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# The Coordination between DNA Replication Initiation and Other Cell Cycle Events

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## 1. Introduction

The cell cycle is the process of accurate self-reproduction and proliferation of a cell. It is the basis of the growth, development, heredity and evolution of organisms. Misregulation of the cell cycle may result in malignant cell proliferation, tumorigenesis or cell death. In this chapter, we mainly discuss the coordination regulations between DNA replication initiation and other cell cycle events that ensure genomic integrity. Recent breakthroughs have uncovered more and more DNA replication licensing machinery proteins (ORC, Cdc6, Cdt1, geminin, etc.) functioning in other cell cycle events, including centrosome replication, mitotic events, transcription and so on.

## 2. The connections between DNA replication and other cell cycle events

DNA replication occurs once and only once per cell cycle mainly regulated by DNA replication initiation factors in eukaryotic cells. The pre-replication complex (pre-RC) assembly or the DNA replication licensing is the first step in DNA replication initiation, characterized by the sequential recruitment of ORCs, Cdc6, Cdt1 and MCMs to the DNA replication origins to form the pre-RC at the end of mitosis (Bell and Dutta 2002). The replication licensing is suppressed during G2 phase and mitosis to prevent DNA re-replication within one cell cycle by down-regulating the Cdt1 activity in metazoans achieved mainly by degradation of Cdt1 or through its inhibitor geminin (Nishitani et al. 2006). Geminin inhibits Cdt1 by binding to Cdt1 and interfering with the interaction of Cdt1 and MCM proteins, thus preventing Cdt1 from recruiting MCM2-7 proteins to the replication origins (Wohlschlegel et al. 2000, Yanagi et al. 2002). The precise regulations of pre-RC protein levels and assembly are effective ways to prevent reassembly of *de novo* MCM2-7 onto the replicated origins to re-license and re-replicate the genomic DNA in the subsequent phases of the same cell cycle (Figure 1). In addition to DNA replication licensing, pre-RC proteins are also involved in the centrosome duplication in the S phase, chromosomes alignment and segregation in mitosis and cytokinesis and RNA transcription throughout the cell cycle.

## 2.1 Coordination of centrosome duplication and DNA replication

The centrosome in an animal cell is composed of two centrioles and the surrounding pericentriolar material (PCM). Centrosome duplication and separation also take place once and only once in one cell division cycle as does DNA replication, and accordingly the process of centrosome duplication and separation is recognized as the centrosome duplication cycle or centrosome cycle. Mis-regulation of centrosome duplication causes multiple centrosomes, multipolar spindle and chromosome misalignment. Centrosome duplication initiates simultaneously with the initiation of DNA replication. Both centrosome duplication and chromosome replication have to be coordinated to produce proper centrosome numbers for a normal cell cycle. Although the coordination between these two cycles has been noticed for a long time (Mazia 1987), the underlying mechanism is still largely unknown.

### 2.1.1 The centrosome cycle

Through electron microscopy (Kuriyama and Borisy 1981), the centrosome cycle has been recognized to comprise the following steps: the centriole disengagement, the centriole duplication and elongation, and the centrosome maturation and separation. From the mitotic exit to the early G1 phase, the centrioles change their orthogonal configuration and are in preparation of a pre-duplication state (Alvey 1985, Piel et al. 2000). The centriole duplication initiates with the nucleation of the daughter centrioles at the late G1 phase and elongates during the S and G2 phases, resulting in two new centrosomes paired in one PCM. The maturation and separation of the two centrosomes occurs during the G2/M transition along with the chromatin condensation. Considering the behaviour of the centrosome cycle, it might be possible that there is a licensing mechanism for regulating that centrosome duplication occurs once per cell cycle similar to the DNA replication licensing (Figure 1). Several proteins possibly function in the licensing process of centrosome duplication. Overexpression of Plk4, nucleophosmin (NPM/B23) and SAS-6 leads to centrosome amplification (Bettencourt-Dias et al. 2005, Habedanck et al. 2005, Leidel et al. 2005, Dammermann et al. 2004). The expression level of the Plk4 protein peaks at mitosis and is minimal in the G1 phase (Fode, Binkert and Dennis 1996). It is possible that the protein level of Plk4 is strictly down-regulated after centrosome duplication starts in order to avoid centrosome re-duplication in one cell cycle, similar to the down-regulations of the DNA replication licensing proteins Cdt1 and Cdc6 after DNA replication initiation in S and G2 phases. B23 (also named as Nucleophosmin (NPM)), a multifunctional nucleolar protein, is also probably involved in the licensing system of the centrosome duplication. B23 partially binds to unduplicated centrosomes in the G1 phase, dissociates from the centrosomes by cyclin E-CDK2 phosphorylation at the late G1 phase and triggers centriole duplication initiation (Okuda et al. 2000, Tokuyama et al. 2001). In S and G2 phases, B23 is prevented from re-association with centrosomes due to phosphorylation until mitosis (Okuda et al. 2000, Tokuyama et al. 2001, Zatsepina et al. 1999). Induction of the unphosphorylated B23 by microinjection of anti-B23 monoclonal antibody or expression of the non-phosphorylated form of B23 results in persistent centrosome binding of B23 and inhibits centrosome duplication initiation at the very early step of the centriole disengagement (Okuda et al. 2000, Tokuyama et al. 2001). SAS-6 is a coiled-coil protein which localizes to centrosomes and is recruited to centrioles at the onset of the centrosome cycle (Leidel et al. 2005). Centrosome duplication once per cell cycle requires the activity of SAS-6. Overexpression of SAS-6 results in excess foci-bearing centriolar markers, while RNAi knockdown of this protein interferes with the normal centrosome duplication (Leidel et al. 2005).

