Cell, Vol. 123, 13–24, October 7, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.cell.2005.09.019

Right Place, Right Time, and Only Once: Replication Initiation in Metazoans

Review

Yuichi J. Machida, Joyce L. Hamlin, and Anindya Dutta*
Department of Biochemistry and Molecular Genetics
University of Virginia School of Medicine
Charlottesville, Virginia 22908

DNA replication is tightly regulated at the initiation step by both the cell cycle machinery and checkpoint pathways. Here, we discuss recent advances in understanding how replication is initiated in metazoans at the correct chromosome positions, at the appropriate time, and only once per cell cycle.

Introduction

The discovery that DNA has a base-paired double-helical structure was a landmark in 20th century biology because it suggested an obvious mechanism for how genetic information is replicated and transmitted to daughter cells. Since then, the basic proteins involved in DNA replication, such as DNA polymerases and helicases, have been discovered and extensively studied. Now we are beginning to reveal the mechanisms by which DNA replication is initiated at particular positions on chromosomes, called origins, and to determine why replication occurs only during S phase and how overreplication of the genome is avoided. Although yeast have been the frequent subject of study in eukaryotic DNA replication, DNA replication in metazoans is not identical to that in yeast and in fact has a number of unique characteristics. These differences may relate to cellular differentiation and to the formation of tumors when DNA replication goes awry. Thus, our primary aim in this review is to focus on recent findings in replication initiation in eukaryotes while emphasizing the components and events that are specific to metazoans.

Loading the Helicase onto Chromatin at Origins of Replication

DNA replication in all eukaryotes starts with the process of loading a replicative helicase onto chromatin in late M phase of the cell cycle. This helicase is a ring-shaped hexamer composed of the proteins Mcm2 to Mcm7. Mcm2 to Mcm7 are closely related proteins that harbor AAA+ ATPase domains, which are required for both elongation and initiation in the *Xenopus in vitro* replication system (Pacek and Walter, 2004). Loading of the MCM2-7 complex is achieved by the concerted action of the origin recognition complex (ORC), Cdc6, and Cdt1 (Figure 1), although metazoans tend to have MCM proteins distributed over the chromosomes instead of being concentrated near origins as in yeast. As discussed later, metazoan ORC also appear to lack a strict sequence preference for binding DNA. Together, these two properties of the metazoan prereplicative complex (pre-RC) might explain the broad initiation zones that are characteristic of many metazoan replication origins.

*Correspondence: ad8q@virginia.edu

Although ORC, Cdc6, and Cdt1 are required to load MCM2-7 helicases onto chromatin, the exact mechanism of action of these proteins is poorly understood. The ring-shaped structure of MCM2-7 encircles the DNA, suggesting that ORC and Cdc6 might act as an ATP-dependent clamp-loader analogous to the RF-C clamp-loader for the proliferating cell nuclear antigen (PCNA) (Mendez and Stillman, 2003). Among the pre-RC components in metazoans, Orc1, Orc4, and Cdc6 have both Walker A and Walker B motifs, whereas Orc5 has only a Walker A motif. The Walker A motif is critical for ATP binding and the Walker B motif is required for ATP hydrolysis. Mutation of the Walker A motif of Orc1, but not of Orc4 and Orc5, reduces the ATPase activity of metazoan ORC (Chesnokov et al., 2001; Giordano-Coltart et al., 2005), suggesting that the Orc1 ATPase domain is responsible for ORC ATPase activity. Mutagenesis studies indicate that ATP binding by ORC (or Cdc6), but not hydrolysis of ATP, is required for DNA binding by ORC or Cdc6 (Chesnokov et al., 2001; Cook et al., 2002; Frolova et al., 2002; Giordano-Coltart et al., 2005; Herbig et al., 1999). Given that a nonhydrolyzable ATP analog inhibits the loading of MCM2-7, but not loading of ORC and Cdc6 (Gillespie et al., 2001; Harvey and Newport, 2003b), it seems likely that ATP hydrolysis is used to load MCM2-7 on chromatin.

The interaction between Cdt1 and MCM2-7 is greatly enhanced by the presence of Cdc6 (Cook et al., 2004). Therefore, it will be interesting to test whether Cdc6 uses the energy of ATP hydrolysis to promote the interaction of Cdt1 and MCM2-7. Alternatively, the energy may be used to release the ORC/Cdc6 complex from MCM2-7 as in the release of the clamp-loader (RF-C) from the clamp (PCNA). Such a release may be required for the reiterative loading of many MCM2-7 complexes that then migrate away from the ORC, spreading out along the chromosomes.

Recently, Mcm8, a protein with similarity to MCM2–7, was shown to exhibit helicase activity in vitro, and its depletion inhibited DNA replication by 50% without affecting pre-RC formation (Maiorano et al., 2005). This result is somewhat contradicted by a study using RNAi that indicates a role for human Mcm8 in loading ORC and Cdc6 (Volkening and Hoffmann, 2005). However, a homolog has not been identified in yeast, suggesting that the primary helicase involved in unwinding of the double helix in DNA replication is MCM2–7, whereas Mcm8 has a specialized role in vertebrates.

Origin Firing

DNA is not detectably unwound until S phase, suggesting that the MCM2-7 loaded onto origins during G1 phase remains inactive. MCM2-7 does not show strong helicase activity in vitro, supporting the possibility that it requires activation to initiate replication. Loading of Cdc45 seems to be a crucial step for the activation of the MCM2-7 helicase (Figure 1). Immunoneutralization of Cdc45 prevents both origin firing and elongation in the *Xenopus* in vitro replication system (Pacek and Wal-

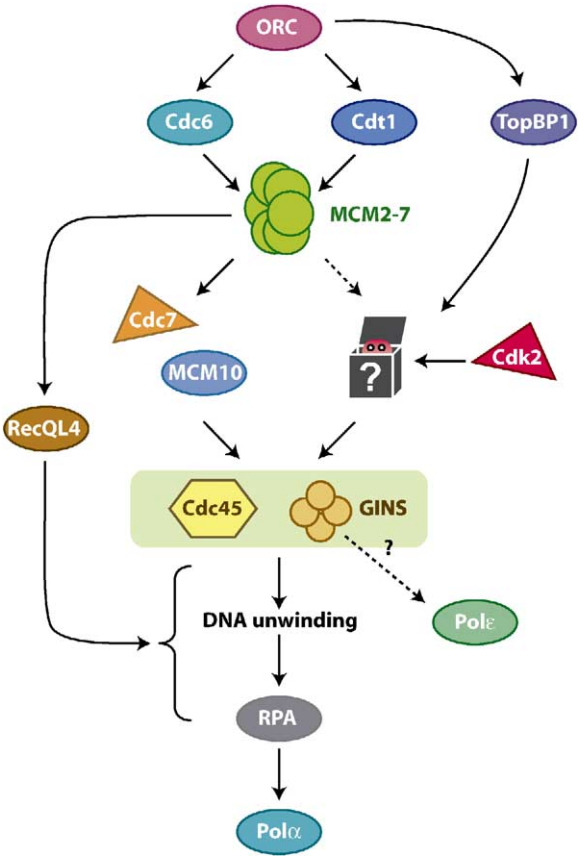

Figure 1. The Order of Protein Loading onto Replication Origins

ORC, Cdc6, and Cdt1 load MCM2-7 helicase complexes on chromatin in late M phase. In S phase, the action of two kinases, Cdc7 and Cdk2, triggers loading of Cdc45 and GINS onto origins, leading to activation of MCM2-7 and replication initiation. TopBP1 is loaded in an ORC-dependent manner and is required for Cdk2 to trigger origin firing. The Cdk2 target, shown as a black box, has not been reported in metazoans. RecQL4 is loaded in an MCM2-7-dependent manner and is required for DNA unwinding or loading of RPA. DNA polymerase α is recruited to single-stranded DNA and initiates DNA synthesis. We speculate that GINS might promote the loading of Polε.

ter, 2004), indicating that Cdc45 is required for both activation of MCM2-7 on origins and chromosome unwinding at the replication forks. Consistent with the idea that Cdc45 is a helicase cofactor, helicase activity was associated with Cdc45 immunoprecipitates from *Xenopus* egg extracts (Masuda et al., 2003).

Although most data support the MCM2-7 activation model, there are some observations that suggest that MCM2-7 is derepressed at the transition from G1 to S phase. Mcm7 interacts with a tumor suppressor protein, pRB, in a yeast two-hybrid assay (Sterner et al., 1998). This interaction has a negative effect on DNA replication in *Xenopus* egg extracts. Furthermore, Cyclin D-Cdk4 prevents binding of pRB to Mcm7 (Gladden and Diehl, 2003). Thus, pRB might suppress MCM2-7 helicase activity until activation of Cdk in a manner similar to how it represses E2F-mediated transcription until S phase. A different step at which pRB might inhibit replication has been reported in *Drosophila* (Bosco et al., 2001). The Mcm3-associated protein (Mcm3AP) is an acetyltransferase that may also be involved in repression of MCM2-7. Mcm3AP binds and acetylates Mcm3 (Takei et al., 2001) and inhibits replication initiation (Takei et al., 2002), suggesting that Mcm3 acetylation might suppress MCM2-7 helicase activation until S phase. The MCM2-7 activation model and the derepression model are not mutually exclusive, and both mechanisms may be required for tight regulation of MCM2-7 activation and coordination of replication initiation with other cell cycle pathways.

