Replication initiation: implications in genome integrity

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Abstract: In every cell cycle, billions of nucleotides need to be duplicated within hours, with extraordinary precision and accuracy. The molecular mechanism by which cells regulate the replication event is very complicated, and the entire process begins way before the onset of S phase. During the G1 phase of the cell cycle, cells prepare by assembling essential replication factors to establish the pre-replicative complex at origins, sites that dictate where replication would initiate during S phase. During S phase, the replication process is tightly coupled with the DNA repair system to ensure the fidelity of replication. Defects in replication and any error must be recognized by DNA damage response and checkpoint signaling pathways in order to halt the cell cycle before cells are allowed to divide. The coordination of these processes throughout the cell cycle is therefore critical to achieve genomic integrity and prevent diseases. In this review, we focus on the current understanding of how the replication initiation events are regulated to achieve genome stability.

1. Pre-replication complex and Origin licensing

Accurate DNA replication is critical for achieving genome integrity and cell survival. The assembly of the pre-replication complex (pre-RC), which is the very first step required for DNA replication, is a stepwise event that starts with the binding of ORC (Origin Recognition Complex) to the origins on the DNA during G1 phase [1, 2]. ORC then recruits Cdc6, Cdt1, and finally the MCM (minichromosome maintenance) 2-7 complex, component of the DNA replicative helicase, to the origin. An origin is considered "licensed" once the MCM2-7 double hexamer is loaded, which can later fire to initiate replication during S phase (Figure 1) [3-5].

Origin Recognition Complex (ORC) is a heterohexameric protein complex that recognizes DNA replication origins and serves as the initiator of DNA replication [6]. Six subunits of ORC were named Orc1 through Orc6 in descending order of their molecular mass in *S. cerevisiae*, where they were first identified. Since their discovery in *S. cerevisiae*, the orthologs of all six subunits have been identified in many other eukaryotic organisms, and ORC's function as the replication initiating factor is highly conserved across species [7-15]. Orc1-5 belong to AAA+ (ATPases Associated with diverse cellular Activities) family proteins with conserved AAA+ fold, including Walker A and Walker B motifs, suggesting their ATP binding ability and ring-shaped protein complex formation [16-18]. Orc1-5 also contain winged-helix (WH) domain at their C-termini. Orc6, albeit a member of ORC, does not contain such structural features. Structural and sequence analysis in *Drosophila* and human revealed that N-terminus of Orc6 contains two cyclin-box folds that are homologous to the DNA binding domain of transcription factor TFIIB [19-21]. The C-terminus contains a helix region that is required for Orc6 binding to ORC complex [22, 23].

The role of ORC proteins in establishing preRC at the origins is critical for the maintenance of genome integrity, as the distribution and density of origins must be sufficient to support the replication of the entire genome so that no region is left un-replicated. However, the origin selection program, especially in higher eukaryotes, is very complicated and remains an active field of research [24]. Early studies in *S. cerevisiae* for replication initiation sites revealed a conserved DNA sequence called autonomously replicating sequence (ARS) [25]. The ARS comprises one A element and three B elements (B1, B2, B3), which are essential for origin function [26]. The cryo-EM structure of ORC binding to ARS explained sequence-specific DNA recognition of ORC [27]. It was further revealed that this specificity lies within a 19-amino acids helix of Orc4 [28]. However, *S. cerevisiae* appears to be the only eukaryote with a well-defined DNA origin consensus sequence. In *S. pombe*, origins are AT-rich clustered stretches [29-31]. Origins in higher eukaryotes do not show any features at the level of a defined DNA sequence. Metazoan ORC binds to DNA promiscuously and replication can initiate from random sequences [32-34], suggesting that origins may be defined by 3-dimensional structures of DNA, chromatin environments, or interactions with other proteins. Before the publication of the first genome-scale study in 2008, only about 20 origins had been identified and categorized in the entire human genome [35, 36]. Certain determinants in metazoan origin selection have been revealed by genome-wide studies in the past decade [37]. Nucleosome positioning is an important factor, as metazoan origins, like in yeast, are maintained as nucleosome-free regions [38-41]. In human cells, it was demonstrated that origins correlate with DNase I-hypersensitive regions [42]. Similarly, genome-wide mapping of origins also discovered that origins are located near transcription start sites (TSSs), active promoters, CpG islands, and G-quadruplexes in metazoan [35, 43-49]. However, none of these factors is strictly required for origin selection. A recent study also defined “core origins” in human and mouse genomes and showed that they shared a G-rich sequence signature [50]. The licensing of origins in different chromatin regions, especially heterochromatin, has been an important area of investigation, as cells must be able to license origins across the entire genome regardless of the chromatin status. It is known that some of the ORC subunits localize to specific heterochromatin regions such as centromere and telomere [51, 52]. In terms of origin licensing, other proteins may also be required in facilitating the licensing in certain heterochromatin regions. TRF2 (telomeric repeat-binding factor 2) is known for protecting telomere as one of the shelterin complex subunits. TRF2 also recruits ORC to telomeric regions for facilitating licensing through the interaction with Orc2 [53]. Another well-known example is ORCA (ORC-associated, also known as LRWD1), which can recruit ORC to chromatin [54]. ORCA facilitates the licensing in certain heterochromatin regions [55]. Moreover, ORCA is also critical for heterochromatin organization as well as homologous recombination at telomeric regions [56, 57]. Thus, ORC and its associated proteins are equally important for the maintenance of genome integrity.