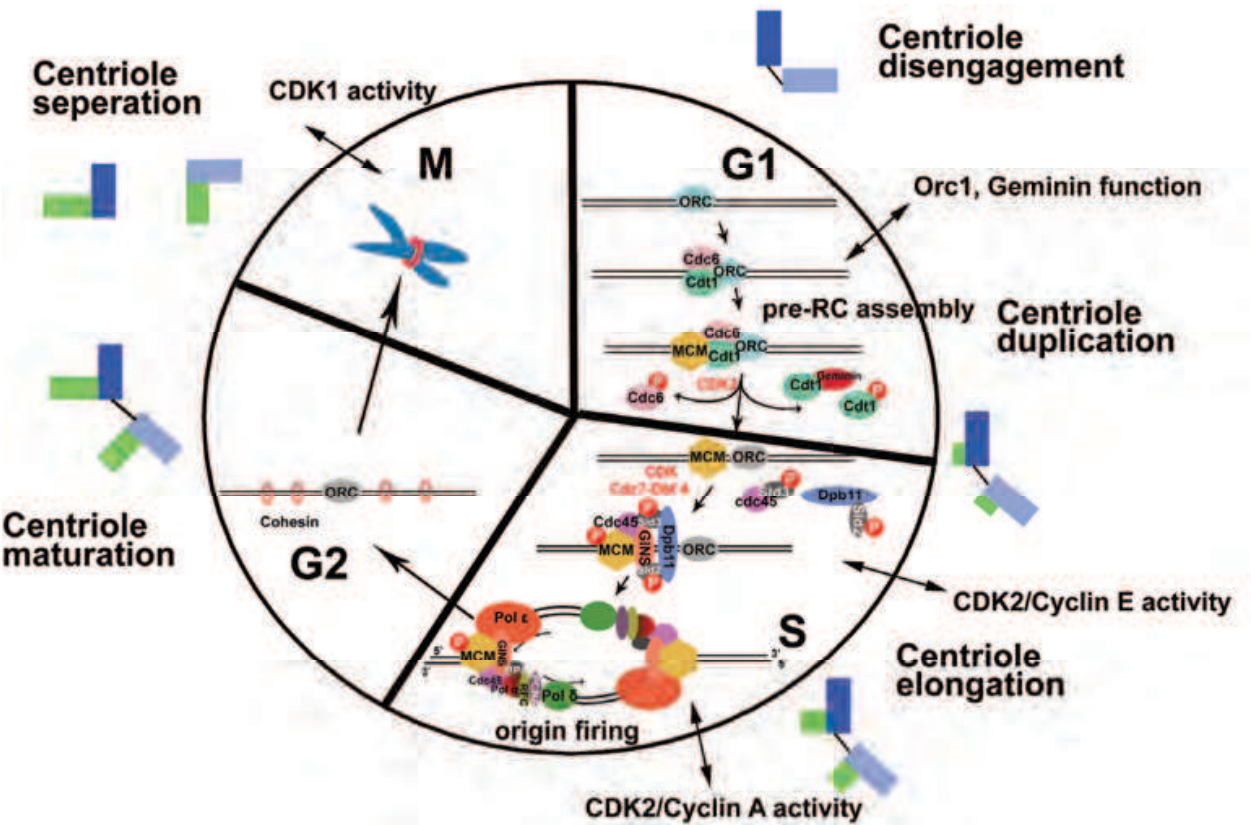


Fig. 1. The DNA replication cycle and the centrosome duplication cycle

The DNA replication cycle is shown inside the circle, while the centrosome replication cycle is shown outside the circle. The proteins with double-arrows denote their function in coordinating both cycles. **Inside the circle.** At the late M and G1 phases, ORC, Cdc6 and Cdt1 recruit MCM helicases to the replication origins to form pre-replication complex (pre-RC). Once the pre-RC is assembled, the origin is licensed to replicate. Upon entry into the S phase, Cdc45 and GINS are recruited to the replication origins dependent on Dpb11, sld2 and sld3 under the regulations of CDK2/cyclin E and Cdc7 kinases. The phosphorylated MCM2-7 helicase, together with Cdc45 and GINS, forms a CMG complex and functions to unwind the DNA replication origin site. Subsequently, Pol ε and Pol δ are recruited to the replication fork, and DNA replication initiates. The replicated DNA duplexes are held together by cohesin in the G2 phase and separate in mitosis; **Outside the circle.** During the G1/S transition in the same cell cycle, centrosome duplication initiates from centriole disengagement and takes place during the S phase. CDK2/cyclin E activity is required for centrosome duplication initiation. The replication licensing factors ORC1, geminin and maybe the others function by preventing the centrosome re-duplication after centrosome duplication initiation. Centriole elongation and maturation occur during the S and G2 phases. At the G2-M phase, the duplicated centrosomes are segregated to form the poles of the bipolar spindles to execute mitosis.

In addition to centrosome-localized proteins which may be involved in the licensing of the centrosome cycle, some other regulators including the DNA replication licensing system proteins may also participate in the licensing process of the centrosome cycle.

### **2.1.2 The roles of the DNA replication licensing system proteins in Centrosome duplication**

Accumulated evidences show that several DNA licensing system proteins and regulators also play important roles in the centrosome duplication licensing system. ORC2, one of the origin recognizing complex proteins for DNA replication initiation is reported to localize to centrosomes throughout the cell cycle. ORC2 depletion leads to abnormal centrosome copy numbers, chromosomes misalignment and multipolar spindle in addition to DNA replication defects (Prasanth et al. 2004). In addition, transfected ORC1 is also found to localize to centrosomes. When co-expressed with the cyclin A in cells, the centrosomal localized proportion of the transfected ORC1 is elevated. ORC1 controls centrosome duplication through cyclin E to prevent reduplication of centriole and centrosomes. Depletion of ORC1 results in increased cyclin E level and cyclin E-dependent centriole reduplication. Accordingly, cyclin E can override the ORC1 inhibition of centrosome reduplication, rather than cyclin A or cyclin B. Simultaneous depletion of cyclin E and ORC1 inhibits the reduplication of centrioles caused by ORC1 depletion (Hemerly et al. 2009). Moreover, MCM5 also localizes to centrosomes depending on its interaction with CLS domain of cyclin E and prevents centrosome over-duplication in S phase-arrested cells by interacting with cyclin E (Ferguson and Maller 2008).

Geminin is a DNA replication licensing inhibitor. Through targeting Cdt1 and interfering with Cdt1-MCM interaction, geminin prevents the recruitment of MCM2-7 by Cdt1 to the chromatin. Geminin is targeted for destruction by APC in M phase (McGarry and Kirschner 1998) and accumulates in late G1 phase, S phase and G2 phase when APC is inactivated. Consequently, DNA replication licensing is prohibited in S phase and G2 phase largely by the geminin inhibiting mechanism in metazoans (Wohlschlegel et al. 2000). Depletion of geminin leads to substantial re-replication in primary cells and mouse embryos (Melixetian et al. 2004, Gonzalez et al. 2006).

Recent studies show that DNA replication licensing inhibitor geminin might also function in the centrosome duplication licensing system as an inhibitor. Geminin-depleted cells show over-duplication of centrosomes without the passage through mitosis, suggesting that geminin might function as a licensing inhibitor of centrosome duplication in a similar manner to its function in DNA replication licensing during S and G2 phase (Tachibana et al. 2005). We further found that geminin is also localized to centrosomes through the mediation of Arp1, one subunit of the dynein-dynactin complex. The centrosomal localization of geminin is dependent on the integrity of the dynein-dynactin complex and intact microtubules. The coiled-coil domain of geminin is responsible for its centrosome localization and interaction with Arp1 and is required for the inhibition of centrosome re-duplication (Lu et al. 2009). Although a number of reports (Hemerly et al. 2009, Ferguson and Maller 2008, Tachibana et al. 2005, Lu et al. 2009) indicate that the same partners in DNA licensing play roles in centrosome duplication, their functional cooperation in centrosome duplication and preventing re-duplication are not yet described.