Two kinases, Cdc7 and Cdk2, are involved in the loading of Cdc45 on origins (Figure 1). Cdc7 phosphorylates the subunits of MCM2-7, and although the exact function of the phosphorylation is not known it may change the conformation of MCM2-7 to facilitate the loading of subsequent factors such as Mcm10 and Cdc45. In contrast, the crucial target of the metazoan Cdk2 in replication initiation is still unknown (Figure 1). In yeast, phosphorylation of an initiation factor, Sld2/Drc1, by Cdk is essential for initiation of DNA replication (Masumoto et al., 2002; Noguchi et al., 2002). The N terminus of human RecQL4, a helicase, shares homology with the N terminus of Sld2/Drc1, suggesting that RecQL4 is a metazoan ortholog of yeast Sld2/Drc1. However, the Cdk phosphorylation sites on Sld2/Drc1 are not conserved in RecQL4, raising a possibility that RecQL4 is not a bona fide Cdk2 target for replication initiation in metazoans. Consistent with this, RecQL4 is required for the loading of RPA, the single-stranded DNA (ssDNA) binding protein that is recruited after Cdc45 loading, whereas RecQL4 is not required for the Cdk2-dependent Cdc45 loading step (Sangrithi et al., 2005) (Figure 1).

In yeast, phosphorylation of the initiation factor Sld2/Drc1 stimulates its binding to Dpb11/Cut5. The metazoan homolog of Dpb11/Cut5, TopBP1, is required for Cdk2 to stimulate replication in *Xenopus* egg extracts (Hashimoto and Takisawa, 2003), suggesting that either TopBP1 is a target of Cdk2 or that it is required for loading an unidentified Cdk2 target protein on origins (Figure 1). In yeast, Sld5 was originally identified as a synthetic lethal gene with Dpb11/Cut5 (TopBP1). Sld5 is one component of a ring-shaped complex, GINS, that is loaded onto origins with Cdc45 in a mutually dependent manner (Kubota et al., 2003). Because TopBP1 interacts with the DNA polymerase, polε, TopBP1 and GINS (containing Sld5) might cooperate in loading polε onto origins to promote formation of an active replication fork. We suspect that additional factors important for this step remain to be identified in both metazoans and yeast, because the conversion of the pre-RC to an active replication fork is a very dynamic area of inquiry.

Restricting DNA Replication to Once per Cell Cycle

The key mechanism employed to ensure that chromosomes are replicated once and only once per cell cycle is separation of pre-RC formation and replication initiation into mutually exclusive phases in the cell cycle (G1 and S, respectively). MCM2-7 remains inactive in G1 phase, and once DNA replication is initiated following MCM2-7 activation, no additional MCM2-7 complexes

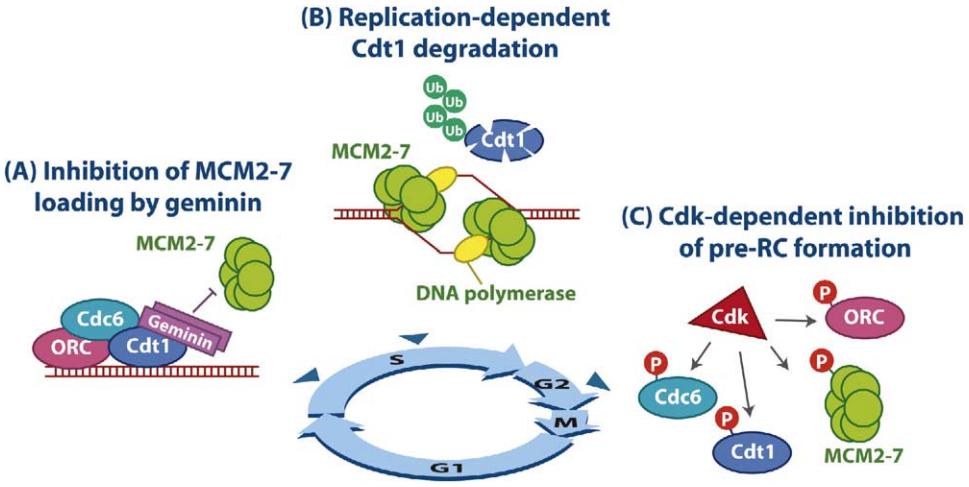

Figure 2. Three Possible Mechanisms for the Inhibition of the Second-Round Pre-RC Formation

Rereplication is prevented by inhibition of pre-RC formation by three mechanisms: (A) An inhibitory complex between geminin and Cdt1; (B) Cdt1 degradation in S phase by Skp2 dependent and independent pathways; (C) High activity of cyclin dependent kinase (Cdk) in G2 and M phases inactivates (or destabilizes) pre-RC components Cdc6, Cdt1, MCM2-7, and/ or ORC.

are loaded onto origins. The lack of activity of MCM2-7 in G1 phase can be explained by the fact that activation requires Cdk activity, which is low in G1 phase. On the other hand, at least three mechanisms might contribute to inhibition of pre-RC assembly after the onset of S phase: (1) prevention of pre-RC formation by Cdk, (2) prevention of pre-RC reformation by geminin, and (3) replication-dependent origin inactivation (Figure 2) (primary references in Blow and Dutta, 2005; Machida and Dutta, 2005).

Cdk-dependent suppression of pre-RC formation is a major (and perhaps only) mechanism for preventing rereplication in yeast. Even in metazoans, phosphorylation of components of pre-RCs by Cdks has a negative effect on pre-RC formation. For example, *Xenopus* Mcm4 loses its chromatin loading ability after phosphorylation by Cdk. Cdk causes nuclear export of excess Cdc6 protein in human cells. SCF(Skp2) is an E3 ubiquitin ligase that regulates cell cycle progression by destabilizing cell cycle regulators such as a Cdk inhibitor, p27. Skp2 binds to the substrates when the substrates are phosphorylated. Several groups reported that Cdk-mediated phosphorylation of Cdt1, the component of pre-RC involved in loading MCM2-7, triggers its ubiquitination by SCF(Skp2) and degradation by the proteasome. Human Orc1, the ATPase subunit of ORC, is also ubiquitinated by SCF(Skp2) and degraded in S phase, although it is not known whether this is Cdk2-dependent. Therefore, at least two pre-RC components are destabilized by the combined action of Cdk2 and SCF(Skp2).

Ever since its discovery as an anaphase-promoting complex (APC) substrate with an inhibitory activity on pre-RC formation, geminin has emerged as a key regulator of metazoan replication. Geminin is conserved from the worm *Caenorhabditis elegans* to humans, but homologs have not been reported in yeast. Geminin is destabilized during G1 phase and accumulates during S, G2, and M phases of the cell cycle. At the metaphase-to-anaphase transition, geminin is ubiquitinated by APC and degraded by the proteasomes to allow pre-RC formation in G1 phase. Geminin binds to Cdt1 and inhibits loading of MCM2-7 onto chromatin.

How does the geminin-Cdt1 interaction inhibit MCM2-7 loading? Geminin is detected in the chromatin fraction in a Cdt1-dependent manner when added to *Xenopus* egg extracts. Therefore, it appears to directly inhibit Cdt1 function at origins. Geminin inhibits Cdt1 binding to both DNA and to Cdc6 and MCM2-7 subunits (Cook et al., 2004; Yanagi et al., 2002), although, despite these disruptions, Cdt1 can still bind to chromatin, perhaps by interacting with ORC. The structures of geminin and Cdt1 provide some insight into how this inhibition is effected (Lee et al., 2004; Saxena et al., 2004).

Geminin dimerizes through its coiled-coil domain in the central region (Figure 3A). Several amino acid residues including four leucines and isoleucines mediate dimerization. Geminin bearing mutations in these residues can no longer dimerize and, importantly, it loses its ability to interact with Cdt1 and inhibit pre-RC formation. The two subunits of the coiled-coil domain together provide a binding interface for a single molecule of Cdt1. The surface of the coiled-coil domain has an array of glutamic acid residues that provides negative charges for the Cdt1 interaction (Figure 3B). Eighteen amino acids in the central region of Cdt1 were necessary and sufficient for interaction with geminin (Figure 3A). Three positively charged residues in this small domain provide ionic interaction with negative charges on the coiled-coil domain of the geminin dimer. Cocrystals of Cdt1 and geminin also revealed van der Waals contact at the binding interface (Lee et al., 2004). An unstructured region adjacent and N-terminal to the coiled-coil domain of geminin provides a second interface for interaction with Cdt1 (Figure 3A). Disruption of either interaction abolishes geminin's ability to inhibit replication (Saxena et al., 2004), suggesting that both interac-

