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Mechanism of chromosomal DNA replication initiation and replication fork stabilization in eukaryotes

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Chromosomal DNA replication is one of the central biological events occurring inside cells. Due to its large size, the replication of genomic DNA in eukaryotes initiates at hundreds to tens of thousands of sites called DNA origins so that the replication could be completed in a limited time. Further, eukaryotic DNA replication is sophisticatedly regulated, and this regulation guarantees that each origin fires once per S phase and each segment of DNA gets duplication also once per cell cycle. The first step of replication initiation is the assembly of pre-replication complex (pre-RC). Since 1973, four proteins, Cdc6/Cdc18, MCM, ORC and Cdt1, have been extensively studied and proved to be pre-RC components. Recently, a novel pre-RC component called Sap1/Girdin was identified. Sap1/Girdin is required for loading Cdc18/Cdc6 to origins for pre-RC assembly in the fission yeast and human cells, respectively. At the transition of G1 to S phase, pre-RC is activated by the two kinases, cyclin-dependent kinase (CDK) and Dbf4-dependent kinase (DDK), and subsequently, RPA, primase-polα, PCNA, topoisomerase, Cdc45, polδ, and polε are recruited to DNA origins for creating two bi-directional replication forks and initiating DNA replication. As replication forks move along chromatin DNA, they frequently stall due to the presence of a great number of replication barriers on chromatin DNA, such as secondary DNA structures, protein/ DNA complexes, DNA lesions, gene transcription. Stalled forks must require checkpoint regulation for their stabilization. Otherwise, stalled forks will collapse, which results in incomplete DNA replication and genomic instability. This short review gives a concise introduction regarding the current understanding of replication initiation and replication fork stabilization.

DNA replication origins, pre-RC assembly, replication fork stability, S phase checkpoint

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The chromosomal DNA in eukaryotes is significantly different from that in prokaryotes. The difference is mainly reflected in size and chromatin structure. The size of genomic DNA in eukaryotic cells ranges from ~1.4×10^7 bp in yeast to 3×10^9 bp in human cells, while it is ~4.6×10^6 bp in *Escherichia coli.* Eukaryotic DNA is associated with a great variety (hundreds to thousands) of proteins while prokaryotic DNA is basically naked. The most abundant proteins associated with genomic DNA in eukaryotes are histone proteins. About every 200 bp of genomic DNA is bound by one nucleosome composed of eight histone polypeptides. These two characteristics make the replication of genomic DNA in eukaryotes highly complicated and strictly regulated.

1 The initiation of chromosomal DNA replication in eukaryotic cells

In eukaryotes, DNA replication initiates at specific sites on

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chromosomal DNA, and these sites are called DNA replication origins. Pre-replication complex (pre-RC) is assembled on DNA origins at late M and G1 phase, and subsequently, pre-RC is activated by the two protein kinases, cyclin-dependent kinase (CDK) and Dbf4-dependent kinase (DDK), at the transition of G1 to S phase. After pre-RC is activated, those proteins acting at replication forks are orderly recruited to DNA origins to create two bi-directional replication forks for starting DNA synthesis [1].

