Cellular Checkpoint Mechanisms Monitoring Proper Initiation of DNA Replication*

Yuichi J. Machida and Anindya Dutta†
From the Department of Biochemistry and Molecular Genetics, University of Virginia School of Medicine, Charlottesville, Virginia 22908

The most basic function of the eukaryotic cell cycle is to duplicate accurately the vast amount of DNA in the chromosomes and then segregate the copies into two daughter cells. These cell cycle events are initiated sequentially at the appropriate times during the cell cycle by successive activation of different types of complexes of cyclin and cyclin-dependent kinase (Cdk). The integrity of each cell cycle event is monitored by checkpoint mechanisms that prevent initiation of the later event until the earlier event is complete (1). Chromosomes have to be replicated not only accurately but also once and only once per cell cycle. Therefore, replication initiation must be under strict control so that origins do not fire more than once in a cell cycle. In higher eukaryotes, checkpoint mechanisms seem to be conserved. Phosphorylation of metazoan Cdc6 by Cdk promotes its ubiquitination by SCF complex and degradation by proteasomes (4–9). Phosphorylation of ORC subunits by Cdk prevents chromatin-loading of Mcm2–7 and Cdt1 is also promoted by Cdk in Saccharomyces cerevisiae, leading to their exclusion from the nucleus (12, 13). In higher eukaryotes, these checkpoint mechanisms ensure genome integrity by monitoring proper initiation of DNA replication.

Initiation of DNA Replication

In eukaryotic cells, initiation of DNA replication is achieved by an ordered assembly of protein complexes at origins of replication. This process consists of two steps; licensing of origins in late M or early G1 phase and firing of origins at appropriate times during the cell cycle. Origin licensing is mediated by the assembly of pre-replicative complexes (pre-RCs) on origins. At the center of the pre-RC is the origin recognition complex (ORC), a six-subunit complex (Orc1–Orc6), which binds origins in late M or early G1 phase and firing of origins at appropriate times during the cell cycle. Components of pre-RC are substrates of Cdk, and their phosphorylation has negative effects on new pre-RC assembly (Fig. 1). In yeasts, phosphorylation of Cdc6 by Cdk promotes its ubiquitination by SCF complex and degradation by proteasomes (4–9). Phosphorylation of ORC subunits by Cdk prevents chromatin-loading of Mcm2–7 and Cdt1 is also promoted by Cdk in Saccharomyces cerevisiae, leading to their exclusion from the nucleus (12, 13). In higher eukaryotes, these checkpoint mechanisms seem to be conserved. Phosphorylation of metazoan Cdc6 promotes its nuclear export in S phase instead of degradation, although a fraction of Cdc6 remains nuclear (14–19). Cdk-dependent phosphorylation of human Cdt1 triggers its ubiquitination and subsequent degradation by proteasomes in S phase (20–22). Xenopus Mcm4 has been shown to be a substrate of Cdk, and hyperphosphorylated Mcm complexes cannot load on chromatin (23). These facts suggest that Cdk-mediated inhibition of pre-RC formation is a conserved mechanism to suppress re-replication in eukaryotes.

Higher eukaryotes have evolved a Cdk-independent mechanism to prevent re-replication. Geminin, first identified as a substrate of anaphase-promoting complex (APC), is an inhibitor of pre-RC formation (24) (Fig. 1). Geminin binds Cdt1 and inhibits chromatin loading of Mcm2–7 complexes (25, 26). Protein levels of geminin are high during S, G2, and M phases, where pre-RC assembly is restricted. Upon activation of APC in M phase, geminin is degraded to allow new pre-RC assembly for the next S phase. Once APC becomes inactive in S phase, geminin is stabilized and inhibits further formation of pre-RCs. In many organisms, perturbation of the mechanisms preventing new pre-RC formation after S phase entry leads to re-replication. In fission yeast, overexpression of a Cdc6 homolog, Cdc18, is sufficient to cause re-replication (27, 28). In contrast, overexpression of Cdc6 does not simply result in re-replication in budding yeast (4, 29). To induce re-replication in this organism, at least three mechanisms preventing re-replication have to be relieved simultaneously: Cdc6 degradation, nuclear export of Mcm2–7, and ORC phosphorylation (10). Interestingly, overexpression of Cdt1, but not Cdc6, is sufficient to induce re-replication in human cells (30). This appears...
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FIG. 1. Initiation of DNA replication in eukaryotes. In G1 phase, ORC recruits Cdc6, Cdt1, and Mcm2-7 complexes to origins of DNA replication to form pre-RCs. Loading of Cdc45, a key step of origin firing, is triggered by two kinases, cyclin E-Cdk2 and Cdc7-Dbf4. Once origins are unwound, RPA and various DNA polymerases are loaded on origins to initiate DNA replication. During S phase, Cdk-mediated phosphorylation of pre-RC components prevents re-formation of pre-RCs on origins that have already fired. Geminin is stabilized upon S phase entry and inhibits pre-RC formation by interaction with Cdt1.