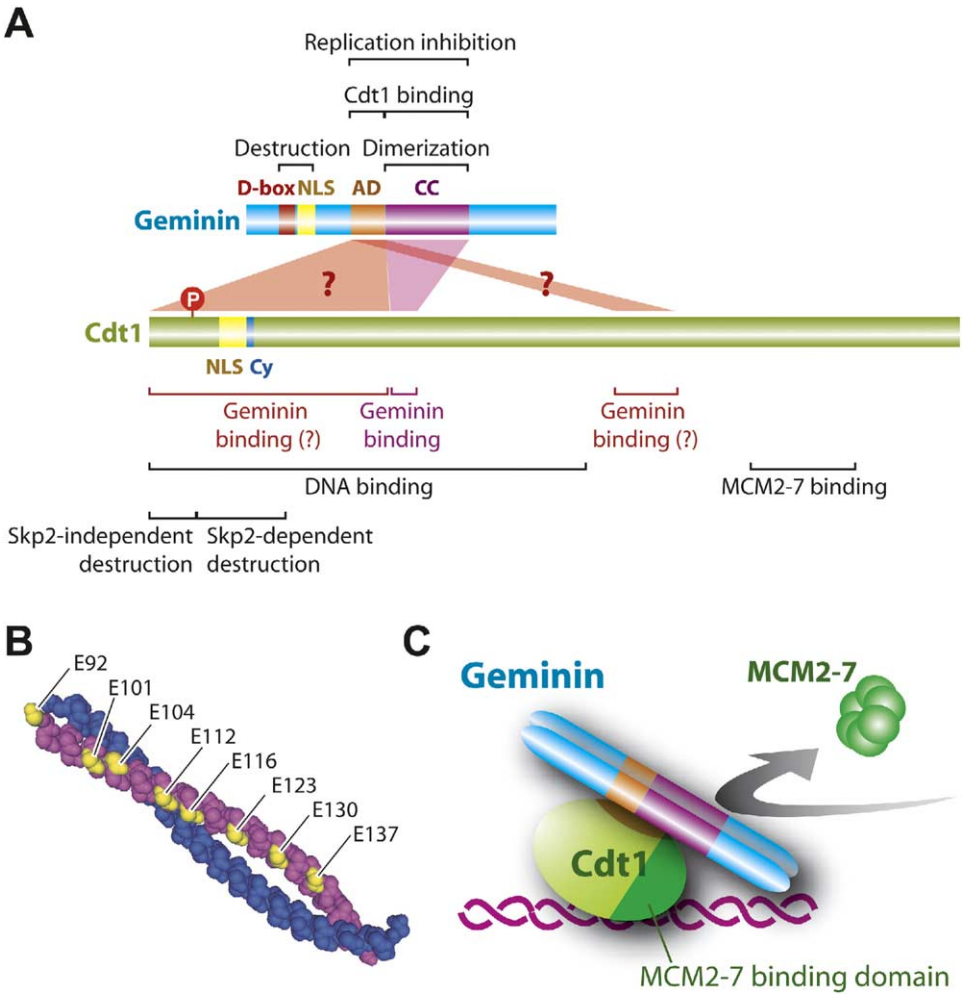

Figure 3. Inhibition of Cdt1 Function by Geminin

(A) A schematic representation of domain structures of geminin and Cdt1. Regions required for specific functions are shown by brackets. Two different portions of Cdt1 are reported to interact with the adjoining domain of geminin and indicated with question marks. AD; adjoining domain, CC; coiled-coil domain, Cy; cyclin binding motif, D-box; destruction box, NLS; nuclear localization signal. The Cdk phosphorylation site (Thr29) on Cdt1 is shown by P.

(B) Crystal structure of the coiled-coil domain of geminin. Only the peptide backbone is shown. Glutamic acid residues, which are important for association of geminin with Cdt1, are indicated in yellow on the purple molecule.

(C) A model for the inhibition of MCM2-7 loading onto chromatin through the geminin-Cdt1 interaction. The C-terminal part of geminin might inhibit the access of MCM2-7 to Cdt1 through steric hindrance.

tions are required for geminin to inhibit pre-RC formation. The structural studies also reveal the potential importance of the C-terminal half of the coiled-coil domain of geminin for inhibition of the interaction between Cdt1 and the MCM2-7 complex (Lee et al., 2004). The data strongly suggest that the N-terminal part of the coiled-coil domain along with the adjacent N-terminal region anchor geminin to Cdt1, whereas the C-terminal half of the coiled-coil domain prevents access of the MCM2-7 complex to the C-terminal portion of Cdt1 by steric hindrance (Figure 3C).

RNAi in mammalian cells unexpectedly revealed that geminin also stabilizes Cdt1 during G2/M phase to promote pre-RC formation in the subsequent G1 phase (Ballabeni et al., 2004). Therefore, at least in mammalian cells, geminin is a master regulator for pre-RC formation, working both as an inhibitor and as an activator depending on the stage of the cell cycle.

Replication-dependent origin inactivation is a relatively new concept. The degradation of Cdt1 during replication in *Xenopus* egg extracts is coupled specifically to DNA synthesis but not initiation because it is blocked by a DNA polymerase inhibitor, aphidicolin (Arias and Walter, 2005). A similar degradation mechanism was also reported in humans (Takeda et al., 2005). Therefore, two different mechanisms degrade metazoan Cdt1 in S phase: Skp2-dependent degradation triggered by Cdk, and Skp2-independent degradation triggered by replication. The portions of Cdt1 required for these degradation modes are different. Skp2-dependent degradation requires a cyclin binding motif and a Cdk2 phosphorylation site whereas Skp2-independent

(replication-dependent) degradation requires the N terminus of Cdt1 (Figure 3A). The ubiquitin ligase involved in the Skp2-independent mechanism remains unknown, but Cul4 may be involved, because in *C. elegans* a Cul4-containing ligase is involved in Cdt1 degradation during S phase. In vertebrates, Cul4 has been implicated only in DNA-damage-induced degradation of Cdt1. Interestingly, the Skp2-independent degradation of Cdt1 is required for normal progression of S phase (Takeda et al., 2005). Although it is not known whether Cdt1 degradation is required for initiation or elongation, an attractive hypothesis is that Cdt1 degradation triggers replication initiation and origin inactivation simultaneously. However, the findings in mammalian cells need to be interpreted in light of the costaining of Cdt1 and replication forks during *Drosophila* gene amplification (Claycomb et al., 2002), which implies that not all Cdt1 is degraded at the onset of DNA replication. Alternatively, it may be that the presence of Cdt1 at replication forks is specific to gene amplification.

Which of these mechanisms is critical for prevention of rereplication in metazoans? In *Drosophila* and human cells, geminin seems essential for preventing rereplication, because geminin depletion by RNAi in cultured cells causes rereplication, as does overexpression of geminin's target, Cdt1 (Blow and Dutta, 2005). In contrast, geminin depletion is not sufficient to induce robust rereplication in vitro in *Xenopus* egg extracts (Arias and Walter, 2005; Li and Blow, 2005). To induce rereplication in *Xenopus* egg extracts, adding excess Cdt1 protein is required to counter Cdt1 degradation, suggesting that the presence of geminin and degradation of Cdt1 are redundant mechanisms that independently prevent rereplication in this system. The common mechanism in all of these experimental systems is suppression of Cdt1 activity after S phase. Elevation or overexpression of Cdt1 is sufficient to induce rereplication in *C. elegans*, *Drosophila*, *Xenopus,* and human cells. The critical role for Cdt1 suppression in preventing rereplication in metazoans appears different than in yeast, where Cdk-mediated prevention of pre-RC formation plays a central role. However, Cdk-mediated suppression is still important in preventing rereplication in metazoans, particularly in G2/M cells (Ballabeni et al., 2004) where Cdk inhibition, but not geminin depletion, is sufficient to induce pre-RC formation (Figure 2). Thus, different mechanisms suppress rereplication in S phase and G2/M phase in metazoans.

Similar to the role of geminin in stabilizing Cdt1 for pre-RC formation, recent work has uncovered a positive role for Cdk activity in pre-RC formation in humans (Mailand and Diffley, 2005). Phosphorylation of Cdc6 by Cdk2 protects it from polyubiquitination by APC, leading to the stabilization of Cdc6. This stabilization is critical when cells enter the cell cycle from a quiescent state. Thus, for both the major inhibitors of rereplication, geminin and Cdk, the exact effect on pre-RC formation depends on the stage of the cell cycle.

Checkpoint Regulation of Replication Initiation

Cellular checkpoint mechanisms ensure the normal order of cell cycle events. Aberrant DNA replication or DNA damage in S phase immediately leads to the activation of an intra-S-phase checkpoint. In addition, checkpoint-mediated pathways are critical for the control of rereplication. The absence of proteins like p53 or geminin in yeast ensures that some of these pathways are unique to metazoans. In addition not all of these pathways have been examined in yeast.

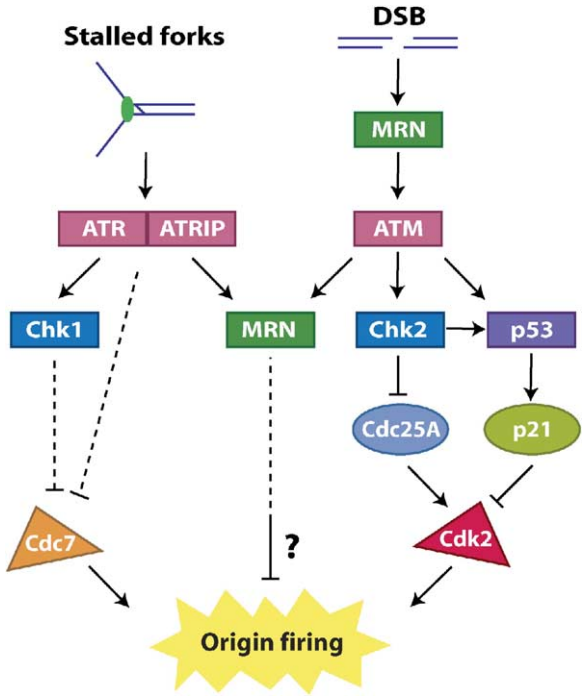

Figure 4. Inhibition of Origin firing by Checkpoint Pathways

Checkpoint pathways that suppress origin firing following fork stalling or DNA damage are shown. The MRN-dependent pathway inhibits origin firing in parallel with Chk1- and Chk2-dependent pathways. The mechanism by which the ATR-dependent pathway inhibits Cdc7 is not known. The downstream target of MRN-dependent pathway for inhibition of origin firing is also unknown. The MRN complex may be required upstream from ATM, but could also be a target downstream from ATM. Other targets of regulation by ATM include Chk2 (which inhibits Cdc25A) and p53 (which activates p21) to control Cdk2. DSB; double-stranded DNA break, MRN; Mre11-Rad50-Nbs1 complex.