### **2.1.3 The roles of the key regulators of the DNA replication licensing system in centrosome duplication**

The key regulators that prevent DNA re-replication, such as the S phase kinase CDK2, also regulate centrosome duplication and reduplication. High CDK2 activity in S phase prevents the pre-RC reassembly through different ways in different organisms. In yeast, the rising

CDK2 activity at the onset of the S phase prevents DNA replication relicensing by targeting and inactivating all the initiation proteins of the licensing system in different ways. ORC2 and ORC6 are phosphorylated and inhibited by CDK2 (Nguyen, Co and Li 2001). Cdc6 in *S. cerevisiae* (or Cdc18 in *S. pombe*) is phosphorylated by CDK2 and subsequently degrades in S phase after licensing (Jallepalli et al. 1997, Elsasser et al. 1999). Cdt1 in *S. pombe* is subject to degradation following CDK2 phosphorylation with a similar fate to Cdc6. In *S. cerevisiae*, MCM2-7 exports out of the nucleus by CDK2 phosphorylation (Nguyen et al. 2000). Cdt1 is also excluded from the nucleus by association with MCM2-7 during S, G2 phases and early mitosis (Tanaka and Diffley 2002). All these CDK2 dependent controls prevent the access of these licensing factors to the chromatin and thus prevent relicensing during the cell cycle effectively. These controls by CDK2 are redundant, for all the controls have to be destroyed simultaneously in order to induce significant re-replication (Nguyen et al. 2000). In metazoans cells, CDK2/cyclin A interacts with and phosphorylates ORC1 (Mendez et al. 2002). Excess Cdc6 is translocated to the cytoplasm in the S phase due to CDK2 phosphorylation (Saha et al. 1998). Cdt1 is targeted for destruction via the SCF<sup>skp2</sup> ubiquitin pathway by CDK2 phosphorylation (Takeda, Parvin and Dutta 2005). There is evidence suggesting that the phosphorylation of proteins by cyclin A-CDK1/CDK2 is responsible for blocking re-replication in Emi (early mitotic inhibitor) depletion induced re-replication (Machida and Dutta 2007).

Cyclin E and cyclin A, the activators of CDK2, have also been implicated in regulating centrosome duplication by targeting likely centrosome duplication licensing proteins and coupling the initiation of centrosome duplication and DNA replication initiation. Cyclin E localizes at the centrosome through its centrosome localization signal (CLS), and CDK2/cyclin E activity is required for centrosome duplication. Studies in S phase frog egg extract support multiple rounds of centrosome reproduction and found that inactivation of CDK2/cyclin E blocks centrosome reduplication (Hinchcliffe et al. 1999, Matsumoto and Maller 2004). Notably, B23 is identified to be a substrate of CDK2/cyclin E in centrosome duplication. CDK2/cyclin E phosphorylates threonine 199 of B23 and releases B23 from the unduplicated centrosomes to initiate centrosome duplication (Okuda et al. 2000, Tokuyama et al. 2001). Besides, cyclin E interacts directly with MCM5 through its CLS domain and recruits MCM5 to the centrosomes. Over-expressing MCM5 or the domain of MCM5 which is responsible for cyclin E interaction inhibits the centrosome re-duplication of S phase arrested cells (Ferguson and Maller 2008). All these data indicate that CDK2/cyclin E functions in centrosome duplication. The possible mechanism will be that, at the late G1 phase, CDK2/cyclin E phosphorylates and releases its substrates including B23 from the unduplicated centrosome to initiate centrosome duplication; during the progress of the centrosome duplication in the S phase, CDK2/cyclin E activity is not needed and suppressed by another series of proteins such as ORC1 and MCM5 as reported (Hemerly et al. 2009, Ferguson and Maller 2008). So far the reported inhibitor proteins for CDK2/cyclin E are mostly DNA replication licensing proteins. These inhibitor proteins, which are either promoted by CDK2/cyclin A to localize at the centrosome, such as ORC1, or directly phosphorylated and recruited to centrosome by CDK2/cyclin E such as MCM5, are enriched at the centrosomes to suppress CDK2/cyclin E activity and prevent centrosome reduplication (Figure 2). Accordingly, depletion of these proteins such as ORC1, ORC2 and geminin leads to centrosome reduplication and multiple centrosome copy numbers

(Hemerly et al. 2009, Prasanth et al. 2004, Tachibana et al. 2005). Notably, it has been demonstrated that centrosome reduplication by ORC1 depletion is in a cyclin E dependent way, and cyclin E could override the prevention of ORC1 on centrosome over-duplication (Hemerly et al. 2009). Depletion of ORC2 and geminin also resulted in centrosome reduplication (Prasanth et al. 2004, Tachibana et al. 2005). It is not clear if ORC2 and geminin cooperate with cyclin E to regulate centrosome duplication. In contrast to their licensing roles in DNA replication, ORC1, geminin, MCM5 and so on, take an inhibitory role for centrosome over-duplication. The fact that overexpression of these proteins could inhibit centrosome re-duplication in S phase arrested cells is probably by suppressing the constant high CDK2/cyclin E activity in S phase-arrested cells.

The possible mechanism of centrosome duplication initiation and prevention of centrosome reduplication involves two subsets of proteins and is likely to be separated into two steps. One subset of the licensing proteins including B23 associates with the unduplicated centrosome and licenses it to duplicate. Upon phosphorylation by CDK2/cyclin E, the licensing proteins are dissociated from the centrosome to allow it to initiate duplication. Persistent association of these proteins with the centrosome will inhibit the initiation of centrosome duplication. Another subset of licensing proteins for centrosome duplication plays an inhibitory role to prevent relicensing of the centrosome duplication, probably by suppressing the kinase activity of CDK2/cyclin E (Figure 2). It is likely that this subset of proteins, mostly the DNA replication licensing proteins including ORC1, ORC2, MCM5 and geminin, coordinates DNA replication and centrosome duplication in the same cell cycle. CDK2/cyclin A is also required for centrosome duplication. Depletion of CDK2 or cyclin A and cyclin E abolishes centriole separation (Lacey, Jackson and Stearns 1999). Cyclin A also directly interacts with MCM5 and ORC1. Persistent centrosome localization of MCM5 is dependent on cyclin A (Ferguson, Pascreau and Maller 2010). These results indicate that cyclin E and cyclin A sequentially function in centrosome duplication. CDK2/cyclin E initiates centrosome duplication by phosphorylating its substrates, such as B23. CDK2/cyclin A subsequently prevents centrosome re-duplication by phosphorylating MCM5, ORC1 and possibly additional DNA replication licensing proteins and targeting them to the centrosome to prevent it from reduplication through suppressing CDK2/Cyclin E activity (Figure 2). Moreover, Rb and E2F are also involved in both DNA replication and centrosome duplication (Meraldi et al. 1999). These functions may be performed by regulating gene transcription of DNA replication licensing proteins.

In summary, the centrosome duplication cycle and the DNA replication cycle in a cell are coordinated tightly to occur once and only once per cell cycle. These two cycles take place in the same time window with initiation during the late G1 phase, proceeding in the S phase and inhibition of re-duplication in S and G2 phases. They use the same licensing proteins and are subject to the same regulators of CDK2/cyclin E and CDK2/cyclin A. The mechanism for DNA replication licensing is well understood, while the detailed mechanism for centrosome duplication licensing and initiation remains unclear. Despite the identification of several DNA replication licensing proteins and kinases involved in centrosome duplication, how these licensing proteins and kinases additionally regulate centrosome duplication licensing and duplication processes, especially how cyclin E and cyclin A in the S phase sequentially regulate the same apparatus of DNA replication licensing proteins to coordinate the DNA replication and the centrosome duplication, remains largely unknown.