In recent years, a debate about whether ORC is an essential factor for origin licensing has been brought up by a study showing that human cells remain viable with no dramatic defect in replication after the depletion of Orc1 or Orc2 by CRISPR-mediated knockout [58]. This study challenged the long-standing view that ORC is the essential initiator of DNA replication. It is possible that partial ORC rings are still functional and able to load MCMs for proper replication; it is also possible that there exists an ORC-independent licensing system, but there is no evidence supporting this idea [59]. A follow-up study depleting another ORC subunit Orc5 or co-depleting Orc2/Orc5 showed that ORC is dispensable for replication [60]. However, other studies continue to suggest the essentiality of ORC using genome-wide or ORC-specific CRISPR screens [61-63]. It is also important to note that these different studies used different approaches and experimental systems to deplete and examine ORC's role. Future studies are necessary to address this critical question.

After ORC binds to the origin, Cdc6 is the next factor to associate with ORC. Cdc6 is part of the pre-RC, that functions downstream of ORC and is important for the loading of MCM onto the chromatin [64-68]. Like Orc1-5, Cdc6 belongs to the AAA+ family and has ATPase activity [16, 69]. Evidence from yeast to human, all point that Cdc6's ATPase activity is critical for the regulation of its pre-RC function [70-73]. It has the most extensive sequence similarity with Orc1 and they are suggested to be paralogs [18, 74]. Cdc6 directly interacts with ORC *in vivo* and *in vitro* [75, 76]. Cdc6 associates with chromatin-bound ORC in an ATP-dependent manner [71, 77, 78]. After ORC binds to the DNA, Cdc6 is recruited and docks into the Orc1/Orc2 gap, forming the ORC-Cdc6 complex and closing the ring to encircle DNA [79-81]. Therefore, Cdc6 provides the sixth AAA+ fold protein to the pentameric ORC1-5, establishing the classic hexameric AAA+ complex toroid configuration.

After the formation of the ORC-Cdc6 complex on origin DNA, MCM2-7 (Minichromosome maintenance 2-7) hexamer can then be loaded with the help of Cdt1 (Cdc10-dependent transcript 1), which acts as a molecular chaperone for MCM [82].