DNA damage and stalled replication forks activate the checkpoint kinases ATM and ATR. These proteins in turn activate many downstream cascades including the Chk1 and Chk2 kinases to execute checkpoint functions. ATM usually responds to double-stranded DNA (dsDNA) breaks, whereas ATR responds to a wide variety of lesions and replication blocks. Cdc45 accumulates on chromatin when the checkpoint pathways are abrogated by caffeine-mediated inhibition of ATM and ATR, (Costanzo et al., 2000, 2003; Falck et al., 2002), indicating that checkpoint pathways inhibit origin firing before Cdc45 loading. Among the many proteins involved in Cdc45 loading, Cdc7 and Cdk2 are critical targets of the intra-S-phase checkpoint. The ATM-mediated checkpoint inhibits Cdk2 activity (Costanzo et al., 2000), whereas the ATR-mediated checkpoint inhibits Cdc7 activity (Costanzo et al., 2003) (Figure 4). Cdk2 inhibition is mediated through Cdc25A

degradation, which leads to an inhibitory phosphorylation of Cdk2 at Tyr15 (Costanzo et al., 2000; Falck et al., 2001). In contrast, the exact mechanism for Cdc7 inhibition is not known, but impaired association between Cdc7 and its regulatory subunit, Dbf4, was observed (Costanzo et al., 2003). In addition to the ATM-Chk2-Cdc25A-Cdk2 pathway, the Mre11/Rad50/Nbs1 (MRN) complex also inhibits DNA synthesis (Falck et al., 2002) (Figure 4). The MRN complex may be required upstream from ATM but could also have targets downstream from and independent of ATM.

Subunits of MCM2-7 are phosphorylated directly by ATM/ATR after DNA damage (Cortez et al., 2004; Yoo et al., 2004). The consequences of phosphorylation are currently unknown, but if a large fraction of MCM2-7 is phosphorylated, it might prevent new origin firing. Alternatively, the MCM2-7 proteins may be only phosphorylated locally, near stalled forks. Given that the uncoupling of the MCM2-7 helicase and DNA polymerase exposes single-stranded DNA (ssDNA)—the signal for ATR activation (Byun et al., 2005)—local phosphorylation of MCM2-7 might modulate the coupling of the helicase with the polymerase thereby regulating the amount of ssDNA exposed at stalled forks. This mechanism would provide a feedback loop for ATR activation.

Cdt1 is rapidly ubiquitinated and degraded by the proteasome pathway after γ- or UV-irradiation (Blow and Dutta, 2005). Following UV-irradiation, Cdt1 degradation is caffeine sensitive, whereas after γ-irradiation it is not. The Cul4-containing complex is the E3 enzyme for Cdt1 ubiquitination in mammalian cells in response to both γ-irradiation and UV-irradiation (Blow and Dutta, 2005). In the case of UV damage, DDB1 is the adaptor protein for Cdt1 (Hu et al., 2004). On the other hand, another E3 ubiquitin ligase, SCF(Skp2), is also shown to associate with Cdt1 after UV-irradiation (Kondo et al., 2004). As depletion of Cul4 or DDB1 by RNAi prevents almost all Cdt1 degradation after UV irradiation (Hu et al., 2004), the Skp2-mediated pathway may not be the major mechanism for the degradation of Cdt1 after DNA damage. DNA damage also results in rapid destabilization of Cdc6 (Duursma and Agami, 2005). Phosphorylation of Cdc6 by Cdk2 protects Cdc6 from ubiquitination by APC and subsequent degradation by proteasomes, a function that was important for cells emerging from quiescence (Mailand and Diffley, 2005). In response to DNA damage, p53-dependent induction of p21 inhibits Cdk2, leading to destabilization of Cdc6. Although degradation of Cdt1 and Cdc6 can occur after DNA damage in G1 phase, it is not yet clear whether this degradation is important for prevention of replication initiation as MCM2-7 has already been loaded on chromatin in late M phase.

Several studies have suggested that checkpoint pathways play a role in preventing rereplication (for review, Machida and Dutta, 2005). Rereplication by depletion of geminin or an excess of Cdt1 is a potent inducer of checkpoint pathways that bring about G2/M arrest or an intra-S phase arrest, respectively. Recently, it has been shown that rereplication in *Xenopus* egg extracts induced by the addition of Cdt1 protein was greatly enhanced by caffeine (Li and Blow, 2005), suggesting that the checkpoint pathway can suppress rereplication even in *Xenopus* egg extracts. One of the effectors of rereplication induced checkpoint pathways, p53, is a guardian of the genome and is frequently mutated or lost in many types of cancers. Overexpression of Cdt1 induces detectable rereplication only in cells lacking p53 (Machida and Dutta, 2005). Consistent with a role of p53 in rereplication control, elevated expression of Cdt1 and Cdc6 in lung cancers was associated with increased tumor growth and a higher frequency of chromosomal instability only when p53 was concurrently mutated (Karakaidos et al., 2004).

The Locations of Origins of Replication

An important issue in the metazoan replication field is whether there is strong sequence specificity in the interaction between initiation proteins and origins of replication. The replicon model for explaining the regulation of DNA replication in bacteria and their episomes has been the guiding paradigm for virtually all studies directed toward the isolation and characterization of origins from eukaryotic genomes (Jacob and Brenner, 1963). In this model, a replicon is defined as a unit of replication that contains two important genetic elements: a gene encoding a *trans*-acting initiator and a mutable replicator that directs the initiator (and initiation) to itself. Experiments guided by this basic paradigm in yeast had been so successful that the model promised to hold throughout the eukaryotic world. However, there has been a significant paradigm shift in the last decade because the model does not adequately describe regulation of initiation in most eukaryotic organisms more evolved than *S. cerevisiae*.

The first mammalian origin to be localized resides in the 240 kb amplified dihydrofolate reductase domain in the methotrexate-resistant CHO cell line, CHOC 400 (Milbrandt et al., 1981). Most of the data on this complex origin have converged into a reasonably unified model in which initiation can occur at as many as 40-50 sites within the spacer, with maxima at *ori-β* and *ori-γ*, spaced about 20 kb apart. At most, 20% of initiations in the spacer occur within the 2 kb encompassing the most active region (*ori-β*; (Dijkwel et al., 2002)). Several origins in other higher eukaryotes are broad zones of inefficient sites, including the hamster rhodopsin origin (Dijkwel et al., 2000), the human rDNA origin (Little et al., 1993), and the *Drosophila* histone and α-polymerase genes (Shinomiya and Ina, 1993, 1994). In contrast to its localized loading on origins in yeasts, MCM2-7 is loaded in a distributive pattern around origins in higher eukaryotes (Edwards et al., 2002). This difference might explain why many metazoan origins correspond to broad zones of potential sites, in contrast to the well-defined initiation sites found in yeast genomes.

However, some origins are clearly more circumscribed and come closer to the bacterial paradigm. These include the human lamin B2 (Paixao et al., 2004) and β-globin (Wang et al., 2004) origins. The human globin origin is actually a mini-zone of initiation, although two genetic elements have been described that behave as redundant replicators when the β-globin origin is assayed at an ectopic chromosomal site (Wang et al., 2004). In contrast, the lamin B2 origin appears to correspond most closely to a single site (Abraham et al., 2003). Thus, there appears to be a spectrum of origin

types ranging from tightly circumscribed to extremely broad initiation zones. Likewise, the efficiency of origin firing can range from nearly 100% [as with human β-globin (Kitsberg et al., 1993) or *Physarum* ardB&C (Benard et al., 1996)] to less than 20% [as with the origins for DHFR (Dijkwel and Hamlin, 1992; Dijkwel et al., 1994); and rhodopsin (Dijkwel et al., 2000)].

In this context, it is noteworthy that mammalian and *Drosophila* ORC, the presumptive initiator, does not appear to have a strong preference for binding to any particular DNA sequences (Remus et al., 2004; Schaarschmidt et al., 2004; Vashee et al., 2003). Instead, *Drosophila* ORC prefers negatively supercoiled DNA, with the DNA topology proving to be a more important determinant than DNA sequence for ORC binding (Remus et al., 2004). In addition, CpG methylation of DNA can inhibit ORC binding (Harvey and Newport, 2003a). It appears that origin specification involves mechanisms other than simple recognition of DNA sequence by ORC in metazoans. We therefore suggest that the genomes of metazoans are peppered at very frequent but random intervals (perhaps every few hundred base pairs) with a hierarchy of potential and degenerate replicators. When activated, these replicators control initiation only in their immediate environments. Theoretically, in the absence of any chromosomal context effects (i.e., in naked DNA), these sites could be more or less active as recognition elements for metazoan initiators in much the same way that micrococcal nuclease exhibits a hierarchic preference for certain sequences in naked DNA when enzyme is limiting (Dingwall et al., 1981).