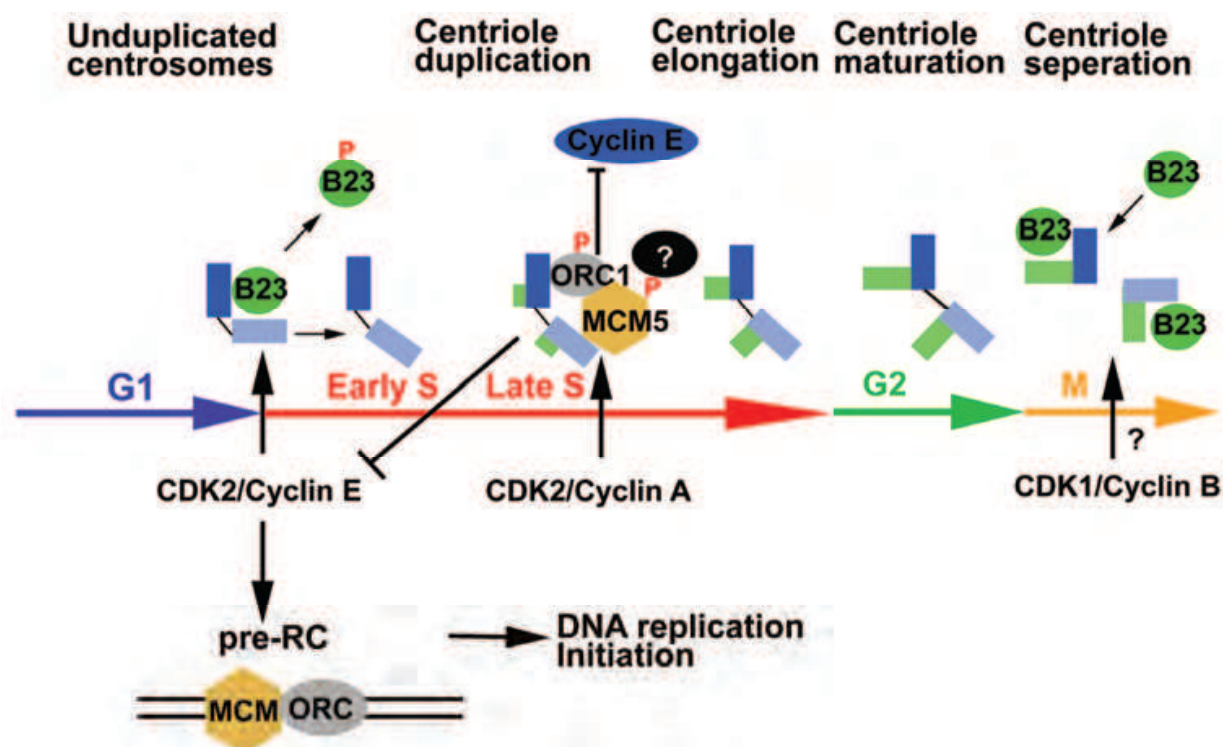


Fig. 2. An emerging model for centrosome duplication licensing

Centrosome duplication initiates during the late G1 phase. CDK2/cyclin E phosphorylates its substrates including B23 to release them from the unduplicated centrosome to initiate centrosome duplication. CDK2/cyclin E also promotes the transition of pre-RC to pre-IC and activates DNA replication initiation along with initiation of centrosome duplication. During the process of centrosome duplication in S phase, CDK2/cyclin A comes to prevent centrosome re-duplication by phosphorylating MCM5, ORC1 and other DNA replication licensing proteins and targeting them to the centrosome to suppress the local CDK2/Cyclin E activity. Once the centrosomes separated after duplication in mitosis, B23 relocates to the centrosomes to prepare for a new round of duplication in the next cell cycle, possibly regulated by CDK1/cyclin B phosphorylation.

## 2.2 The coordination of mitotic events and DNA replication

To ensure genomic integrity, the cell enters mitosis only when it has finished its DNA replication. This coordination between DNA replication and mitosis is controlled partially by checkpoints, including the "intra-S phase checkpoint" when DNA damage occurs and the "S-M checkpoint" in a normal cell cycle which ensures DNA replication completes before mitotic entry. Accumulating evidences show that lack of DNA replication licensing proteins causes aberrant mitotic cells and implicate that the DNA replication licensing proteins directly coordinate both DNA replication and mitosis.

### 2.2.1 The mitosis events and their key regulators

Mitosis is the process by which a eukaryotic cell segregates its chromosomes in its nucleus into two genetically identical daughter sets to two nuclei. The mitosis is generally followed by cytokinesis to faithfully separate the two nuclei and the cytoplasm with its organelles and cell membranes into daughter cells. This process of the mitosis is achieved by elaborate

regulatory mechanisms and apparatus assembly during the process. The central molecular engines coordinating mitosis are a series of mitotic kinases, including CDK1/cyclin B, Aurora kinase and Polo-like kinase (Plks) and their partner phosphatases (Figure 3).