Cdt1 was recognized as an initiator of replication due to its ability to induce re-replication [83]. Cdt1 is conserved in eukaryotes from yeast to human and is required for MCM2-7 loading in all eukaryotes tested [84-87]. Cdt1 forms a stable complex with MCM2-7, primarily by interacting with MCM6 subunit [88-91], and helps maintain the integrity of MCM2-7 [91-95]. Moreover, structural studies in *S. cerevisiae* suggested that the binding of Cdt1 to MCM2-7 prevents MCM2/5 gate closure [96-98] which in turn allows the DNA to pass and enter the central channel. In *S. cerevisiae*, Cdt1 has also been reported to be critical for MCM loading to ORC-Cdc6 through its interaction with Orc6 [99-102]. However, it is unknown if this interaction occurs in metazoans. Other reports suggest that in *S. cerevisiae* MCM2-7 itself directly engages with ORC-Cdc6 [95, 103], but Cdt1 helps to overcome an MCM6-dependent autoinhibitory mechanism which would otherwise sterically hinder the MCM docking to ORC-Cdc6 [103]. Together, these studies demonstrated the critical role of Cdt1 in origin licensing.

MCM2-7 hexamer loading marks the final step of the pre-RC assembly on the chromatin [104]. MCM2-7 loading requires the coordinated function of ORC, Cdc6, and Cdt1. MCM proteins were first identified in genetic screens designed to uncover genes that are required for replication in *S. cerevisiae* [105]. The orthologs were subsequently identified in other eukaryotes and shown to have an essential function in replication [106-108]. Biochemical characterization soon revealed that MCM2-7 assemble into a hexameric complex *in vivo* and *in vitro* [109-111]. Similar to Orc1-5 and Cdc6, MCM2-7 belongs to AAA+ family [112]. MCM hexamer is formed in the order of MCM5-MCM3-MCM7-MCM4-MCM6-MCM2, with an open gate between MCM2 and 5 to allow the passage of DNA [113, 114]. As discussed above, Cdt1 helps to stabilize MCM integrity and prevent gate closure. The interaction of Cdc6 to the C-terminus of MCM3 has also been reported to be critical for MCM loading [95]. Following the loading of MCM, both Cdc6 and Cdt1 are ejected in an ATP hydrolysis-dependent manner. An ORC-Cdc6-Cdt1-MCM (OCCM) intermediate can be stabilized by using a nonhydrolyzable ATP analog [81], in a state prior to the loading of the next MCM2-7 hexamer.

The defining step of origin licensing is the formation of stable head-to-head MCM2-7 double hexamer around DNA [93, 115]. The formation of double hexamer has been extensively examined using *S. cerevisiae* proteins [116]. However, there were conflicting results observed using different methods regarding how the loading of the second MCM is achieved. Biochemical studies suggested that two hexamers are loaded independently on two inverted ORC binding sites near each other [95, 117]. In contrast, single molecular experiments showed that two single hexamers are loaded in a sequential way [78], and the loading of the second MCM requires a distinct Cdc6 molecule [96]. The latest cryo-EM experiments identified a key intermediate after OCCM, called MCM-ORC (MO), demonstrating that double hexamer loading is indeed coupled and sequential [118]. In OCCM, the MCM C-terminal domains are in contact with ORC. In the newly observed MO structure, however, ORC binds in an inverted configuration with N-terminal sides of MCM through Orc6 N-terminus. Therefore, it was concluded that the first MCM hexamer loading, coupled with the release of the first set ORC-Cdc6 and Cdt1, creates a distinct binding site and allows the second ORC binding in an inverted orientation. This configuration then recruits the second Cdc6 and allows for the loading of the second Cdt1-MCM2-7 using the same mechanism.

2. Pre-initiation complex and origin firing

Origins are licensed in G1 phase and that makes them ready to fire in S phase. At the onset of S phase, a wave of protein modifications and sequential recruitment regulate the conversion of inactive MCM2-7 complex to active CMG (Cdc45, MCM2-7, GINS) helicase. The CMG helicase, together with polymerases, PCNA, RPA (Replication Protein A), and other essential proteins, initiates DNA unwinding and replication fork establishment (Figure 1). The activation of helicase, like pre-RC assembly, is regulated by a series of events that is highly conserved in eukaryotes.