1.1 DNA replication origins and origin selection in eukaryotes

The structures of DNA origins among eukaryotes are not conserved. In the budding yeast *Sacchoromryces cerevisiae*, DNA origins have a size of ~100 bp and contain a 11-bp consensus sequence called "A" element (5′-(A/T)TTTA(T/C)(A/G)TTT(A/T)-3′). This 11-bp consensus sequence is recognized and bound by origin recognition complex (ORC) and is essential for origin activity. Besides the "A" element, DNA sequence close to the "A" element is also contacted with ORC and therefore, is required for origin activity, but the sequence is not conserved [2–4]. In the fission yeast *S. pombe*, the size of DNA origins ranges from ~500 to 1500 bp, which is about 5–10 times larger than the budding yeast DNA origins. *S. pombe* DNA origins do not have a consensus sequence in a strict sense, but they do contain two or more DNA sequences that are asymmetric AT-rich and required for origin activity. *S. pombe* ORC binds to these AT-rich sequences through the nine AT-hook motifs of Orc4 subunit [5–8]. In general, *S. pombe* DNA origins possess one, two or more ORC binding sites in each origin, while the DNA origins in *S. cerevisiae* often have only one ORC binding site. Besides the ORC binding site, *S. pombe* DNA origins contain an additional origin element that is essential for origin activity. This element is bound by the recently discovered pre-RC component–Sap1 protein. Sap1 is an essential protein for cell growth, but the essential function of Sap1 for cell growth was not yet determined before [9,10]. We recently demonstrated that Sap1 interacted with ORC and bound to all DNA origins. ORC and Sap1 physically interact with each other and act to load Cdc18 (the Cdc6 homologue in the fission yeast) to origins for the assembly of pre-RC. In metazoans, DNA origins have a size similar to the fission yeast DNA origins (~1000 to 2000 bp) and they also lack a consensus sequence [11,12]. Like DNA origins in the fission yeast, AT-rich sequences are also present in metazoans DNA origins and these AT-rich sequences appear important for origin activity [13,14]. Recently, the Sap1 homologue in metazoans was identified. It is Girdin protein that has a region highly homologous to Sap1 protein. Like Sap1, Girdin interacts with ORC and binds to DNA origins. At the G1 phase, Girdin acts to recruit Cdc6 to origins for pre-RC assembly. Hence, DNA origins from the fission yeast to metazoans contain two essential origin elements, one is bound by ORC and the other bound by Sap1/Girdin. ORC and Sap1/Girdin physically interact with each other and act together to recruit Cdc18/Cdc6 to origins for pre-RC formation.

Besides the ORC and Sap1/Girdin binding sites, it appears that some other factors influence the origin activity. These factors include chromatin structure, histone modification, DNA secondary structure, and transcription activity [15,16]. In general, replication initiation takes place at euchromatin region. The reason is probably that euchromatin regions are easily accessible by replication proteins. Therefore, any factors that are able to influence chromatin structures could affect origin activity. Currently, little is known regarding what the other factors are and how these factors affect origin choice and origin activity.

1.2 The initiation process of chromosomal DNA replication in eukaryotes

From the budding yeast to the fission yeast and human, the origin structure gets more complicated. This indicates that one or more steps in the process of replication initiation may be different among eukaryotes, even though the fundamental mechanism of initiation should be the same. As we understand at present, the process of replication initiation is divided into two steps: the first step is the selection of replication origins and assembly of pre-RC; the second step is the activation of pre-RC, recruitment of replication proteins to origins, and subsequent firing of origins for starting DNA synthesis [17–19].

As shown in Figure 1, from the budding yeast to human cells, ORC always binds to DNA origins during cell growth cycles. At G1 phase, ORC in the budding yeast acts as a platform to recruit Cdc6 and Cdt1 to origins, and Cdc6 and Cdt1 act together to recruit MCM to origins for the pre-RC formation [20]. The *in vitro* assembly of pre-RC with the purified ORC, Cdc6, Cdt1 and MCM has been achieved in the budding yeast, indicating that these four proteins are sufficient for pre-RC assembly [21–23]. From the fission yeast to human cells, the recruitment of Cdc18/Cdc6 to DNA origins is accomplished by the combined action of ORC and Sap1/Girdin proteins. After MCM is loaded onto origins, the pre-RC assembly is completed. The activation of MCM as a replicative helicase requires the recruitment of Cdc45 and GINS to origins. Cdc45, GINS and MCM interact with each other to form a CMG complex that is an active DNA helicase and functions at replication forks [24]. The recruitment of Cdc45 and GINS to origins needs the pre-RC activation that is catalyzed by Cdc7-Dbf4 (DDK) and CDK at the G1 to S phase transition [25]. Further recruitment of DNA polymerases, RPA, and primase to DNA origins results in replication initiation.