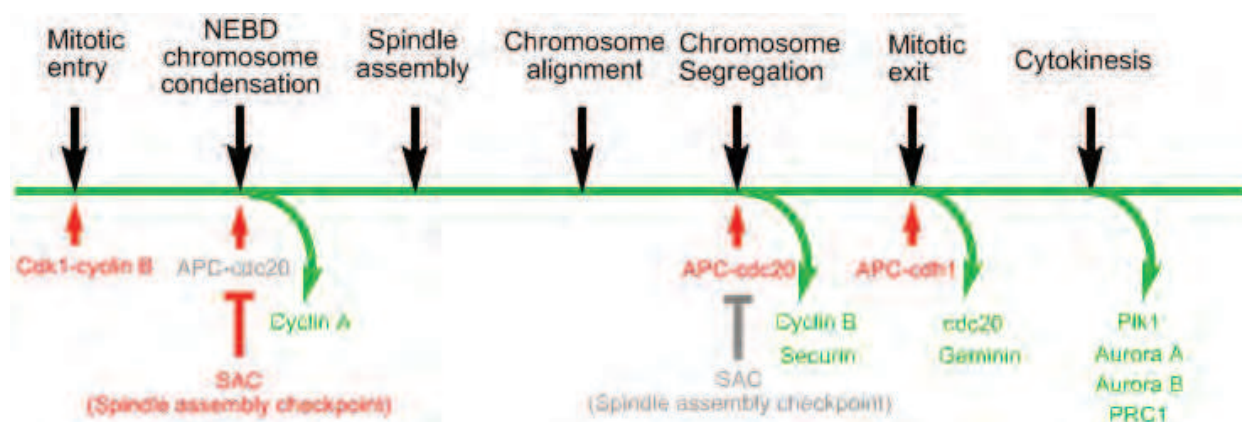


Fig. 3. The mitotic events and its key regulators

The orderly activation and destruction of different regulators and accordingly the orderly progression of the mitotic events are shown. Red arrows denote the activated CDK or APC; green arrows denote degraded proteins. At mitotic entry, CDK1-cyclin B is activated. During the nuclear envelope breakdown (NEBD), APC-cdc20 is partially activated to degrade cyclin A, yet is mostly inhibited by Spindle assembly checkpoint (SAC) (the red SAC stands for the activated SAC and the grey APC-cdc20 stands for the inhibited APC-cdc20). After the onset of the anaphase, most SAC proteins are inactivated, while the APC-cdc20 is activated to degrade its substrate proteins including cyclin B and securin (the red APC-cdc20 stands for the activated APC-cdc20 and the grey SAC for the inactivated SAC). Soon after securin degradation, separase activity is released to cleave cohesin and initiate segregation of the sister-chromatids. Cyclin B1 degradation inactivates CDK1 and APC-cdc20, and activates the second APC activator cdh1. APC-cdh1 then targets additional substrates including cdc20 and geminin for degradation during mitotic exit. During cytokinesis, Plk1, Aurora kinases and PRC1 are degraded by APC-cdh1.

CDK1 is activated by cyclin B. Cyclin B translocates into the nucleus after the G2 phase checkpoint prior to mitosis and triggers mitotic entry initiation (Toyoshima-Morimoto et al. 2001). Cyclin B is not destroyed until anaphase by the anaphase promoting complex or cyclosome (APC/C). During the interval from mitotic entry to anaphase, active CDK1/cyclin B promotes chromosome condensation, spindle assembly and chromosome segregation sequentially, and prevents the onset of cytokinesis until anaphase. Through cyclin B1 degradation by APC/C during the transition from metaphase to anaphase, CDK1 is inactivated, and accordingly, mitotic exit and cytokinesis of the cell take place to generate the two genetically identical daughter cells (Malumbres and Barbacid 2009).

Aurora kinases are a family of serine-threonine protein kinases, consisting of Aurora A, Aurora B and Aurora C in mammals. Aurora A localizes to centrosomes during the G2 phase and mitosis and distributes to the mitotic spindle in mitosis. In contrast, Aurora B localizes to centromeres and chromosome arms in early mitosis, concentrates further on centromeres in prometaphase, relocates to spindle midzone in anaphase and concentrates at the midbody of chromosomes at telophase and cytokinesis. Both Aurora A and Aurora B are targets of APC/C and are destroyed during mitotic exit (Littlepage and Ruderman 2002,

Stewart and Fang 2005). Aurora A promotes centrosome maturation and separation, spindle assembly and mitotic entry, while Aurora B regulates chromosome condensation and segregation, metaphase to anaphase transition and cytokinesis. Despite the high similarity in protein sequences and structures between Aurora A and B, Aurora kinases exhibit different subcellular localization and correspondingly divergent functions in mitotic events. We found that a single amino acid residue change is adequate to convert Aurora A to Aurora B in partners binding and cellular function (Fu et al. 2009).

Polo-like kinases (Plks) also comprise a family of serine/threonine kinases. The family members include Plk1, Plk2, Plk3 and Plk4 in vertebrates, although they differ in structure and function. The Plk protein consists of a Polo-box domain (PBD) responsible for substrate recognition and a kinase domain for catalyzing its substrate. Plk1 is the best known member of the Plks (Sunkel and Glover 1988, Llamazares et al. 1991, Strebhardt and Ullrich 2006). It functions essentially in mitosis, regulating a variety of the mitosis events including bipolar spindle formation, chromosome segregation, centrosome maturation, CDK1 activation, APC regulation and cytokinesis execution. During mitotic exit, Plk1 is degraded by APC-cdh1 (Lindon and Pines 2004). Plk1 recognizes its substrates by consensus recognition sequences in the PBD domain and usually requires a phosphopriming by prime kinases such as CDKs and mitogen-activated protein kinase. From prophase to metaphase, Plk1 phosphorylates INCENP, BUB1 and Neddl after phosphopriming by CDK1 (Zhang et al. 2009). However, from anaphase to cytokinesis when CDK1 is inactive, Plk1 promotes its own recognition of the substrates such as central spindle proteins MKLP2 and PRC1 (Carmenta et al. 1998).

### **2.2.2 The roles of the checkpoints in coordination of DNA replication with mitosis**

Coordination between DNA replication and mitosis is executed by different checkpoints. When the DNA double strand break (DSB) occurs in S phase, unfired replication origins are specifically inhibited by “intra-S phase checkpoint” to acquire S phase delay. Two pathways are involved in the intra-S phase checkpoint. One is the ATM-MDC1-MRN (especially NBS1) dependent phosphorylation of SMC1 (structural maintenance of chromosomes-1) (Yazdi et al. 2002), although how the phosphorylated SMC1 interferes with DNA replication is unclear. Another one is ATM/ATR-mediated Cdc25A phosphatase proteolysis that inhibits CDK2-cyclin E/cyclin A kinase activity. The inhibited CDK2-cyclin E/cyclin A in turn prevents the loading of Cdc45, the key co-activator of DNA helicase MCM 2-7, to the unfired replication origins and thereby inhibits the DNA replication (Falck et al. 2002).

Another “DNA replication checkpoint” also functions during DNA damage in S phase by stalling the replication fork to delay the S phase. This checkpoint is mainly mediated by ATR/CHK1 activation. Although the mechanism for this checkpoint and the substrates of ATR/CHK1 are poorly understood, the ATR/CHK1-dependent CDK2-cyclin E/cyclin A inhibition through Cdc25A degradation at least partially contributes to the slow down of the overall replication rates. Many DNA replication proteins at the replication forks including RPC (replication factor C complex), RPA1/2, the MCM2-7 complex, MCM10 and several DNA polymerases are phosphorylated by ATR (Cortez, Glick and Elledge 2004, Liu, Kuo and Melendy 2006); however, the functions of these phosphorylation events are largely unclear.

Besides the checkpoints in response to DNA damage mentioned above, the “S-M checkpoint” is also an intrinsic mechanism required for normal cell cycle progression. The S-M checkpoint ensures that cells faithfully finish genome replication before entry into mitosis. The S-M checkpoint is mediated by ATR and prevents mitotic entry by inhibiting

CDK1/cyclin B kinase activity. In *Xenopus* egg extracts, ATR depletion may result in early mitotic entry without completing DNA replication (Hekmat-Nejad et al. 2000). CHK1-Knocking out in ES cells also causes premature mitotic entry with incomplete DNA replication (Niida et al. 2005). Although the precise pathway by which ATR functions in the unperturbed S phase is largely unknown, it is noticeable that the ATR/CHK1 pathway can limit excessive firing of replication origins (Shechter, Costanzo and Gautier 2004) and that the inhibition of CHK1 causes increased origin firing and Cdc45 loading (Syljuasen et al. 2005). It will be intriguing in the future to reveal how the checkpoint proteins which function in DNA damage checkpoint regulate normal DNA replication and prevent premature entry into mitosis.