MCM2-7 hexamer itself has very limited helicase activity. It was later identified that the full helicase activity requires the assembly of Cdc45 and GINS (from the Japanese go-ichi-ni-san that stands for 5-1-2-3, representing Sld5, Psf1, Psf2, and Psf3 subunits) complex with MCM2-7, forming CMG helicase [119-122]. CMG contains a copy of MCM2-7, a single Cdc45, and one GINS tetramer forming a stable 11 subunits helicase. The assembly begins at the G1 to S transition, where MCM double hexamer serves as a platform to recruit DDK (Dbf4-dependent Cdc7 kinase) via interaction by MCM2 and MCM4 [123, 124]. DDK is a cell cycle regulated kinase [125, 126]. Together with S-phase CDK, they are two essential kinases that regulate the assembly of other initiation factors to MCM2-7. DDK phosphorylates multiple MCM subunits including MCM2, 4 and 6 [127-131]. Notably, DDK only targets chromatin bound MCM in the context of a double hexamer but not a single hexamer [132-136], suggesting only the double hexamer configuration is allowed to initiate productive replication. The phosphorylated MCMs then recruit Cdc45 [128]. The recruitment process is facilitated by Sld3 and Sld7 in *S. cerevisiae*, or their functional homologs Treslin and MTBP in humans [137-139]. In addition to Cdc45, GINS is also associated with MCM2-7. The assembly of GINS complex into MCM requires CDK activity and several chaperone proteins. Sld3 and Dpb11 (TopBP1 in vertebrates) facilitate a complex containing GINS, Sld2 (RecQ4 in vertebrates), and Polε to bind to MCM [140-142]. In this process, CDK phosphorylates Cdc45, Sld2, Sld3 and Sld7 to facilitate protein interaction [142, 143]. The protein complex formed at this point is defined as a pre-initiation complex (pre-IC) [144]. In the pre-IC, MCM double hexamer splits into two single hexamers, suggesting they are ready to form bi-directional replication forks [145]. MCM10 is also a critical protein required for origin firing. Although tightly coupled, the pre-IC formation can be separated from the subsequent firing event *in vivo* in cells lacking MCM10 [145], or when MCM10 is omitted in the *in vitro* reconstitution reactions [146].

After the pre-IC formation, MCM10 is recruited by interacting with CMG [147-149]. MCM10 is essential for origin firing not only for activating CMG but also stabilizing the replisome [150-152]. Meanwhile, the single strand DNA binding ability of budding yeast MCM10 is important for stabilizing the origin melting reaction [153]. A recent study further suggested that MCM10 is required for CMG to transit between dsDNA and ssDNA [154]. Importantly, in addition to its role in helicase activation, MCM10 also travels along at the replication fork and stimulates replication elongation [155, 156]. On the other hand, extensive structural and single-molecule studies have elucidated that active CMG translocates on DNA with its N-terminal domain in front and C-terminal motor domain pushing from behind, and ssDNA passing through the middle channel making contact with several MCM subunits [157-160].

3. Dormant origins

An important concept of DNA replication regarding the maintenance of genome stability is that in each G1 phase, there are way more origins that are licensed than the ones that actually fire in the subsequent S phase [161]. These origins, which are inactive during normal replication, are called “dormant origins”. It has been known for a long time that MCM is loaded in excess onto chromatin. Studies from yeast to human pointed out that MCM can be loaded up to 20-fold in excess over the numbers of loaded ORC or replication origins [162-164]. Indeed, it has been shown that DNA replication can occur normally with significantly reduced levels of MCM proteins in the cells [162, 165, 166]. Meanwhile, it was also found that though the replication efficiency is maintained in these MCM-depleted cells, they showed defects in the S-phase checkpoint. Critically, the MCM-depleted cells became hypersensitive to low levels of replication stress that would otherwise be tolerated by normal cells [163, 167]. Thus, the role of these excess licensed dormant origins was demonstrated as a safeguard system when cells are facing replication stress, and the dormant origins only fire when other replication forks fail to finish replication. The amount of dormant origins, or level of origin licensing, is not only correlated to the tolerability of cells to replication stress, but also shown to be critical for the maintenance of stem cell pluripotency [168].