Re-replication Activates G2/M Checkpoint

Integrity of DNA replication during S phase is monitored by checkpoint mechanisms in eukaryotes. If replication forks are stalled during S phase, mitotic entry is blocked by G2/M checkpoint until DNA replication is complete. ssDNA created at stalled forks recruits a checkpoint kinase ATR (ATM- and Rad3-related) and Rad17-Rfc2–5, which in turn loads the Rad9-Rad1-Hus1 complex, to initiate the checkpoint pathway (35). Activated ATR stimulates activation of a checkpoint kinase, Chk1, which transduces checkpoint signal to the cell cycle machineries. One of the critical targets of this checkpoint is a dual specificity phosphatase, Cdc25C, which is sequestered in cytoplasm after phosphorylation by Chk1. As a result, the phosphorylation of mitotic Cdk at Tyr-15 is increased, and entry into mitosis is inhibited (35). Recently, it was also reported that Cdc6 plays a role in coupling of DNA replication and the following mitosis in human cells (36). DNA damage by UV or alkylating agents such as methyl methanesulfonate activates a similar checkpoint pathway to create time to repair those lesions before mitotic entry (35). On the other hand, damage response to double-stranded DNA breaks (DSBs) is primarily mediated by another checkpoint kinase, ATM (ataxia telangiectasia-mutated), and results in the activation of Chk2 in mammalian cells (35).

Activation of checkpoint proteins was observed in re-replicating cells (30, 32–34). Re-replication induced by Cdt1 overexpression or geminin RNAi results in the activation of Chk1 and Chk2 in human cells (30, 32, 34). Chk1 activation after geminin depletion was also reported in Drosophila and Xenopus cells (33, 37). There are two possible mechanisms for checkpoint activation after re-replication. The first possibility is that re-replication produces DSBs and activates ATM-mediated DNA damage checkpoint. DSBs could be generated by abnormal tension of aberrant structure of re-replicated DNA. Alternatively, double-stranded DNA ends mimicking DSBs might be created when the second replication fork catches up with the previously formed fork with unligated Okazaki fragments. Although DSBs were not directly detected using a comet assay in Cdt1-overexpressed human cells, activation of Chk2, which preferentially mediates signals from DSBs, suggests that re-replication creates low levels of DSBs and results in activation of the DNA damage checkpoint. Consistent with this is foci formation of Rad51, a protein involved in homologous recombination during DSB repair, in re-replicating cells (34). The second possibility is that re-replication causes fork stalling and activates checkpoint proteins. Re-replication eventually ceases after geminin depletion (32), suggesting that forks are stalled after re-replication. Indeed, ssDNA, which is sensed by the ATR-Chk1 pathway after fork perturbation, was detected in re-replicating cells (34).

Checkpoint proteins activated in response to re-replication by geminin RNAi cause G2/M arrest in Xenopus and human cells (32, 34, 37). Overexpression of a dominant-negative form of Chk1, treatment with an inhibitor of Chk1 (UCN-01), or depletion of Chk1 by RNAi can abolish the cell cycle arrest (32, 34, 37). These data suggest that Chk1 activation is critical for inhibition of mitotic entry in re-replicating cells. Abrogation of Chk1 function results in a decrease in re-replicating cells after geminin RNAi (32, 33). Without checkpoint activation, re-replicating cells undergo premature mitosis and subsequent cell death (32, 34). Cdc25C was phosphorylated at Ser-216 and sequestered in cytoplasm after re-replication in human cells (32). Overexpression of a mutant Cdc25C lacking a Chk1 phosphorylation site overcomes the G2/M arrest caused by geminin depletion in Xenopus, suggesting that Cdc25C is a critical Chk1 target in the G2/M checkpoint pathway in response to re-replication (37). Consistent with this, the cell cycle arrest is accompanied by accumulation of cyclin B1 and an increase in inhibitory phosphorylation at Tyr-15 of Cdk1 (32, 34, 37).

\footnote{Y. Machida and A. Dutta, unpublished data.}
expression of Cdk1AF, a mutant Cdk1 lacking inhibitory phosphorylation sites, bypasses the cell cycle arrest in Xenopus after re-replication (37). In summary, re-replication results in activation of the G2/M checkpoint pathway to inhibit mitotic entry with overreplicated chromosomes (Fig. 2).

