyzed, APC activity is essential for the *Drosophila* endocycle to keep levels of mitotic cyclins low (Schaeffer et al. 2004). Thus, APC-mediated destruction of Geminin in the Gap phase could be important to allow temporal regulation of pre-RC assembly.

During placental development, mammalian giant trophoblast cells enter into an endocycle and acquire DNA contents of up to 1000C (Zybina and Zybina 1996). The unique function of Cyclin E in the endocycle is highlighted by the fact that the only major defect observed in Cyclin E mutant mice is a lack of endoreduplication in trophoblast giant cells and megakaryocytes (Geng et al. 2003). As seen in flies, Cdk2–Cyclin E activity must oscillate during endoreduplication (MacAuley et al. 1998; Hattori et al. 2000). Another similarity is that Cdk2–Cyclin E is required for MCM2–7 recruitment onto chromatin (Geng et al. 2003). Finally, recent evidence suggests that Geminin is down-regulated during mammalian endoreduplication (Gonzalez et al. 2006), and it will be interesting to understand how this and other mechanisms, such as Cdt1 destruction, contribute to the endocycle.

Consequences of rereplication

It is important to consider what happens when the mechanisms aimed at preventing rereplication fail. What is the nature of the DNA structures generated during rereplication and how do cells respond to these structures? When *S. cerevisiae* cells undergo rereplication, their chromosomes become fragmented (as seen by pulsed field gel electrophoresis), and they launch a rad9-dependent, mrc1-independent checkpoint (Archambault et al. 2005; Green and Li 2005). Importantly, rad9 normally responds to DNA damage whereas Mrc1 signals replication stress, indicating that chromsome breaks, and not stalled replication forks trigger the checkpoint. In mammalian cells that are rereplicating due to the absence of Geminin, Cdt2, or Ddb1, γ-H2AX staining and Comet assays indicate the presence of dsDNA breaks (Vaziri et al. 2003; Melixetian et al. 2004; Zhu et al. 2004; Jin et al. 2006; Lovejoy et al. 2006; Zhu and Dutta 2006). Single-stranded DNA is also detected, but it colocalizes with γ-H2AX and therefore likely reflects resection of dsDNA ends. These cells launch a classical G2/M checkpoint: chk1 and chk2 are activated, and Cdk1 activity is down-regulated, preventing entry into mitosis. Cdk2 is probably also inhibited because cells eventually cease rereplicating. Interestingly, inhibition of Chk1 in this setting drives cells into mitosis, causing mitotic catastrophe and apoptosis. Rereplication is also suppressed by Chk1 inhibition, probably because cells rapidly enter mitosis. Generally, similar results were obtained in *Drosophila* tissue culture cells (Mihaylov et al. 2002). In *Xenopus* egg extracts, addition of recombinant Cdt1 after the first round of replication induces rereplication and Chk1 phosphorylation (Li and Blow 2005). Unlike what is seen in cells, inhibition of the checkpoint in egg extracts *enhances* the amount of rereplication observed. Because the extracts used are not competent to enter mitosis, the only effect of blocking the checkpoint in this setting is to prevent the inhibition of Cdk2 activity that normally occurs during checkpoint activation (Costanzo et al. 2000, 2003). In summary, rereplication appears to give rise to dsDNA breaks, which in turn, triggers a DNA damage response, arresting cells in G2. The checkpoint effectively causes a permanent cell cycle arrest, and eventual apoptosis.

A critical question is whether checkpoint activation in rereplicating cells functions to rescue these cells from genomic instability, or, if rereplication is truly an irreparable genetic insult, whether checkpoint activation represents the first step in the initiation of programmed cell death. Interestingly, one report showed that a p53-dependent checkpoint reduces the amount of rereplication that is observed upon Cdt1 overexpression (Vaziri et al. 2003). However, a substantial amount of DNA damage was likely required to trigger the checkpoint, and it remains uncertain whether this damage could be repaired. More work is clearly required to resolve these important questions.