Although the importance of dormant origins is well appreciated, the selection mechanism regarding which subset of licensed origins should be activated while others should stay dormant is poorly understood. It was suggested that a fraction of pre-RC is multi-mono-ubiquitinated on Orc3/Orc5 by an E3 ligase OBI1, which in turn marks those pre-RCs (out of all available pre-RC) for activation [169]. However, the mechanism of how OBI1 selects its substrate is still unknown. Recently, one study made an interesting observation that the parental MCMs inherited from the previous cell cycle have a distinct function from the nascent MCMs [170]. It was shown that the parental MCMs are preferred for forming active CMGs even though both parental and nascent MCMs can form pre-RC on chromatin. On the other hand, nascent MCMs serve to adjust the pace of active CMGs, possibly by acting as physical resistance of replication fork progression. It was therefore proposed that the surplus of licensed MCMs not only provides a backup system upon replication stress, but also acts to manage replication fork speed to actively prevent replication stress-associated DNA damage. Nevertheless, the mechanism that differentiates these two groups of MCMs remains to be elucidated.

4. Licensing checkpoint

As mentioned above, to prevent genome instability, cells should only enter S phase with a sufficient number of licensed origins. It was suggested that there is a "licensing checkpoint" that senses the number of licensed origins and delays or stops S phase entry if there is an insufficient number of origins licensed [171-173]. In untransformed cells, reducing MCM loading by different methods causes the cells to arrest at G1 phase. Importantly, the licensing checkpoint is often defective in cancer cells; where the inhibition of origin licensing leads to genome instability and apoptosis in several cancer cell lines due to their inability to halt from entering S phase [174-176]. Several of these reports link licensing checkpoint to the p53 status as well as cyclinE/CDK2 activity. However, the detailed mechanism of how cells sense the number of licensed origins remains elusive and requires further study. Recently, a study focusing on the re-entry of the cell cycle from quiescence, or G0, showed that MCM loading is slow and reduced in the first G1 phase, suggesting the licensing checkpoint is largely inactive when cells re-enter cell cycle [177]. Further, the chromatin environment was shown to impact origin utilization when quiescent cells reenter the cell cycle [178]. This under licensed condition in the first G1 phase leads to naturally occurring replication stress, as well as hypersensitivity towards genotoxic drugs. Together, the licensing checkpoint is critical for cells to maintain genome integrity and aberrant licensing can lead to cancer development.

5. Mechanisms preventing re-replication

One of the major challenges of DNA replication is to ensure that replication happens once and only once per cell cycle. Refiring of origins during the same S phase can result in re-replication leading to genome instability and chromosomal breakage during the following mitosis. Cells must exert elaborate regulatory events to prevent re-replication from happening, and the major regulatory point is to prevent re-licensing during S phase [179].

In budding yeast, all six ORC subunits remain chromatin-associated throughout the cell cycle [180]. However, in mammalian cells, Orc1 is released from chromatin upon S phase entry in a CDK-dependent manner, gets ubiquitinated by SCF^{Skp2} and degraded via ubiquitin-mediated proteasomal degradation [181-184]. This prevents ORC from executing a second round of licensing. Only at M phase to G1 transition is Orc1 able to re-associate with chromatin and enable origin licensing [185-188]. The cell cycle dependent regulation of Orc1 proves to be one of many mechanisms that allow replication to occur once and only once per cell cycle [189]. As mentioned previously, Orc3 and Orc5 are also ubiquitinated in a cell cycle dependent manner that is critical for origin function. The role of other E3 ligases in regulating pre-RC/pre-IC function, origin firing, and maintenance of genome stability are an area of intense research [190, 191]. In addition, a recent report suggested that budding yeast ORC dimerizes in a cell cycle dependent manner to control licensing [192]. It remains to be seen if human ORC is regulated in a similar way.

S. cerevisiae Cdc6 and its ortholog, Cdc18 in *S. pombe*, are rapidly degraded when cells enter S phase via ubiquitin-mediated proteasomal degradation, after being phosphorylated by S and M-phase CDKs [193-197]. Human Cdc6 is also regulated by CDK but through a different mechanism. Human Cdc6 is targeted for degradation by APC/C-dependent proteolysis [198]. During G1 phase where licensing takes place, Cdc6 is protected from destruction by cyclinE/Cdk2-dependent phosphorylation [199]. In S phase, it was proposed that Cdc6 is phosphorylated by cyclinA/Cdk2 and exported from the nucleus [75, 200, 201]. However, this model was challenged by other studies showing that a fraction of Cdc6 remains chromatin-bound even in S phase [202-204]. In addition, a more recent study showed that Cdc6 is also targeted for degradation by $SCF^{cyclinF}$ complex late in the cell cycle [205]. Therefore, the detailed mechanism regulating Cdc6 during cell cycle remains to be delineated.