The probability that any given site will efficiently attract an initiation complex will depend upon whether it finds itself in a permissive environment. Thus, in a euchromatic chromatin domain, the overall architecture would be permissive, allowing a non-transcribed (intergenic) region to initiate with more or less efficiency, whereas sites within an active transcription unit, in the same euchromatic domain, would not. Non-permissive heterochromatic regions and/or active transcription units may therefore be replicated passively from active upstream or downstream origins before they fire their own origins.

In this general model, lengthy intergenic spacers such as DHFR correspond to broad initiation zones because they contain many degenerate replicators. Conversely, very narrow intergenic spacers might isolate only one or a few active initiation sites (as in the lamin B2 and human β-globin loci). In fact, the one or two sites that are found in narrow spacers may have evolved particularly high affinities for the initiation complex to ensure that they are activated in every cell cycle.

It is already clear that there are no recognizable consensus sequences among the known origins except for the presence of neighboring AT-rich elements and DNA unwinding elements [reviewed in (Gilbert, 2004)]. However, for some narrowly defined origins of replication, such as lamin B2 or *ori-β*, useful knowledge can still be gathered from both mutagenesis and by moving elements of origins (in the form of cassettes) to ectopic chromosomal sites (Altman and Fanning, 2004; Wang et al., 2004). These mutagenic studies will likely define the nature of sequence characteristics that have increased affinity for ORC and/or other proteins involved in the initial melting of the helix.

Regulation of Replication Initiation by Epigenetic Phenomena

It is widely accepted that chromatin structure around a promoter modulates the transcriptional activity of genes. Activators and suppressors of transcription often remodel chromatin by modifying or repositioning histones. Histone acetylation is linked to opening chromatin for gene activation. Notably, histones around ORC binding sites are hyperacetylated during gene amplification in *Drosophila* follicle cells (Aggarwal and Calvi, 2004). Tethering histone acetyltransferase (HAT) and histone deacetylase to origins can increase and decrease origin activity, respectively (Aggarwal and Calvi, 2004). The best candidate for the HAT working at origins is HBO1 (histone acetyltransferase binding to ORC). HBO1 is a MYST family histone acetyl transferase and associates with pre-RC components such as Mcm2 and Orc1 (Burke et al., 2001; Iizuka and Stillman, 1999). Interaction of HAT with pre-RC components suggests that histone acetylation around origins is an active process in which chromatin is remodeled by replication initiators. Alternatively, origins could be determined passively—replication factors likely have easier access to DNA in open chromatin around transcriptionally active genes. Supporting this model, the formation of active transcription complexes on chromatin can specify an origin on a plasmid in *Xenopus* extracts (Danis et al., 2004). This process does not require active transcription, but the histones around the transcription complex are acetylated, suggesting that changes in chromatin structure by transcription complexes nucleate origins. Intriguingly, origin specification was not at the level of ORC binding, because ORC was not enriched at origins in this experiment. Thus, origin specification by histone acetylation might occur after ORC binding. Consistent with this, the origin decision point, the time at which origins become delimited and specific, is in mid-G1, whereas ORC binds to chromatin much earlier (Okuno et al., 2001). Intersecting genome-wide analysis of histone modifications, ORC binding sites and actual origins will be required to determine the interplay between histone modifications and ORC binding in the specification of origins (MacAlpine et al., 2004).

In addition to histone modifications, nucleosome repositioning is involved in general chromatin remodeling events. One of the ATP-dependent chromatin remodeling complexes, ACF1-SNF2h, is localized to pericentromeric heterochromatin during its replication in late S phase (Collins et al., 2002). Cells depleted of ACF1 delay progression of replication in late S phase, indicating that ACF1-SNF2h is required for DNA replication at heterochromatin. The replication defect of ACF1-depleted cells was rescued by 5-aza-2-deoxycytidine treatments, which causes decondensation of heterochromatin by inhibition of DNA methylation. Therefore, it seems ACF1-SNF2h remodel heterochromatin for replication. Although it is not known whether SNF2h plays a role in chromatin remodeling at replication origins or forks, it is worth noting that SNF2h is also recruited to remodel

chromatin at the Epstein-Barr virus origin where host cell initiation machinery is utilized (Zhou et al., 2005).

Although open chromatin is generally associated with early replication in both metazoans and yeast, it would be overreaching to draw definitive comparisons between the two groups because the molecules and enzymes involved have not been worked out in sufficient detail. In *S. cerevisiae*, molecules equivalent to ACF1-SNF2h or HBO1 have not been implicated in replication initiation, but the nucleosome rearrangement around an origin by ORC has been shown to facilitate pre-RC formation (Lipford and Bell, 2001). Considering that metazoan ORC does not have a specific DNA binding sequence, a similar role of ORC-dependent nucleosome rearrangement in pre-RC formation may have been missed in metazoans up to now. In addition, a histone acetyl transferase of the MYST family, Sas2, has been genetically linked to ORC, although it is not clear yet whether it plays a role in regulating replication initiation (Ehrenhofer-Murray et al., 1997).

Replication Timing

Although S phase-promoting kinases (Cdk2 and Cdc7) are highly active after S phase entry, activation of those kinases does not trigger all origins immediately. Origins instead fire at different times during S phase. Yeast released from a hydroxyurea block can complete the replication of the entire genome under conditions where the late-firing origins do not fire. In contrast, the larger size of metazoan genomes, makes it very unlikely that all late-firing origins will be completely dispensable for the complete replication of metazoan chromosomes. Recent large scale profiling of replication in *Drosophila* and human cells provides insight into how replication timing is determined in vivo. Replication timing correlates with a range of genomic features. Chromosomal regions that are gene-rich, and rich in GC or alu repeat sequences replicate earlier (Jeon et al., 2005; White et al., 2004; Woodfine et al., 2004). In contrast, gene-poor regions, heterochromatin and regions with high concentrations of LINE repeats tend to replicate late (Jeon et al., 2005; Schubeler et al., 2002; Woodfine et al., 2004). Although heterochromatin replicates in late S phase, it is not yet clear whether this is because replication timing is influenced by heterochromatin or whether heterochromatin is a consequence of late-replication. Mutation of the histone deacetylase (HDAC) Rpd3 in *Drosophila* induces genome-wide hyper-acetylation and stimulates origin activity (Aggarwal and Calvi, 2004), supporting the notion that heterochromatin can suppress replication. The second model, in which late-replication plays a role in heterochromatin formation, was proposed based on the observation that HDAC is recruited to replication foci only during late S phase (Rountree et al., 2000). In this model, late-replicating chromosomal regions form heterochromatin simply because heterochromatin is the only type of chromatin that is assembled in late S phase. Consistent with this idea, DNA injected in early S phase is packaged into acetylated histones and transcriptionally active, whereas DNA injected in late S phase harbors deacetylated histones and is transcriptionally inactive (Zhang et al., 2002). Both these possibilities may be correct; heterochromatin may make itself replicate late in S phase when heterochromatin is normally assembled. By this mechanism, heterochromatin persists as heterochromatin through multiple cell divisions until a signal comes to form open chromatin (such as transcriptional activation). It will be interesting to see whether a late-replicating region can be experimentally forced to replicate early in S phase and whether such replication forces the region to become open chromatin and replicate early in the next cell cycle.

There is a positive correlation between replication timing and the probability of gene expression in *Drosophila* and humans (MacAlpine et al., 2004; Schubeler et al., 2002; White et al., 2004; Woodfine et al., 2004). This is in contrast to replication profiling in yeast where there is no correlation between replication timing and the likelihood of gene expression (Raghuraman et al., 2001). Similarly, conflicting results relate replication time with level of gene expression, with one group finding some correlation between increased gene expression and early replication, whereas others fail to observe this correlation (Jeon et al., 2005; Schubeler et al., 2002; Woodfine et al., 2004). Superimposition of the genome-wide distribution of origins and ORC binding sites in *Drosophila* revealed significant colocalization of ORC and RNA polymerase II (MacAlpine et al., 2004). On the other hand, many of the origins mapped so far in humans are located in intergenic regions. The two results might agree with each other if most of the bound RNA pol II in ChIP-on-chip studies is stalled near promoters. Together, these results suggest that transcription can dictate both origin choice and initiation timing. Nevertheless, it is still not clear whether transcription factors help recruit initiation factors to chromatin or whether open chromatin at active genes increases the chance of loading initiation factors. In any case, the positive effect of transcription on replication timing is seen over large domains (>100 kb) rather than with individual genes (MacAlpine et al., 2004), suggesting that the effect is mediated through large scale chromatin structure changes.

Since the gene expression pattern is different in cells from different tissues, one might predict that the replication profile also differs between cells from different tissues. However, recent comparison between lymphoblast and fibroblast cells showed that >99% of the chromosome regions examined in the study replicated with similar timing, although the study was limited to chromosome 22, which is early replicating (White et al., 2004). Furthermore, comparison of replication timing of differentially expressed genes did not detect a correlation between gene expression and replication timing (White et al., 2004). Therefore, it seems that changes in gene expression are associated with changes in replication timing only in restricted loci like the β-globin locus. Indeed, differentiation-induced replication time changes tend to be restricted to either AT-rich or LINE-rich regions (Hiratani et al., 2004). Since AT-rich (GC-poor) and LINE-rich sequences are related to late replication, it seems that when transcription is activated in these regions, they replicate early instead of replicating during the default state in late S phase.