### **2.2.3 The roles of DNA replication licensing proteins in coordination of DNA replication with mitosis**

Many DNA replication licensing proteins play multiple roles in coordinating DNA replication and mitosis in addition to DNA replication licensing. As reported, depletion of the DNA licensing machinery proteins ORC1, ORC2, ORC6 and geminin results in aberrant mitosis. Depletion of ORC1 by siRNA leads to mitotic arrest and centrosome amplification. ORC1 depletion also results in reduced MCM3 loading onto chromatin and activates DNA damage responses (Hemerly et al. 2009). Depletion of ORC2 also leads to increased mitotic cells and over-amplified centrosomes, abnormal chromosomes condensation, defects of chromosomes alignment and multipolar spindles in mitosis (Prasanth et al. 2004). ORC6, also an origin recognition complex protein, localizes to the kinetochores during mitosis and to the midbody region of the chromosome during cytokinesis (Prasanth, Prasanth and Stillman 2002). ORC6 depletion leads to decreased DNA replication, multipolar spindles, misalignment chromosomes, cytokinesis failure and multinucleated cells (Prasanth et al. 2002, Bernal and Venkitaraman 2011), indicating that ORC6 might coordinate DNA replication, chromosomes segregation and cytokinesis.

Geminin and Cdc6 also play important roles in coordinating DNA replication and mitosis. Depletion of geminin results in multiple mitotic defects in addition to DNA replication defects. Geminin inactivation causes overduplicated centrosomes in one cell cycle (Melixetian et al. 2004). When using caffeine to override the G2-M checkpoint and induce mitosis, geminin-depleted cells showed bipolar spindles with multiple centrosomes and unattached chromosomes or multipolar spindles with multiple centrosomes (Tachibana et al. 2005). Cdc6 in yeast can prevent cells from progressing into mitosis before maturation by directly interacting with CDK1 and inhibiting its kinase activity (Weinreich et al. 2001). Cdc6 also coordinates DNA replication and mitosis in human cells. Overexpressed Cdc6 causes checkpoint kinase Chk1 to be phosphorylated and activated to prevent premature mitotic entry before DNA replication is completed (Clay-Farrace et al. 2003) (Figure 4). Cdc6 also plays important roles in mitosis. Depletion of Cdc6 causes abnormal spindles, misaligned chromosomes and multinucleated cells in addition to defects of DNA replication; however, no Chk1 activation was detected upon Cdc6 depletion (Lau et al. 2006). Depletion of Cdc6 in mouse oocytes also leads to spindle assembly defects (Anger, Stein and Schultz 2005). Cdc6 is phosphorylated by Plk1 at T37 in mitosis and colocalizes with Plk1 to the central spindle in anaphase. Phosphorylation of Cdc6 by Plk1 promotes its interaction with CDK1 and inhibition of CDK1 activity, releases the separase activity and chromosome segregation (Yim and Erikson 2010) (Figure 4).

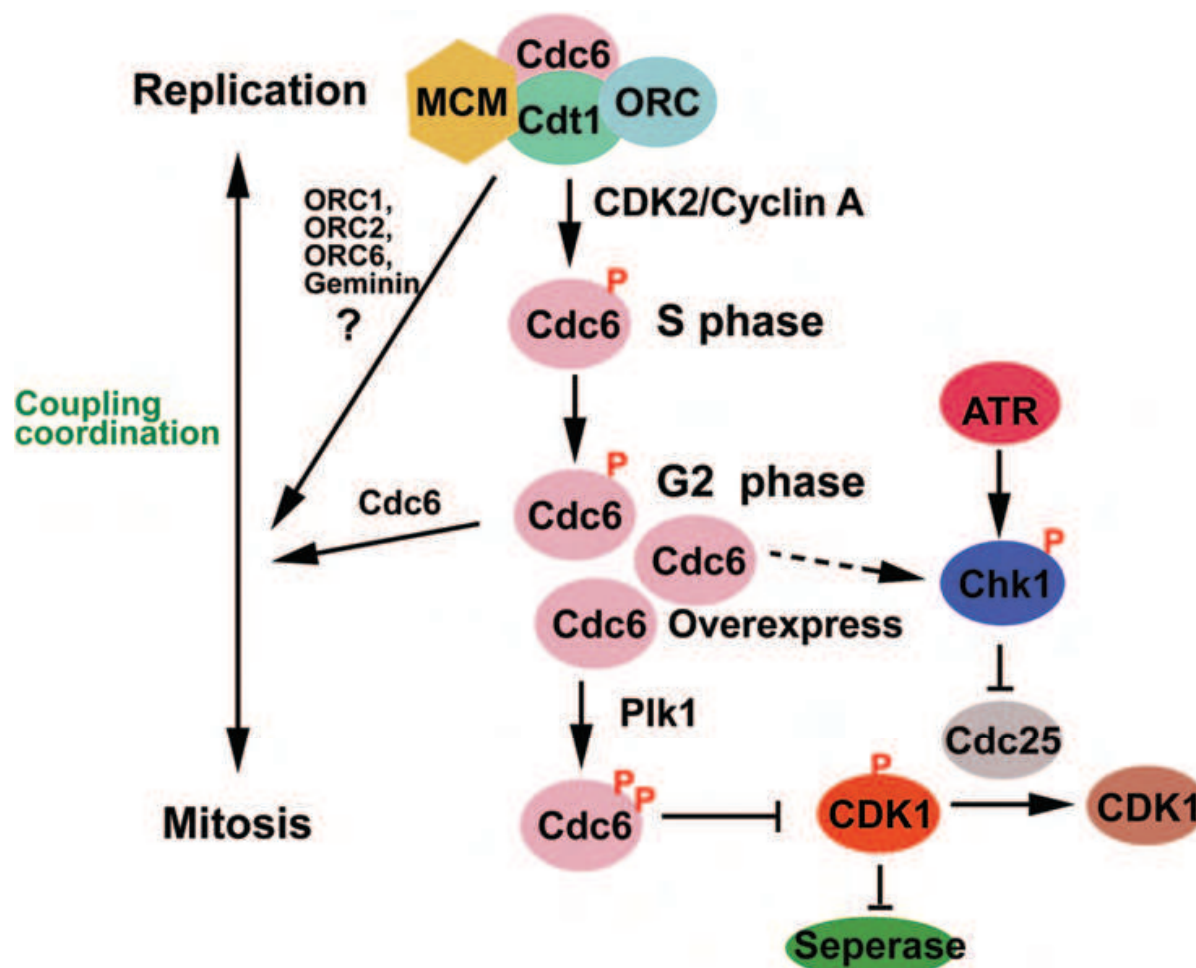


Fig. 4. The coordination of DNA replication and mitosis by DNA replication licensing proteins

DNA replication licensing protein Cdc6 is phosphorylated during the S phase by CDK2/cyclin A. Aberrant overexpression of Cdc6 in the G2 phase activates Chk1 and prevents mitotic entry. In mitosis, Plk1 phosphorylates Cdc6 and promotes the interaction between Cdc6 and CDK1. The phosphorylated Cdc6 promotes chromosome segregation by inhibiting CDK1 and the release of separase activity. Depletion of the other DNA replication licensing proteins can also lead to multiple mitotic defects.