How does rereplication generate DNA damage? When reinitiation is relatively infrequent, the resulting forks would travel a relatively large distance before encountering another fork (Fig. 6, left arrow, right side of chromosome). As a result, rereplication forks may stall and ultimately collapse, potentially generating DSBs or chicken foot structures. At higher initiation frequencies, the same locus may undergo multiple rapid initiation events, such that two replication forks travel in the same direction (Fig. 6, left arrow, left side of chromosome). If the second fork overtakes the first, both forks will likely collapse with generation of a dsDNA break (see expanded view). This phenomenon has recently been observed when rereplication in G2 phase is promoted in *Xenopus* egg extracts by overexpression of Cdt1 (Davidson et al. 2006). Other potential sources of DNA damage can be envisioned. Replicated sister chromatids are held together from S phase until anaphase by the cohesin complex, which is thought to physically encircle the two sister chromatids in a proteinaceous ring (Losada and Hirano 2005). Thus, it is conceivable that rereplication imposes a physical stress on the cohesin ring, which could be signaled to checkpoint pathways. Alternatively, in G2 phase the histone pool may be insufficient to package the rereplicated DNA, leading to chromosome breakage.

A critical question, which has not been widely addressed, is what happens when only a very small number of reinitiation events take place (Fig. 6, right arrow). The resulting stalled forks may go undetected by the checkpoint machinery, and mitosis should proceed normally unless centromeric DNA is reduplicated. In the following S phase, there are two possibilities. If replication initiation occurs on the rereplicated region (Fig. 6, right arrow, right chromosome arm), then the local increase in ploidy will be maintained after replication is complete. If, however, new initiation events do not occur on the

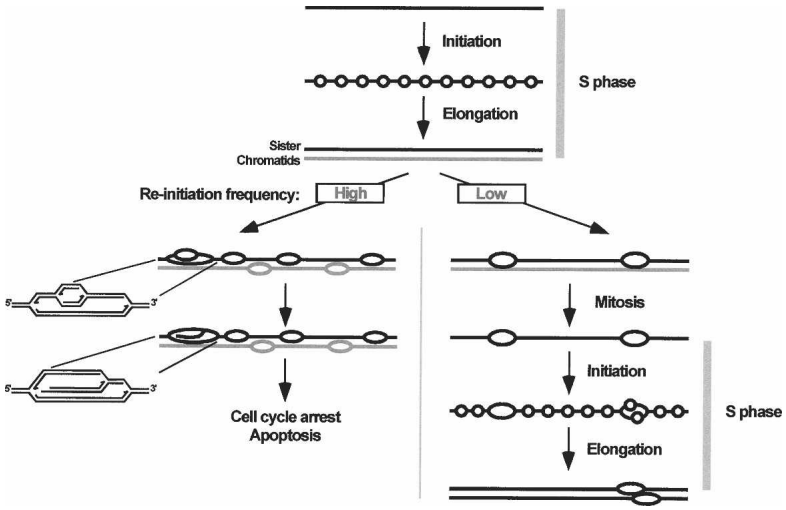


Figure 6. Consequences of rereplication. (*Top*) After one normal round of DNA replication, different amounts of rereplication can occur. (*Left* arrow) During extensive rereplication, there is a significant probability that DNA replication will repeatedly initiate from the same site within a short time frame, which leads to the "bubble-within-a-bubble" structure shown on the left arm of the chromosome. If the younger DNA replication fork catches up with the older fork, double-stranded DNA breaks will arise, as shown in the expanded view. (*Right* arrow) When very low levels of rereplication take place, the rereplicated DNA can experience two fates as cells continue to cycle. First, as shown for the reinitiation bubble on the right arm, it will be propagated if, after mitosis and entry into the next S phase, DNA replication initiates on the rereplicated segment, and this will likely lead to recombination or chromosome fragmentation. Second, as shown for the reinitiation bubble on the left arm, it might be resolved in the subsequent S phase if it does not support replication initiation.

affected rereplicated segment, the problem might resolve itself (Fig. 6, right arrow, left chromosome arm). Therefore, it is tempting to speculate that cells have evolved a strategy to avoid replication initiation on regions that rereplicated in a pervious cell cycle, although it is not obvious how this might be accomplished. Because refiring of a small number of origins is likely to be the most common and most dangerous situation, it will be critical to understand the consequences of such subtle misregulation.