However, not all regions of chromosomes follow these trends. There are early-replicating heterochroma-

tin regions and highly transcribed but late-replicating regions (Hiratani et al., 2004; White et al., 2004). Furthermore, a significant percentage of chromosomal regions in a cancer cell line actually do not even show a defined replication time in S phase (Jeon et al., 2005). It is not known whether this pan-S phase pattern of replication is because many origins on different alleles fire at random times in S phase (inter-allelic variation), or because the sharply defined replication time of a gene differs from cell to cell (inter-cellular variation). Either way, these results suggest that there is significant flexibility in origin usage and replication timing.

Recent studies suggest that the ATM/ATR-mediated checkpoint regulates origin firing even in unperturbed metazoan cells (Shechter et al., 2004). Abrogation of the checkpoint pathway stimulates Cdc45 loading and origin firing in *Xenopus* egg extract and human cells (Marheineke and Hyrien, 2004; Shechter et al., 2004; Syljuasen et al., 2005). Thus, ATR, which is probably activated by ssDNA at normal replication forks, suppresses other unfired origins through the intra-S-phase checkpoint pathway. Chances are that factors such as chromatin structure determine which origins fire early and efficiently, and then checkpoint pathways suppress inefficient origins once efficient ones fire. Increasing the number of nuclei in *Xenopus* extracts prolongs S phase, which is partially reversed by inhibition of the checkpoint, suggesting that the number of firing origins is restricted by checkpoint pathways (Marheineke and Hyrien, 2004; Shechter et al., 2004). Why do cells need a mechanism to prevent firing of too many origins at the same time? One reason is that firing too many origins is deleterious to cells because it causes fork stalling and dsDNA breaks (Marheineke and Hyrien, 2004; Syljuasen et al., 2005). Checkpoint pathways could restrict the number of origins that fire in a given time-interval so that fork density is maintained at an optimal level.

New Directions

DNA replication is tightly coupled to the progression of the cell cycle, partly because of the requirement of Cdk2 activity for origin firing. How do cells know the right time for Cdk2 activation? Since pre-RC formation is inhibited once Cdk2 is activated, theoretically Cdk2 activation should happen only after enough pre-RCs are formed, so that replication is not initiated with too few licensed origins. Cdk2 activation is indeed somehow coupled to replication initiation (Machida and Dutta, 2005). For example, depletion of Orc2 in human cells arrests the Cdk cycle before S phase due to inhibition of cyclin E-Cdk2 (Machida et al., 2005). Overexpression of a stable form of geminin also arrests cells with hypo-phosphorylated pRB (suggesting low Cdk activity). Degradation of a Cdk inhibitor is coupled to replication initiation in *Xenopus* egg extracts. Further investigation is needed to find the molecular link between initiation factors and Cdk2. In addition, other studies implicate replication initiator proteins in other cell cycle events. Pre-RC is required for cohesin loading on chromatin in G1 (Gillespie and Hirano, 2004; Takahashi et al., 2004). ORC subunits are also involved in chromatin condensation, cytokinesis and/or heterochromatin formation (Chesnokov et al., 2003; Prasanth et al., 2004, 2002). It will be interesting to determine whether the processes of chromatin cohesion, condensation, cytokinesis or heterochromatin formation are extensively linked with replication initiation through the use of shared proteins.

Studying regulation of DNA replication is also important for understanding human disease and its treatment. Deregulation of replication initiation could be involved in gene amplification seen in tumor cells. In addition, some viruses utilize host initiation proteins for their replication (Dhar et al., 2001; Stedman et al., 2004). These viruses could not easily evolve resistance to a drug designed to target the replication step. As a potential complication, the drug would need to limit virus replication at concentrations that allow host cell chromosomal replication. However, Geminin provides a good model for such a drug because overexpression of wild-type geminin does not affect host cell replication but does affect virus origin replication (Wohlschlegel et al., 2002). As seen with geminin, structural studies on replication initiator proteins should give new insights into the basic mechanism of DNA replication and may provide new targets for drug development.

Although much has been learned about replication initiation from studies in yeast, parallel studies in metazoans reveal sufficient similarities and dissimilarities in the process to encourage the continued pursuit of replication initiation in all species. Last year, we witnessed the first landmark in 21st century biology, the completed sequence of the human genome. The advent of genome tiling array technology will likely provide an additional boost to our understanding of replication initiation in mammalian cells, allowing us to correlate the replication dynamics of human chromosomes with other features such as DNase hypersensitive sites, chromosome domains, translocation and amplification breakpoints, fragile sites, recombination hot-spots, and other aspects of chromosome biology directly relevant to human disease.

Acknowledgments

DNA replication and cell cycle related work in the authors' laboratories is supported by grants CA60499, CA89406 and HG003157 (to A.D.) and GM26108 (to J.L.H.) from the NIH. We apologize to all our colleagues whose publications could not be individually cited because of length restrictions.

References

Abraham, J., Lemmers, B., Hande, M.P., Moynahan, M.E., Chahwan, C., Ciccia, A., Essers, J., Hanada, K., Chahwan, R., Khaw, A.K., et al. (2003). Eme1 is involved in DNA damage processing and maintenance of genomic stability in mammalian cells. EMBO J. *22*, 6137–6147.

Aggarwal, B.D., and Calvi, B.R. (2004). Chromatin regulates origin activity in Drosophila follicle cells. Nature *430*, 372–376.

Altman, A.L., and Fanning, E. (2004). Defined sequence modules and an architectural element cooperate to promote initiation at an ectopic mammalian chromosomal replication origin. Mol. Cell. Biol. *24*, 4138–4150.

Arias, E.E., and Walter, J.C. (2005). Replication-dependent destruction of Cdt1 limits DNA replication to a single round per cell cycle in Xenopus egg extracts. Genes Dev. *19*, 114–126.

Ballabeni, A., Melixetian, M., Zamponi, R., Masiero, L., Marinoni, F., and Helin, K. (2004). Human Geminin promotes pre-RC formation

and DNA replication by stabilizing CDT1 in mitosis. EMBO J. *23*, 3122–3132.

Benard, M., Lagnel, C., Pallotta, D., and Pierron, G. (1996). Mapping of a replication origin within the promoter region of two unlinked, abundantly transcribed actin genes of Physarum polycephalum. Mol. Cell. Biol. *16*, 968–976.

Blow, J.J., and Dutta, A. (2005). Preventing re-replication of chromosomal DNA. Nat. Rev. Mol. Cell Biol. *6*, 476–486.

Bosco, G., Du, W., and Orr-Weaver, T.L. (2001). DNA replication control through interaction of E2F-RB and the origin recognition complex. Nat. Cell Biol. *3*, 289–295.

Burke, T.W., Cook, J.G., Asano, M., and Nevins, J.R. (2001). Replication factors MCM2 and ORC1 interact with the histone acetyltransferase HBO1. J. Biol. Chem. *276*, 15397–15408.

Byun, T.S., Pacek, M., Yee, M.C., Walter, J.C., and Cimprich, K.A. (2005). Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint. Genes Dev. *19*, 1040–1052.

Chesnokov, I., Remus, D., and Botchan, M. (2001). Functional analysis of mutant and wild-type Drosophila origin recognition complex. Proc. Natl. Acad. Sci. USA *98*, 11997–12002.

Chesnokov, I.N., Chesnokova, O.N., and Botchan, M. (2003). A cytokinetic function of Drosophila ORC6 protein resides in a domain distinct from its replication activity. Proc. Natl. Acad. Sci. USA *100*, 9150–9155.

Claycomb, J.M., MacAlpine, D.M., Evans, J.G., Bell, S.P., and Orr-Weaver, T.L. (2002). Visualization of replication initiation and elongation in Drosophila. J. Cell Biol. *159*, 225–236.

Collins, N., Poot, R.A., Kukimoto, I., Garcia-Jimenez, C., Dellaire, G., and Varga-Weisz, P.D. (2002). An ACF1-ISWI chromatin-remodeling complex is required for DNA replication through heterochromatin. Nat. Genet. *32*, 627–632.

Cook, J.G., Chasse, D.A., and Nevins, J.R. (2004). The regulated association of Cdt1 with minichromosome maintenance proteins and Cdc6 in mammalian cells. J. Biol. Chem. *279*, 9625–9633.

Cook, J.G., Park, C.H., Burke, T.W., Leone, G., DeGregori, J., Engel, A., and Nevins, J.R. (2002). Analysis of Cdc6 function in the assembly of mammalian prereplication complexes. Proc. Natl. Acad. Sci. USA *99*, 1347–1352.

Cortez, D., Glick, G., and Elledge, S.J. (2004). Minichromosome maintenance proteins are direct targets of the ATM and ATR checkpoint kinases. Proc. Natl. Acad. Sci. USA *101*, 10078–10083.

Costanzo, V., Robertson, K., Ying, C.Y., Kim, E., Avvedimento, E., Gottesman, M., Grieco, D., and Gautier, J. (2000). Reconstitution of an ATM-dependent checkpoint that inhibits chromosomal DNA replication following DNA damage. Mol. Cell *6*, 649–659.

Costanzo, V., Shechter, D., Lupardus, P.J., Cimprich, K.A., Gottesman, M., and Gautier, J. (2003). An ATR- and Cdc7-dependent DNA damage checkpoint that inhibits initiation of DNA replication. Mol. Cell *11*, 203–213.