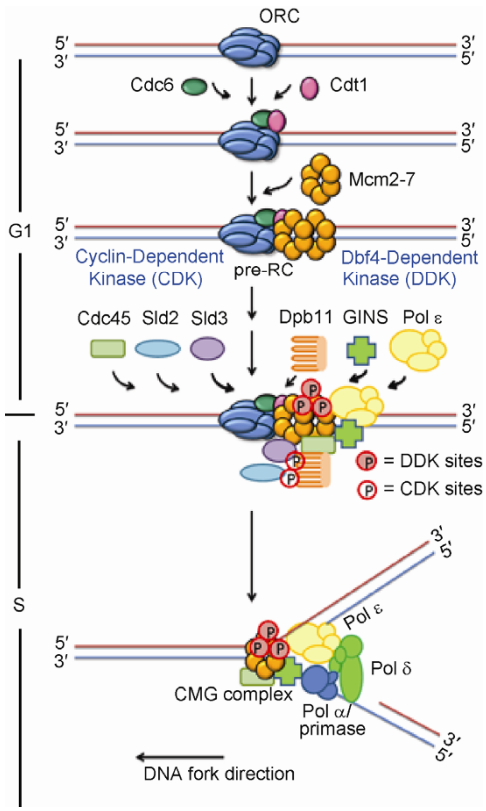

Figure 1 The initiation of DNA replication in *S. cerevisiae*.

1.3 The regulation of chromosomal DNA replication initiation

Chromosomal DNA replication in eukaryotic cells is strictly regulated for maintaining genomic stability. This strict regulation includes two aspects: one is to restrict the assembly of pre-RC to late M and G1 phase; the other is to ensure that each segment of chromosomal DNA gets duplication once in each cell cycle. Through millions of years of evolution, eukaryotic cells have developed a sophisticated regulation system to ensure precise DNA replication.

By restricting the assembly of pre-RC to late M and G1 phase, it ensures that each origin can fire only once per cell cycle. Now, the question is how eukaryotic cells restrict pre-RC assembly to late M and G1 phase? Based on our current understanding, it appears that eukaryotic cells use two kinds of regulation. One is to control the availability of some pre-RC components in nuclei. The other is to regulate the activity of pre-RC components through chemical modification such as phosphorylation. For example, the Cdc18/Cdc6 and Cdt1 proteins in the budding and fission yeast are present only in late M and G1 phases. These two proteins are phosphorylated when pre-RC is activated at the G1 to S phase transition and degraded quickly when cells get into the S phase [26–30]. It is also reported that some pre-RC components are transported out of nuclei when cells are in the S, G2 and M phase, such as MCM in the budding yeast and Cdc6 in human cells [31,32]. Besides the protein degradation and the trans-localization of some pre-RC components, the phosphorylation of each of pre-RC components appears to disrupt their activity to assemble pre-RC [33]. A number of studies have indicated that CDK is the major driving force behind the above regulations [34]. At G1 phase, the CDK activity is low, the pre-RC components in nuclei are either not phosphorylated or under-phosphorylated and so they are competitive for pre-RC assembly. When the CDK activity gets high as cells progress to the S phase, pre-RC is activated and its components are phosphorylated. For those phosphorylated pre-RC components, some of them are degraded or transported into cytoplasm, the rest lose their functions to assemble pre-RC. Thus, the assembly of pre-RC is restricted to the G1 phase and each origin is restricted to fire once per cell cycle to avoid re-replication of DNA in one cell cycle.

Although it is important to avoid DNA re-replication, it is even more important to avoid under-replication (the term "under-replication" is defined here as no DNA replication of a region) of DNA segments. Otherwise, DNA breaks occur in those under-replication regions during two sister chromosomes' segregation. Checkpoint control is thought to be the regulation system to ensure that each segment of DNA gets duplication once in one cell cycle. Checkpoint is activated when DNA replication is in stress such as that DNA lesions occur or replication forks stall. The activated checkpoint blocks cell progression, which gives cells the time to fix DNA lesions and then resume DNA replication. In normal cell growth cycles, eukaryotic cells very possibly use the same checkpoint regulation to ensure that each segment of DNA is replicated. It is proved that even in a normal cell cycle, a basic level of checkpoint is required for precise DNA replication [35].

2 The stabilization of replication forks

2.1 Replication forks and the factors that stall forks

A replication fork is a "Y" shape of DNA structure that is associated with replication proteins composed of several dozens of polypeptides, including CMG (Cdc45/Mcm2-7/GINS) complex, DNA polymerases (Pol α, Polδ, Polε), PCNA (proliferating cell nuclear antigen), Mrc1 (mediator of replication checkpoint 1), Tof1 (topoisomerase 1-associated factor 1), topoisomerases, Okazaki fragment maturation proteins Dna2 and Fen1, and DNA ligase [36]. These replication proteins must act synergistically for the coordinated synthesis of the leading and lagging strand of DNA. As forks move along chromatin DNA, they frequently stall due to the existence of various replication barriers, such as DNA repeat sequences, DNA fragile sites, replication termination sites, and replication slow zones [37–41]. The other factors that stall forks include transcription forks [42], the absence of dNTPs in the presence of hydroxyurea [43],

DNA lesions [44], etc. Stalled forks will activate checkpoint, and the activated checkpoint affects actions back onto the stalled forks.