In conclusion, in addition to the roles in DNA replication, most DNA licensing machinery proteins function in multiple mitotic events to coordinate DNA replication and mitosis. These functions are summarized in Table 1. As shown in Table 1, the defects of DNA replication and mitosis progression caused by depletion of DNA replication licensing proteins indicate that DNA replication and mitotic events are coordinated directly by the same machinery, although the mechanisms are largely unknown. Moreover, the Rb-E2F pathway which targets transcription of DNA replication licensing proteins was important for coupling DNA replication with mitosis. Rb depletion in the adult mouse liver led to aberrant accumulation of DNA replication licensing proteins, over-replication of DNA without mitotic condensation and decreased cyclin B1 level at G2/M checkpoint (Bourgo et al. 2011).

Depletion of DNA replication licensing proteins	Increase of mitotic cells	Centrosome amplification	Abnormal chromosomes condensation	Misalignment chromosomes	Multipolar spindles	Cytokinesis	Multi-nucleated cells
ORC1	+	+	*	*	*	*	*
ORC2	+	+	+	+	+	*	*
ORC6	*	*	*	+	+	+	+
Cdc6	*	*	*	+	+	+	+
geminin	*	+	*	+	+	*	*

Table 1. Mitotic defects caused by the depletion of DNA replication licensing proteins (+ denotes “Yes”; \* denotes “Not reported”)

It has been reported that depletion of many DNA replication licensing proteins can lead to multiple mitotic defects. The phenomena caused by depletion of the respectively DNA replication licensing proteins are summarized in Table 1.

2.3 The coordination of transcription and DNA replication

DNA replication and transcription are fundamental processes essential for normal cell cycle progression and cell proliferation. They are both carried out by assembled protein complexes machinery proceeding at DNA templates.

2.3.1 The pre-initiation complex assembly of RNA transcription and DNA replication

DNA replication can be divided into two stages: the pre-RC assembly with licensing at replication origins; and the pre-IC (pre-initiation complex) assembly with replication origin firing. ORC, Cdc6 and Cdt1 are assembled at origins to recruit MCM helicases to form pre-RC at origins. After replication initiation in S phase, MCM2-7 proteins are displaced from origins and proceed along with replication forks as the DNA helicase (Labib, Tercero and Diffley 2000). The pre-RC assembly and the licensing on origins mark these origins as candidates for DNA replication initiation. Activation of DNA replication initiation on origins, which is also named “origin firing”, requires additional factors assembled to pre-RC to form pre-IC. CDK2 and DDK (Dbf4-dependent cdc7 kinase) promote MCM to form a CMG complex with GINS (a complex of Sld5-Psf1-Psf2-Psf3) and Cdc45 at origins. With the assembly of the CMG complex, the DNA helicase activity is performed, the DNA replication origin is melted and DNA unwinding is initiated (Figure 5). Recruitment of Cdc45 to replication origins plays a key role for the subsequent initiation complex formation and DNA polymerase loading. DNA unwinds and RPA binds to the single strand DNA only in the presence of Cdc45. RPA binding is required for DNA polymerase  $\alpha$  to load to the chromatin. The interaction between Cdc45 and DNA polymerase  $\alpha$  is also important for the loading of DNA polymerase  $\alpha$  (Mimura et al. 2000). The loading of leading strand processive polymerase  $\epsilon$  also depends on Cdc45 (Mimura et al. 2000, Masumoto, Sugino and Araki 2000). In the lagging strand, after recruitment, DNA polymerase  $\alpha$  begins to synthesize short nascent DNA segments following primer RNA synthesis. Then, replication factor C (RFC) recognizes nascent DNA 3’ end and functions as a clamp loader to load PCNA (proliferating cell nuclear antigen). Finally, the lagging strand processive polymerase  $\delta$  is loaded to chromatin by PCNA. Subsequently, DNA replication proceeds from origins as replication forks with processive DNA polymerase (Figure 5).

Transcriptions of chromatin includes the transcription of rRNA genes by RNA polymerases (Pols) I, protein-encoding genes by Pol II and short untranslated genes of 5S rRNA, tRNA and so on by Pol III. Pol I transcription contributes up to about 70% of the nuclear transcription in the growing cells, Pol II transcription takes up to about 20% and Pol III transcription takes up to about 10%. Pols execute transcription of genes from the promoter regions of respective genes. Similar to DNA polymerases, RNA polymerases have no intrinsic ability to recognize specific DNA sequences of the promoters. A pre-initiation complex (PIC) which is made up of transcription factors is required to assemble at the promoter to recruit RNA polymerases. Complexes of TBP (TATA-box binding protein) and TBP-associated factors (TAF) assembled at the promoter regions are required for the initiation of transcription by all three Pols, despite the variation of respective TAFs. In pol II transcription, the TBP-TAF complex TFIID recognizes the TATA boxes and the promoter sequences. Interactions between TAFs and Pol II recruit Pol II and other factors to form the PIC and then Pol II transcription initiates (Verrijzer and Tjian 1996). Pol III is recruited to the promoters by its TBP-TAF complex TFIIIB and the PIC-containing Pol III is assembled to initiate the Pol III transcription (Geiduschek and Kassavetis 2001, Schramm and Hernandez 2002). Distinct from Pol II and Pol III transcription, Pol I transcription is confined to the nucleolus and is activated by PIC assembly. UBF (upstream binding factor), which binds to the UCE (upstream control element) and core promoter, appears to be the first step in PIC formation, followed by the recruitment of the TBP-TAF complex (Learned et al. 1986, Bell et al. 1988). SL1 (promoter-selectivity factor, mouse TIF-IB) is the TBP-TAF complex of Pol I. SL1 recruits Pol I to the promoter through the interaction of TIF-IA with Pol I (Miller et al. 2001). After PIC assembly, Pol I transcription initiates from the promoter of the rRNA genes. Therefore, DNA replication initiation and RNA transcription initiation share the mechanisms that recruit polymerases by an orderly assembled protein complex (Figure 5). Coordination between the two fundamental assembly events and the coupling between DNA replication and RNA transcription in cycling cells to coordinate cell growth are an intriguing issue; however, the mechanism remains to be elucidated.