Danis, E., Brodolin, K., Menut, S., Maiorano, D., Girard-Reydet, C., and Mechali, M. (2004). Specification of a DNA replication origin by a transcription complex. Nat. Cell Biol. *6*, 721–730.

Dhar, S.K., Yoshida, K., Machida, Y., Khaira, P., Chaudhuri, B., Wohlschlegel, J.A., Leffak, M., Yates, J., and Dutta, A. (2001). Replication from oriP of Epstein-Barr virus requires human ORC and is inhibited by geminin. Cell *106*, 287–296.

Dijkwel, P.A., and Hamlin, J.L. (1992). Initiation of DNA replication in the dihydrofolate reductase locus is confined to the early S period in CHO cells synchronized with the plant amino acid mimosine. Mol. Cell. Biol. *12*, 3715–3722.

Dijkwel, P.A., Mesner, L.D., Levenson, V.V., d'Anna, J., and Hamlin, J.L. (2000). Dispersive initiation of replication in the Chinese hamster rhodopsin locus. Exp. Cell Res. *256*, 150–157.

Dijkwel, P.A., Vaughn, J.P., and Hamlin, J.L. (1994). Replication initiation sites are distributed widely in the amplified CHO dihydrofolate reductase domain. Nucleic Acids Res. *22*, 4989–4996.

Dijkwel, P.A., Wang, S., and Hamlin, J.L. (2002). Initiation sites are distributed at frequent intervals in the Chinese hamster dihydrofolate reductase origin of replication but are used with very different efficiencies. Mol. Cell. Biol. *22*, 3053–3065.

Dingwall, C., Lomonossoff, G.P., and Laskey, R.A. (1981). High sequence specificity of micrococcal nuclease. Nucleic Acids Res. *9*, 2659–2673.

Duursma, A., and Agami, R. (2005). p53-Dependent regulation of Cdc6 protein stability controls cellular proliferation. Mol. Cell. Biol. *25*, 6937–6947.

Edwards, M.C., Tutter, A.V., Cvetic, C., Gilbert, C.H., Prokhorova, T.A., and Walter, J.C. (2002). MCM2–7 complexes bind chromatin in a distributed pattern surrounding the origin recognition complex in Xenopus egg extracts. J. Biol. Chem. *277*, 33049–33057.

Ehrenhofer-Murray, A.E., Rivier, D.H., and Rine, J. (1997). The role of Sas2, an acetyltransferase homologue of Saccharomyces cerevisiae, in silencing and ORC function. Genetics *145*, 923–934.

Falck, J., Mailand, N., Syljuasen, R.G., Bartek, J., and Lukas, J. (2001). The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. Nature *410*, 842–847.

Falck, J., Petrini, J.H., Williams, B.R., Lukas, J., and Bartek, J. (2002). The DNA damage-dependent intra-S phase checkpoint is regulated by parallel pathways. Nat. Genet. *30*, 290–294.

Frolova, N.S., Schek, N., Tikhmyanova, N., and Coleman, T.R. (2002). Xenopus Cdc6 performs separate functions in initiating DNA replication. Mol. Biol. Cell *13*, 1298–1312.

Gilbert, D.M. (2004). In search of the holy replicator. Nat. Rev. Mol. Cell Biol. *5*, 848–855.

Gillespie, P.J., and Hirano, T. (2004). Scc2 couples replication licensing to sister chromatid cohesion in Xenopus egg extracts. Curr. Biol. *14*, 1598–1603.

Gillespie, P.J., Li, A., and Blow, J.J. (2001). Reconstitution of licensed replication origins on Xenopus sperm nuclei using purified proteins. BMC Biochem. *2*, 15.

Giordano-Coltart, J., Ying, C.Y., Gautier, J., and Hurwitz, J. (2005). Studies of the properties of human origin recognition complex and its Walker A motif mutants. Proc. Natl. Acad. Sci. USA *102*, 69–74.

Gladden, A.B., and Diehl, J.A. (2003). The cyclin D1-dependent kinase associates with the pre-replication complex and modulates RB.MCM7 binding. J. Biol. Chem. *278*, 9754–9760.

Harvey, K.J., and Newport, J. (2003a). CpG methylation of DNA restricts prereplication complex assembly in Xenopus egg extracts. Mol. Cell. Biol. *23*, 6769–6779.

Harvey, K.J., and Newport, J. (2003b). Metazoan origin selection: origin recognition complex chromatin binding is regulated by CDC6 recruitment and ATP hydrolysis. J. Biol. Chem. *278*, 48524–48528.

Hashimoto, Y., and Takisawa, H. (2003). Xenopus Cut5 is essential for a CDK-dependent process in the initiation of DNA replication. EMBO J. *22*, 2526–2535.

Herbig, U., Marlar, C.A., and Fanning, E. (1999). The Cdc6 nucleotide-binding site regulates its activity in DNA replication in human cells. Mol. Biol. Cell *10*, 2631–2645.

Hiratani, I., Leskovar, A., and Gilbert, D.M. (2004). Differentiation-induced replication-timing changes are restricted to AT-rich/long interspersed nuclear element (LINE)-rich isochores. Proc. Natl. Acad. Sci. USA *101*, 16861–16866.

Hu, J., McCall, C.M., Ohta, T., and Xiong, Y. (2004). Targeted ubiquitination of CDT1 by the DDB1–CUL4A-ROC1 ligase in response to DNA damage. Nat. Cell Biol. *6*, 1003–1009.

Iizuka, M., and Stillman, B. (1999). Histone acetyltransferase HBO1 interacts with the ORC1 subunit of the human initiator protein. J. Biol. Chem. *274*, 23027–23034.

Jacob, F., and Brenner, S. (1963). On the regulation of DNA synthesis in bacteria: the hypothesis of the replicon. C. R. Hebd. Seances Acad. Sci. *256*, 298–300.

Jeon, Y., Bekiranov, S., Karnani, N., Kapranov, P., Ghosh, S., Macalpine, D., Lee, C., Hwang, D.S., Gingeras, T.R., and Dutta, A. (2005). Temporal profile of replication of human chromosomes. Proc. Natl. Acad. Sci. USA *102*, 6419–6424.

Karakaidos, P., Taraviras, S., Vassiliou, L.V., Zacharatos, P., Kastri-

nakis, N.G., Kougiou, D., Kouloukoussa, M., Nishitani, H., Papavassiliou, A.G., Lygerou, Z., and Gorgoulis, V.G. (2004). Overexpression of the replication licensing regulators hCdt1 and hCdc6 characterizes a subset of non-small-cell lung carcinomas: synergistic effect with mutant p53 on tumor growth and chromosomal instability–evidence of E2F–1 transcriptional control over hCdt1. Am. J. Pathol. *165*, 1351–1365.

Kitsberg, D., Selig, S., Keshet, I., and Cedar, H. (1993). Replication structure of the human beta-globin gene domain. Nature *366*, 588–590.

Kondo, T., Kobayashi, M., Tanaka, J., Yokoyama, A., Suzuki, S., Kato, N., Onozawa, M., Chiba, K., Hashino, S., Imamura, M., et al. (2004). Rapid degradation of Cdt1 upon UV-induced DNA damage is mediated by SCFSkp2 complex. J. Biol. Chem. *279*, 27315–27319.

Kubota, Y., Takase, Y., Komori, Y., Hashimoto, Y., Arata, T., Kamimura, Y., Araki, H., and Takisawa, H. (2003). A novel ring-like complex of Xenopus proteins essential for the initiation of DNA replication. Genes Dev. *17*, 1141–1152.

Lee, C., Hong, B., Choi, J.M., Kim, Y., Watanabe, S., Ishimi, Y., Enomoto, T., Tada, S., and Cho, Y. (2004). Structural basis for inhibition of the replication licensing factor Cdt1 by geminin. Nature *430*, 913–917.

Li, A., and Blow, J.J. (2005). Cdt1 downregulation by proteolysis and geminin inhibition prevents DNA re-replication in Xenopus. EMBO J. *24*, 395–404.

Lipford, J.R., and Bell, S.P. (2001). Nucleosomes positioned by ORC facilitate the initiation of DNA replication. Mol. Cell *7*, 21–30.

Little, R.D., Platt, T.H., and Schildkraut, C.L. (1993). Initiation and termination of DNA replication in human rRNA genes. Mol. Cell. Biol. *13*, 6600–6613.

MacAlpine, D.M., Rodriguez, H.K., and Bell, S.P. (2004). Coordination of replication and transcription along a Drosophila chromosome. Genes Dev. *18*, 3094–3105.

Machida, Y.J., and Dutta, A. (2005). Cellular checkpoint mechanisms monitoring proper initiation of DNA replication. J. Biol. Chem. *280*, 6253–6256.

Machida, Y.J., Teer, J.K., and Dutta, A. (2005). Acute reduction of an origin recognition complex (ORC) subunit in human cells reveals a requirement of ORC for Cdk2 activation. J. Biol. Chem. *280*, 27624–27630.

Mailand, N., and Diffley, J.F. (2005). CDKs promote DNA replication origin licensing in human cells by protecting Cdc6 from APC/C-dependent proteolysis. Cell *122*, 915–926.

Maiorano, D., Cuvier, O., Danis, E., and Mechali, M. (2005). MCM8 is an MCM2–7-related protein that functions as a DNA helicase during replication elongation and not initiation. Cell *120*, 315–328.

Marheineke, K., and Hyrien, O. (2004). Control of replication origin density and firing time in Xenopus egg extracts: role of a caffeine-sensitive, ATR-dependent checkpoint. J. Biol. Chem. *279*, 28071–28081.