2.2 The S phase checkpoint control

The term "checkpoint" was initially coined to describe the regulation of cell cycle. It is found that cells cannot progress into the next phase only after the previous phase is completed. For example, only these cells that have finished the S phase can get entry into the G2 and M phases. Thus, it appears that eukaryotic cells have a system to check whether a cell cycle phase has been finished or not, and people call this system as checkpoint. As a lot more is known about checkpoint at the biochemical level, it shows that checkpoint is more related to DNA metabolism including replication, recombination and repair, chromosome segregation and genomic stability. Because DNA replication and chromosome segregation are two core biological events during cell growth cycles, it could be the reason that checkpoint initially described the regulation of cell cycles. There are two major checkpoint pathways. One is the S phase checkpoint regulating DNA metabolism; the other is the M phase checkpoint that regulates chromosome segregation. The replication stress such as replication fork stalling is regulated by the S phase checkpoint. Here, we give a brief review regarding how checkpoint arises from stalled forks and how checkpoint subsequently affects its actions on stalled forks to prevent fork collapse.

(i) The signal in the stalled forks to be sensed for activating the S phase checkpoint. It is clear that checkpoint is activated when replication forks are stalled in the presence of hydroxyurea (HU) or MMS in the medium. Now, the question here is what in the stalled forks is sensed for activating checkpoint. A definite answer to the question still lacks, but the increased amount of ssDNA region and the exposed ssDNA-dsDNA junction in the stalled forks is generally thought to be the signal to activate checkpoint. It has been reported that the length of single-stranded region at stalled forks increases [45]. The ssDNA regions are bound by RPA. Subsequently, ATR-ATRIP is recruited to RPA-bound ssDNA regions through the specific protein-protein interaction between ATR-ATRIP and RPA. The interaction between ATR-ATRIP and RPA could be direct or indirect. It appears that some other proteins are involved in this step [46–48]. At the meantime, RFC-like complex binds to the joint region of ssDNA and dsDNA, and subsequently the 9-1-1 complex and TopBP1 are recruited through their interaction with the RFC-like complex. After TopBP1 and ATR-ATRIP are brought together through the above steps, their physical interaction results in the activation of ATR kinase (Figure 2). The above description of ATR activation could be overly simple, and the actual checkpoint activation may be more complicated. One reason is that the ssDNA region and ssDNA-dsDNA junction may not be the sole factor to determine the checkpoint activation. MMS-induced DNA adducts and HU-caused depletion of dNTPs both stall replication forks, and subsequently longer ssDNA region at forks is generated and checkpoint is activated. But in these two cases, the activated checkpoint pathways are certainly different in some aspects even though some are the same. For example, in the MMS- mediated checkpoint activation, some checkpoint pathway(s) must be activated for regulating the repair of base adducts. Thus, the difference in checkpoint activation cannot be explained if the ssDNA region and the ssDNA-dsDNA junction is the sole signal to be sensed. If the ssDNA region and the ssDNA-dsDNA junction is not the sole signal, what else is sensed? Currently, we know little about the other signal factors. In the future study, this aspect of research should be paid particular attention in order to elucidate the checkpoint-mediated regulation of a variety of DNA lesion repair and stabilization of stalled replication forks.