The cell cycle-dependent regulation of Cdt1 is the major regulatory point for preventing re-replication. *S. cerevisiae* Cdt1 is controlled by CDK phosphorylation that inhibits its interaction with Orc6 [101], and subject to nuclear export during S phase [206]. In other eukaryotes including humans, however, Cdt1 protein is degraded upon S phase entry [207-209]. Cdt1 is controlled by two independent E3 ligase pathways to ensure its destruction after S phase entry. First, Cdt1 is phosphorylated by CDKs, which leads to its recognition by SCF^{Skp2} E3 ligase for ubiquitination and degradation [210-213]. Second, degradation of Cdt1 is further restricted to S phase by “replication-coupled destruction” through interacting with chromatin-bound PCNA (Proliferating Cell Nuclear Antigen), which is the processivity factor for DNA polymerase at the replication fork [214, 215]. Cdt1 contains a conserved PCNA-interacting protein (PIP) box that mediates its interaction with PCNA; the interaction, in turn, triggers Cdt1 ubiquitination and degradation by $CRL4^{Cdt2}$ E3 ligase [216]. In addition, metazoan Cdt1 is inhibited by direct binding of another cell cycle-oscillating protein, Geminin [217-221]. Geminin levels are elevated in S phase and drastically reduced upon entry in M phase due to degradation by APC/C [222], thus allowing Cdt1 to be active only during G1. Moreover, a recent study uncovered that Cdt1 is also hyperphosphorylated in G2 phase by Cyclin A/CDK1, which inhibits the loading of MCM during G2 phase [223]. This finding provided yet another independent mechanism to prevent re-replication within one cell cycle and maintain genome integrity.

6. Replication initiation and replication stress

A significant threat to genomic and chromosomal stability comes from replication stress. Replication stress is referred to conditions that cause the slowing or stalling of replication fork progression and perturbation of the dynamics of DNA synthesis [224, 225]. Replication stress can be induced by exogenous or endogenous sources. Exogenous causes include ionizing radiation (IR), ultraviolet (UV) irradiation, and chemotherapeutic drugs that lead to DNA damage, interstrand crosslinks, and DNA breaks. These DNA lesions block CMG unwinding or polymerases, causing the fork to stall. Chemical compounds such as hydroxyurea (HU) that result in dNTP depletion/imbalance also induce replication stress by causing uncoupling of DNA polymerase with CMG helicase [226]. Similarly, endogenous DNA damage such as naturally occurring depurination or oxidation can lead to replication stress. A great fraction of endogenous causes of replication stress has resulted from sequence or chromatin features that make replication intrinsically difficult. These include short tandem repeats and microsatellites regions [227], secondary structures such as hairpins or G-quadruplexes [228, 229], centromeres and telomeres regions [230, 231]. Collisions of replication and transcription machinery and R-loop, the three-stranded structures containing a DNA-RNA duplex and the excluded ssDNA, also impede replication progression [232, 233]. Common fragile sites, the chromosomal regions prone to experience replication stress and break upon replication inhibition, usually contain one or several of the above features [234-236]. Importantly, common fragile sites also correlate with origin-poor and ORC-poor regions, indicating that inefficient origin activity and reduced dormant origins cause genome instability [237, 238].