Rereplication and disease

It is striking that no mutations in the components of pre-RCs, or in the regulatory pathways that govern their assembly, have been identified in hereditary human diseases (DePamphilis 2006). However, as one might expect, there are some hints that deregulation of the licensing system can cause cancer. Thus, overexpression of Cdt1 confers on NIH3T3 cells the ability to form tumors in mice (Arentson et al. 2002), and overexpression of Cdt1 in thymocytes enhances tumor formation in the absence of p53 (Seo et al. 2005). Moreover, Cdt1 is overexpressed in several human cancers (Karakaidos et al. 2004; Xouri et al. 2004). How high-level Cdt1 expression arises, whether it plays a causal role in cancer, and how it might promote cancer remains to be determined. It has also been suggested that overexpression of Cyclin E may contribute to cancer by restricting origin licensing (Ekholm-Reed et al. 2004). However, this model awaits further confirmation of the role of this kinase in regulating pre-RC assembly. In general, major defects in the regulation of DNA replication are expected to cause cell death. Therefore, developing methods to detect very limited DNA rereplication in individual cells is of paramount importance. Such approaches will likely be essential to identify and characterize naturally occurring mutations in the licensing system and to understand their effects on genome stability and disease.

Conclusions and outlook

After an exciting decade of research, we now understand that eukaryotic cells regulate DNA replication via multiple mechanisms, all of which restrict access of the replicative DNA helicase to DNA from late G1 until the exit from mitosis. In budding yeast, a staggering array of overlapping pathways, all of them CDK dependent, conspire to block MCM2–7 chromatin binding. Some of these pathways, such as the proteolytic destruction of Cdc6 and the nuclear export of MCM2–7 are well understood, whereas others, such as the inhibition of ORC, remain obscure. Although the relative contributions of each pathway are uncertain, it is clear that several pathways are by themselves able to cause significant inhibition of rereplication, and that tight regulation results from the multiplicative effects of several mechanisms. An interesting question is what effect the ablation of individual inhibitory mechanisms has on rereplication.

With only one potential exception (worms), all metazoans utilize Geminin to prevent rereplication. Recently, PCNA-dependent Cdt1 destruction has emerged as a second, apparently universal mechanism to block licensing in S phase. The precise mechanism of how Cdt1 proteolysis is coupled to chromatin-bound PCNA, and whether this system is regulated by phosphorylation or other means, is an interesting topic for future study. Although the role of CDKs in preventing rereplication in metazoans is still unclear, and no pre-RC components have been clearly validated as targets, there is evidence that Cdk1 inhibits pre-RC formation late in the cell cycle. The role of Cdk2 in regulating rereplication during the mitotic cell cycle is even less certain, whereas it clearly blocks licensing during the endocycles. Ironi-

cally, inducing rereplication by deregulating specific pre-RC components turned out to be much simpler in metazoans than in budding yeast. Thus, manipulations of single inhibitory mechanisms often cause massive rereplication. While this probably reflects the fact that multiple mechanisms target the same protein (Cdt1), we must consider the possibility that positive cross-talk between mechanisms causes catastrophic levels of rereplication when any one system is compromised. Why would evolution produce inhibitors of licensing that rely on each other for activity? Perhaps it is more advantageous to undergo massive rereplication, which induces cell death, than small amounts of rereplication, which could lead to heritable genomic instability. Even if they depend on each other, the existence of multiple pathways that inhibit pre-RC assembly is still advantageous, because under normal conditions, when all mechanisms are operational, they will cooperate to minimize relicensing. So far, there is no clear evidence of interdependent mechanisms, but testing this idea would require a thorough analysis of all known mechanisms under different conditions. A major challenge in the future is thus to carefully study the interplay of multiple inhibitory pathways in a single cell type.

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