(ii) The effect of checkpoint on stalled replication forks. After replication forks stall and ATR is activated, it will phosphorylate downstream kinases or proteins for signal transduction. One of the ATR kinase targets is the Chk2 kinase in yeast or Chk1 kinase in metazoans. The activated Chk2 or Chk1 will phosphorylate a series of protein targets and these targets exert their functions to regulate DNA replication and stabilize stalled replication forks. In *S. cerevisiae*, when DNA is damaged by MMS, the activated $Chk2^{Rad53}$ directly phosphorylates Sld3 to inhibit replication initiation at late DNA origins [49]. In the fission yeast, $Cds1^{Chk2}$ directly regulates Dna2 flap endonuclease through phosphorylation on S220 site to prevent stalled forks from reversing [50]. We found that the ATR-deficient cells were more sensitive to HU than the Cds1-deficeint cells and the Cds1-deficient cells were more sensitive to HU than Dna2-deficient cells. This suggests that ATR has other targets besides Cds1 target and Cds1 has other targets besides Dna2 to stabilize stalled replication forks. So far, only a few proteins are identified to be the S-phase checkpoint targets in regulating replication and stabilizing replication forks. Therefore, the future work will be to identify the S-phase checkpoint targets and elucidate their functions in the field of checkpoint-mediated DNA replication and fork stability

3 Perspectives

In the last two decades, the understanding of the mechanism of eukaryotic DNA replication initiation is significantly advanced, but some critical questions still remain. In the next decade, the following questions should be addressed. (i) How is pre-RC assembled in the fission yeast and metazoans at the molecular terms? (ii) How are replication origins chosen in metazoans? (iii) How is pre-RC assembly regulated and what is the mechanism to activate pre-RC? (iv) How do the reactions at replication forks take place? (v)

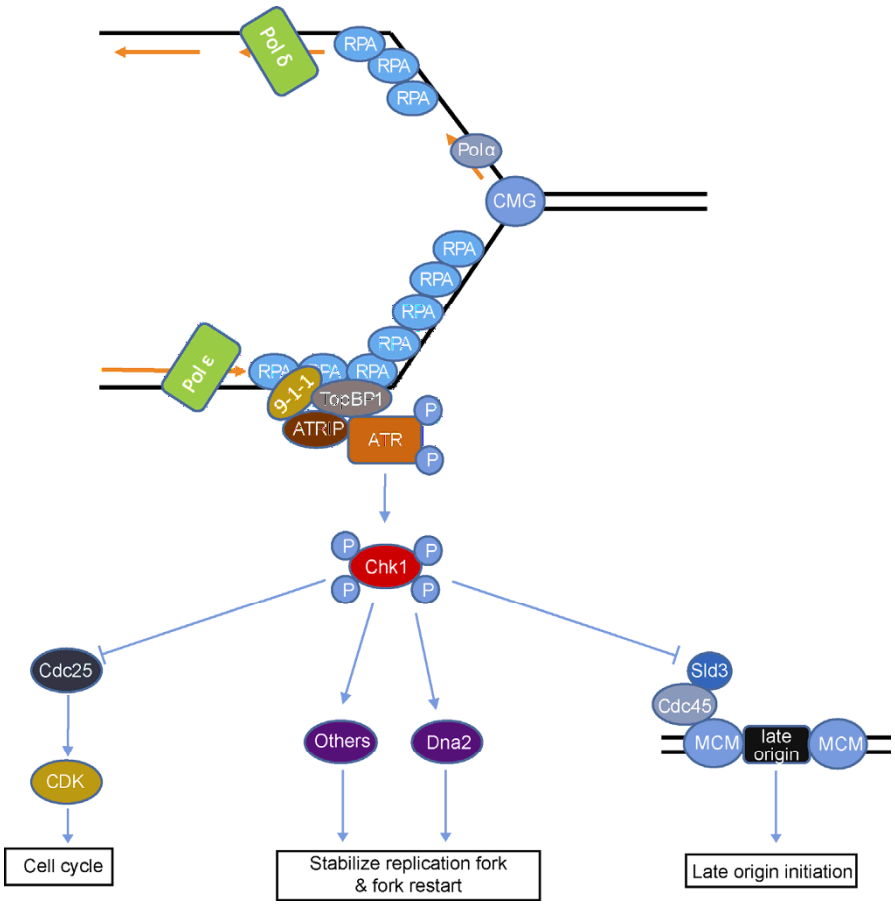

Figure 2 The ATR-Chk1 signaling pathway.

How does checkpoint regulate DNA replication when replication is in stress? (vi) How are replication forks stabilized by checkpoint? All these questions are critical for us to understand eukaryotic DNA replication, fork stability, checkpoint control, and genomic stability. With the development of advanced technology such as deep sequencing and high-resolution imaging, the studies in this research field should be greatly facilitated.

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