Masuda, T., Mimura, S., and Takisawa, H. (2003). CDK- and Cdc45-dependent priming of the MCM complex on chromatin during S-phase in Xenopus egg extracts: possible activation of MCM helicase by association with Cdc45. Genes Cells *8*, 145–161.

Masumoto, H., Muramatsu, S., Kamimura, Y., and Araki, H. (2002). S-Cdk-dependent phosphorylation of Sld2 essential for chromosomal DNA replication in budding yeast. Nature *415*, 651–655.

Mendez, J., and Stillman, B. (2003). Perpetuating the double helix: molecular machines at eukaryotic DNA replication origins. Bioessays *25*, 1158–1167.

Milbrandt, J.D., Heintz, N.H., White, W.C., Rothman, S.M., and Hamlin, J.L. (1981). Methotrexate-resistant Chinese hamster ovary cells have amplified a 135-kilobase-pair region that includes the dihydrofolate reductase gene. Proc. Natl. Acad. Sci. USA *78*, 6043–6047.

Noguchi, E., Shanahan, P., Noguchi, C., and Russell, P. (2002). CDK phosphorylation of Drc1 regulates DNA replication in fission yeast. Curr. Biol. *12*, 599–605.

Okuno, Y., McNairn, A.J., den Elzen, N., Pines, J., and Gilbert, D.M. (2001). Stability, chromatin association and functional activity of mammalian pre-replication complex proteins during the cell cycle. EMBO J. *20*, 4263–4277.

Pacek, M., and Walter, J.C. (2004). A requirement for MCM7 and Cdc45 in chromosome unwinding during eukaryotic DNA replication. EMBO J. *23*, 3667–3676.

Paixao, S., Colaluca, I.N., Cubells, M., Peverali, F.A., Destro, A., Giadrossi, S., Giacca, M., Falaschi, A., Riva, S., and Biamonti, G. (2004). Modular structure of the human lamin B2 replicator. Mol. Cell. Biol. *24*, 2958–2967.

Prasanth, S.G., Prasanth, K.V., Siddiqui, K., Spector, D.L., and Stillman, B. (2004). Human Orc2 localizes to centrosomes, centromeres and heterochromatin during chromosome inheritance. EMBO J. *23*, 2651–2663.

Prasanth, S.G., Prasanth, K.V., and Stillman, B. (2002). Orc6 involved in DNA replication, chromosome segregation, and cytokinesis. Science *297*, 1026–1031.

Raghuraman, M.K., Winzeler, E.A., Collingwood, D., Hunt, S., Wodicka, L., Conway, A., Lockhart, D.J., Davis, R.W., Brewer, B.J., and Fangman, W.L. (2001). Replication dynamics of the yeast genome. Science *294*, 115–121.

Remus, D., Beall, E.L., and Botchan, M.R. (2004). DNA topology, not DNA sequence, is a critical determinant for Drosophila ORC-DNA binding. EMBO J. *23*, 897–907.

Rountree, M.R., Bachman, K.E., and Baylin, S.B. (2000). DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. Nat. Genet. *25*, 269–277.

Sangrithi, M.N., Bernal, J.A., Madine, M., Philpott, A., Lee, J., Dunphy, W.G., and Venkitaraman, A.R. (2005). Initiation of DNA replication requires the RECQL4 protein mutated in Rothmund-Thomson syndrome. Cell *121*, 887–898.

Saxena, S., Yuan, P., Dhar, S.K., Senga, T., Takeda, D., Robinson, H., Kornbluth, S., Swaminathan, K., and Dutta, A. (2004). A dimerized coiled-coil domain and an adjoining part of geminin interact with two sites on Cdt1 for replication inhibition. Mol. Cell *15*, 245–258.

Schaarschmidt, D., Baltin, J., Stehle, I.M., Lipps, H.J., and Knippers, R. (2004). An episomal mammalian replicon: sequence-independent binding of the origin recognition complex. EMBO J. *23*, 191–201.

Schubeler, D., Scalzo, D., Kooperberg, C., van Steensel, B., Delrow, J., and Groudine, M. (2002). Genome-wide DNA replication profile for Drosophila melanogaster: a link between transcription and replication timing. Nat. Genet. *32*, 438–442.

Shechter, D., Costanzo, V., and Gautier, J. (2004). ATR and ATM regulate the timing of DNA replication origin firing. Nat. Cell Biol. *6*, 648–655.

Shinomiya, T., and Ina, S. (1993). DNA replication of histone gene repeats in Drosophila melanogaster tissue culture cells: multiple initiation sites and replication pause sites. Mol. Cell. Biol. *13*, 4098–4106.

Shinomiya, T., and Ina, S. (1994). Mapping an initiation region of DNA replication at a single-copy chromosomal locus in Drosophila melanogaster cells by two-dimensional gel methods and PCR-mediated nascent-strand analysis: multiple replication origins in a broad zone. Mol. Cell. Biol. *14*, 7394–7403.

Stedman, W., Deng, Z., Lu, F., and Lieberman, P.M. (2004). ORC, MCM, and histone hyperacetylation at the Kaposi's sarcoma-associated herpesvirus latent replication origin. J. Virol. *78*, 12566–12575.

Sterner, J.M., Dew-Knight, S., Musahl, C., Kornbluth, S., and Horowitz, J.M. (1998). Negative regulation of DNA replication by the retinoblastoma protein is mediated by its association with MCM7. Mol. Cell. Biol. *18*, 2748–2757.

Syljuasen, R.G., Sorensen, C.S., Hansen, L.T., Fugger, K., Lundin, C., Johansson, F., Helleday, T., Sehested, M., Lukas, J., and Bartek, J. (2005). Inhibition of human Chk1 causes increased initiation of

DNA replication, phosphorylation of ATR targets, and DNA breakage. Mol. Cell. Biol. *25*, 3553–3562.

Takahashi, T.S., Yiu, P., Chou, M.F., Gygi, S., and Walter, J.C. (2004). Recruitment of Xenopus Scc2 and cohesin to chromatin requires the pre-replication complex. Nat. Cell Biol. *6*, 991–996.

Takeda, D.Y., Parvin, J.D., and Dutta, A. (2005). Degradation of Cdt1 during S phase is Skp2-independent and is required for efficient progression of mammalian cells through S phase. J. Biol. Chem. *280*, 23416–23423.

Takei, Y., Assenberg, M., Tsujimoto, G., and Laskey, R. (2002). The MCM3 acetylase MCM3AP inhibits initiation, but not elongation, of DNA replication via interaction with MCM3. J. Biol. Chem. *277*, 43121–43125.

Takei, Y., Swietlik, M., Tanoue, A., Tsujimoto, G., Kouzarides, T., and Laskey, R. (2001). MCM3AP, a novel acetyltransferase that acetylates replication protein MCM3. EMBO Rep. *2*, 119–123.

Vashee, S., Cvetic, C., Lu, W., Simancek, P., Kelly, T.J., and Walter, J.C. (2003). Sequence-independent DNA binding and replication initiation by the human origin recognition complex. Genes Dev. *17*, 1894–1908.

Volkening, M., and Hoffmann, I. (2005). Involvement of human MCM8 in prereplication complex assembly by recruiting hcdc6 to chromatin. Mol. Cell. Biol. *25*, 1560–1568.

Wang, L., Lin, C.M., Brooks, S., Cimbora, D., Groudine, M., and Aladjem, M.I. (2004). The human beta-globin replication initiation region consists of two modular independent replicators. Mol. Cell. Biol. *24*, 3373–3386.

White, E.J., Emanuelsson, O., Scalzo, D., Royce, T., Kosak, S., Oakeley, E.J., Weissman, S., Gerstein, M., Groudine, M., Snyder, M., and Schubeler, D. (2004). DNA replication-timing analysis of human chromosome 22 at high resolution and different developmental states. Proc. Natl. Acad. Sci. USA *101*, 17771–17776.

Wohlschlegel, J.A., Kutok, J.L., Weng, A.P., and Dutta, A. (2002). Expression of geminin as a marker of cell proliferation in normal tissues and malignancies. Am. J. Pathol. *161*, 267–273.

Woodfine, K., Fiegler, H., Beare, D.M., Collins, J.E., McCann, O.T., Young, B.D., Debernardi, S., Mott, R., Dunham, I., and Carter, N.P. (2004). Replication timing of the human genome. Hum. Mol. Genet. *13*, 191–202.

Yanagi, K., Mizuno, T., You, Z., and Hanaoka, F. (2002). Mouse geminin inhibits not only Cdt1–MCM6 interactions but also a novel intrinsic Cdt1 DNA binding activity. J. Biol. Chem. *277*, 40871–40880.

Yoo, H.Y., Shevchenko, A., and Dunphy, W.G. (2004). Mcm2 is a direct substrate of ATM and ATR during DNA damage and DNA replication checkpoint responses. J. Biol. Chem. *279*, 53353–53364.

Zhang, J., Xu, F., Hashimshony, T., Keshet, I., and Cedar, H. (2002). Establishment of transcriptional competence in early and late S phase. Nature *420*, 198–202.

Zhou, J., Chau, C.M., Deng, Z., Shiekhattar, R., Spindler, M.P., Schepers, A., and Lieberman, P.M. (2005). Cell cycle regulation of chromatin at an origin of DNA replication. EMBO J. *24*, 1406–1417.