A more direct relationship between replication initiation and replication stress lies in the context of oncogene activation or overexpression-induced replication stress [239, 240]. Early studies identified that the overexpression of cyclin E induces chromosomal instability due to defects in pre-RC chromatin loading [241, 242]. This could be due to short G1/premature S phase entry before sufficient origins are licensed. As discussed above, a reduced number of dormant origins due to insufficient licensing makes cells more vulnerable to replication stress. The common fragile sites represent regions where dormant origins are either inefficiently licensed/activated resulting in replication fork failure and increased susceptibility to breakage [243]. Moreover, overexpression of oncogenes, including cyclin E, Ras, and Myc, have been proven to drive excessive usage of origins that can lead to acute depletion of dNTPs in the cells, resulting in replication stress and genome instability [244, 245]. Increased replication initiation by oncogenes also increased the chance of replication-transcription collision [246]. A recent study using genome-wide mapping further revealed that oncogene overexpression induces a subset of intragenic origins to be fired early due to short G1/premature S phase entry, which greatly increases the replication-transcription collision and DNA breakage [247]. The regulation of transcription activity at origins is also critical for genomic integrity [248].

One direct consequence of replication stress is the formation of exposed ssDNA due to fork stalling. Thus, replication stress is tightly associated with the activation of ATR pathway of the DDR. The activation of ATR starts from RPA loading onto exposed ssDNA, which can be generated by end-resection of DSBs, replication stress, or intermediates during the DNA repair processes [249-255]. RPA-ssDNA then serves as a platform to recruit ATR via its partner protein ATRIP (ATR interacting protein) [256]. Two independent mechanisms then function to activate ATR. The binding of Rad17/RFC and Rad9-Rad1-Hus1 (9-1-1) complexes to the junction of RPA-ssDNA and dsDNA recruits TopBP1, the first ATR-activating domain (AAD)-containing protein, to activate ATR kinase activity [257-260]. On the other hand, ETAA1, the second protein identified with an AAD, binds to RPA directly and activates ATR in parallel to TopBP1 [261-263]. Subsequently, the activated ATR phosphorylates its downstream effector kinase Chk1 [264]. Like the ATM-Chk2 axis, ATR-Chk1 controls many cellular events during DNA damage. One proteomic study identified more than 700 ATM/ATR downstream substrates in human cells, highlighting the complexity of the DDR network [265]. Importantly, many pre-RC and pre-IC proteins have been identified in this study as ATM/ATR substrates, suggesting the existence of intricate regulatory mechanisms in replication initiation or perhaps playing other non-canonical roles. DNA Polymerase epsilon playing a critical role in checkpoint pathway during S phase to sense stalled replication confirms that a link between DNA replication machinery and S-Phase checkpoint exists in eukaryotes [266-268]. Further, Pol ϵ binds replication origins early in S phase and the essential function of Pol ϵ was not dependent on its DNA synthesis activity [269].

ATR signaling also has critical roles in the regulation of replication during S phase. Under genotoxic conditions, checkpoints are activated and the cell cycle is halted. It is well established that replication initiation is targeted for repression by ATR-Chk1 pathway in order to prevent S phase progression and further damage to the DNA until DNA damages are repaired [270-273]. The repression of origin firing by checkpoint also prevents DNA topological stress [274]. Interestingly, the ATR-Chk1 activation inhibits the global origin firing, yet promotes local origins to initiate in order to support the completion of those regions of DNA where replication forks are stalled [275]. However, the detailed mechanism remains elusive. On the contrary, even without exogenous sources of DNA damage, the basal level of ATR-Chk1 activity is critical to control origin activity. Many studies discovered that ATR or Chk1 inhibition causes increased global origin firing and abnormal early activation of late origins [276-281], suggesting an essential function of ATR pathway in regulating unperturbed S phase progression and its importance in genome integrity. On the other hand, deregulation of many pre-RC and pre-IC proteins directly affects ATR signaling pathway activation. Cdc6 has been shown to directly interact with ATR and activate replication-checkpoint [282]. MCM7 reduction leads to ATR activation defects [165]. Further, TopBP1, one of the pre-IC proteins, is also an essential protein for ATR activation [257]. The regulation of DNA replication initiation events and checkpoint signaling pathways remains an active field of study.