**Inhibition of Re-replication by Checkpoint Proteins**

Several lines of evidence suggest a role for checkpoint pathways in blocking re-replication. Treatment of cells with an ATM/ATR inhibitor, caffeine, or with a Chk1 inhibitor, UCN-01, promotes re-replication by Cdt1 overexpression in human cells, suggesting that checkpoint proteins have an inhibitory effect on re-replication. Deletion of one of the checkpoint proteins, Rad17, in human cancer cells results in re-replication (38), indicating that checkpoint pathways involving Rad17 monitor and suppress spontaneous re-replication. Interestingly, overexpression of Cdt1 and Cdc6 causes re-replication in p53-deficient but not p53+ human cells (30). In p53+ cells, p21 is stabilized, and its downstream targets including p21 and PIG3 are induced (Fig. 2). Overexpression of Mdm2, an E3 ubiquitin ligase for p53, in p53+ cells allows re-replication by Cdt1 and Cdc6 overexpression, suggesting that p53 has a role in preventing re-replication. The best candidate for re-replication block among p53 targets is p21. It is possible that p21 suppresses re-replication through inhibition of Cdk2, which is required for new origin firing (Fig. 2). p53 may also induce apoptosis through PIG3 induction (Fig. 2). On the other hand, geminin depletion causes re-replication regardless of p53 status (32, 34). The difference could be due to the overexpression of Cdc6 in the experiments that induced p53 or due to a direct role of geminin in directing the checkpoint enzymes to p53 so that when geminin is depleted, the checkpoint enzymes fail to phosphorylate and stabilize p53.

Another possible pathway by which checkpoint proteins could suppress re-replication is through activation of intra-S phase checkpoint. One of the important functions of intra-S phase checkpoint is to prevent firing of late origins in response to fork stalling or DNA damage during S phase (35). Like other type of checkpoints, the intra-S phase checkpoint pathway includes activation of ATM/ATR and Chk1/Chk2 checkpoint kinases. Genetics in yeasts and biochemistry using Xenopus extracts have accumulated evidence that Cdc7-Dbf4 is one of the targets of the intra-S phase checkpoint pathway to prevent further firing of origins (35). Given that re-replication is a process with repeated firing of the same origins in an S phase, it is possible that the intra-S phase checkpoint pathway is utilized to suppress further reinitiation of replication when

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3 W. Zhu and A. Dutta, unpublished data.
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Pre-RN members, Cdc6/Cdc18, Cdt1, and Orc1, are defective in DNA replication initiation and therefore lethal (43–47). Examination of the spores lacking Cdc6, Cdt1, and Orc1 revealed that these cells undergo mitosis without DNA replication leading to cells with fractional DNA content and fragmented chromosome. Because generation of checkpoint signal requires formation of DNA replication forks, mutants of replication initiation genes cannot generate the inhibitory signal necessary to stop entry into mitosis. In contrast, conditional yeast mutants of replication initiation proteins show a different phenotype in the restrictive conditions. Haploid cells carrying a temperature-sensitive mutation in Cdc18 arrest with a 2C DNA content (44). Pulse-field gel electrophoresis revealed that these cells have not completed replication. This result suggests that the cells could initiate DNA replication from a fraction of origins, but they could not complete DNA replication. Mitotic entry was successfully inhibited in these cells because cells that have created enough replication forks to generate a checkpoint signal. Therefore, it seems that premature entry of cells into mitosis is dependent on the extent of impairment of initiator protein function in yeasts. In *Drosophila*, a null mutant of DCP, a Cdt1 homolog, is also defective in DNA replication (48). Cells are arrested in an abnormal mitosis with overcondensed and fragmented chromosomes. Importantly, these cells are positive for a mitotic marker, phosphohistone H3, suggesting that DCP cells undergo premature mitosis. This is similar to the null phenotype of initiation protein mutants in yeasts. A similar result was obtained in geminin-overexpressing *Drosophila* cells. These cells are deficient in DNA replication and undergo premature mitosis and apoptosis (49). In addition chromosome condensation was abnormal in *Drosophila* mutants of replication proteins Orc2, Orc5, Mcm4, and proliferating cell nuclear antigen (50, 51).

Interestingly, the situation seems different in higher eukaryotes. Orc2 RNAi in human cells impairs DNA replication and arrests them in G2 phase with low cyclin E-Cdk2 activity. Blocking pre-RC assembly by overexpression of a stable form of geminin in primary cells affects DNA replication, and Cdk is inhibited based on the appearance of hypophosphorylated pRB (52). These results suggest that there is a mechanism that prevents Cdk activation until enough replication-competent origins are formed in G1. At this moment, it is not clear whether pre-RC formation is a signal for S phase entry. The Cdk inhibitor, p27, is stabilized after Orc2 depletion and associated with the cyclin E-Cdk2 complex in human cells. In *Xenopus* egg extracts, replication-coupled degradation of Xic1, a homolog of p27, was reported (53, 54). Xic1 was degraded at origins of replication, and this degradation was coupled with replication initiation. These results suggest a possible role for p27 as an effector in this checkpoint-like response to prevent progression of the cell cycle when there are insufficient initiator proteins.

### Concluding Remarks

Initiation of DNA replication is under strict control and coordinated with other cell cycle processes by checkpoint pathways. Regulation of DNA replication and cell cycle progression is critical for maintenance of genome integrity. Therefore, an interesting future question will be whether impairment of cellular mechanisms that normally ensure proper initiation of DNA replication is a potential cause of human tumors. For example, sporadic re-replication might contribute to gene amplification or polyploidy seen in many types of cancer cells. High levels of cyclin E, reduced levels of p27, and impairment of pRB seen in some cancers might override the checkpoint at the G1/S transition and cause deletion or loss of chromosomes.

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4 Y. J. Machida and A. Dutta, unpublished data.