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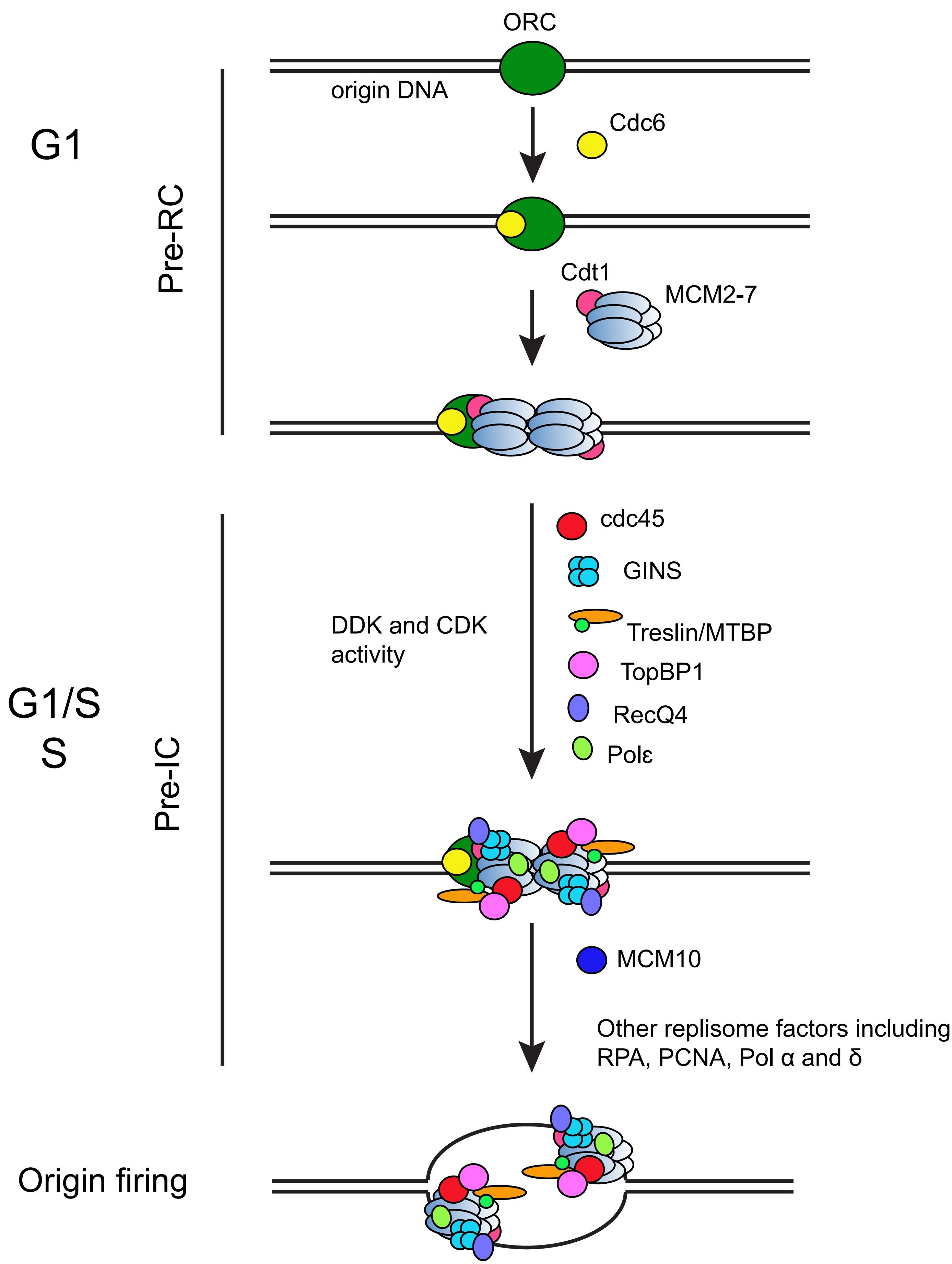

ORC
origin DNA
G1
Cdc6
Pre-RC
Cdt1
MCM2-7
cdc45
GINS
DDK and CDK
activity
Treslin/MTBP
TopBP1
G1/S
S
RecQ4
Pre-IC
Polε
MCM10
Other replisome factors including
RPA, PCNA, Pol α and δ
Origin firing