### 2.3.2 The roles of DNA replication initiation proteins in RNA transcription

It has been noticed that MCM proteins are involved in RNA transcription, which implicates the coordination of RNA transcription and DNA replication (Figure 5). MCM proteins might be components of the Pol II transcriptional apparatus, as MCM2 and other MCMs can be co-purified with Pol II and other general transcription factors in the holoenzyme complex of *Xenopus* oocytes and HeLa cells. Moreover, microinjection of MCM2 antibody specifically inhibits Pol II transcription in *Xenopus* oocytes. The association of MCMs with the holoenzyme partly depends on its amino acids 168-230 and the C-terminal domain of Pol II (Yankulov et al. 1999). Mutations in amino acids 169-212 of MCM2 disrupt its binding to Pol II and to general transcription factors *in vivo* (Holland et al. 2002). MCM2 and MCM5 are also required for general transcription, and their depletion may lead to transcription defects. MCM2-7 proteins also co-localize with Pol II on constitutively transcribing genes. Notably, MCM5 is required for the elongation of Pol II. Moreover, MCM5 functions in Pol II transcription and requires integrity of the MCM complex and helicase activity of MCM5 (Snyder, Huang and Zhang 2009). MCM also functions in cytokine-induced gene transcription activation. Stat1 translocates into the nucleus in response to IFN- $\gamma$  and recruits MCM3 and MCM5 to enhance the stat1-mediated transcription activation. The amino acids R732 and K734 of MCM5 are important residues required for the interaction with Stat1 and stat1-mediated transcription

activation. The enhancement of Stat1-mediated transcription activation also requires ATPase activity and helicase activity of MCM5 (DaFonseca, Shu and Zhang 2001). Further study (Snyder, He and Zhang 2005) shows that MCM5 and other members of MCMs are recruited directly to the gene promoters targeted by Stat1 upon cytokine stimulation. MCMs move along with Pol II during transcription elongation. Furthermore, MCM5 is essential for Stat1-targeted gene transcription elongation. The domain responsible for MCM5 and stat1 interaction is also identified, and expression of this domain interferes with the interaction between MCM5 and Stat1 and represses Stat1 mediated transcription (Snyder et al. 2005). In conclusion, the DNA licensing machinery MCM proteins also play important roles in the activation of RNA transcription. It is possible that other DNA licensing machinery proteins are also involved in the transcription process and coordinate DNA replication and RNA transcription.

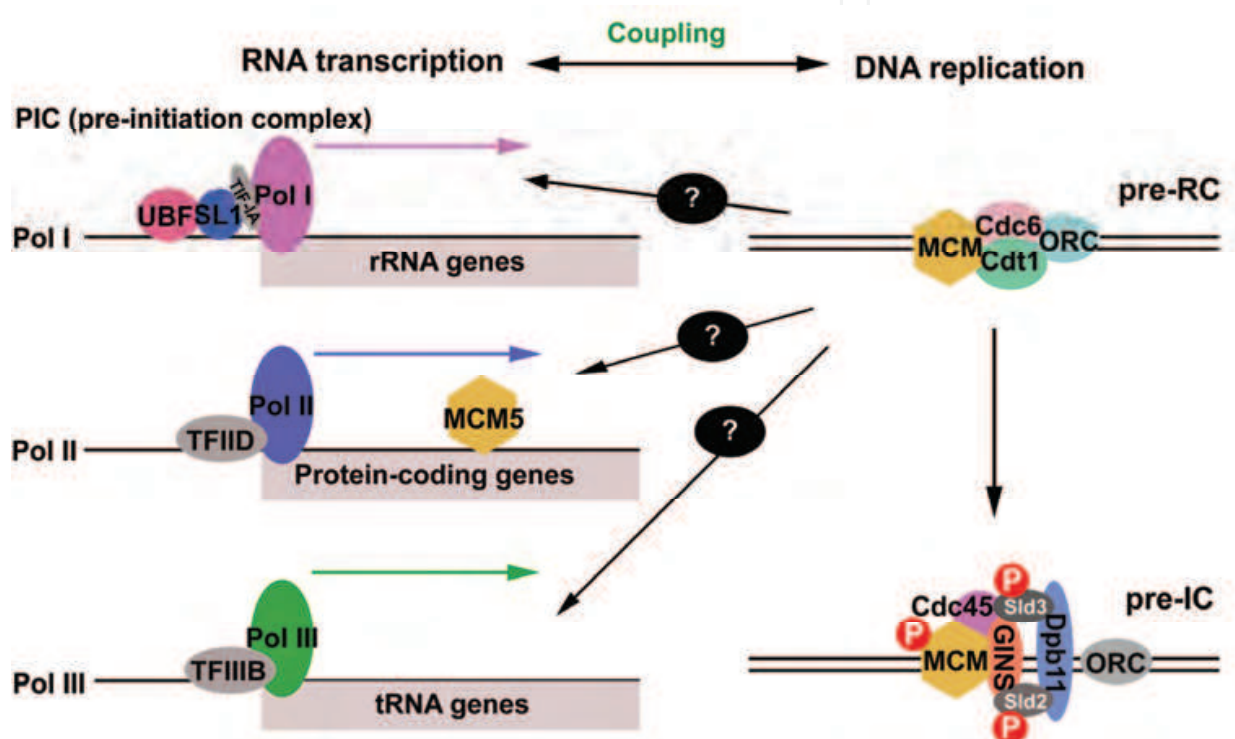


Fig. 5. Coordination of RNA transcription and DNA replication by DNA replication licensing proteins

ORCs, Cdc6 and Cdt1 are assembled at the DNA replication origins to recruit MCM helicases to form the pre-RC. The activation of DNA replication initiation at the origins, known as the "origin firing", requires additional factors to be recruited to the pre-RC to form the pre-IC. Polymerases are recruited by an orderly assembled protein complex in similar ways in both DNA replication and RNA transcription. The pre-initiation complex (PIC) assembly at the promoter is required for the recruitment of the RNA polymerases. The complex assembly of TBP and TBP-associated factors (TAF) at the promoter regions are required for the initiation of transcription by all three Pols, with variation of respective TAFs of SL1 in Pol I transcription, TFIID in Pol II transcription and TFIIB in Pol III transcription. Pol I is recruited by UBF and SL1 through interaction with TIF-IA of the SL1 complex. DNA replication licensing protein MCM5 is required for Pol II transcription and elongation. It is possible that other DNA replication licensing machinery proteins are involved in RNA transcription and couple these two fundamental events of RNA transcription and DNA replication (indicated in by the question marks).

### 3. Conclusion

DNA replication, centrosome duplication and mitosis are the basic events in a cell cycle to ensure proper cell division and proliferation. RNA transcription is also a basic event which takes place throughout the whole cell cycle to provide continuous protein synthesis. In this chapter, we reviewed evidence for coordination between these basic events. Centrosome duplication and DNA replication use the same licensing proteins and are subject to the same regulators of CDK2/cyclin E and CDK2/cyclin A. Similarly, in correlation with mitosis, several DNA licensing machinery proteins have been demonstrated to function in multiple mitotic events and coordinate DNA replication and mitotic entry. Besides, DNA replication initiation proteins such as MCM proteins are involved in RNA transcription and might coordinate RNA transcription and DNA replication. In summary, accumulated evidence shows that the same set of regulators is implied in regulating these connected cell cycle events to ensure genomic integrity and sheds lights on the molecular mechanisms connecting these cell cycle events.

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The study of DNA advanced human knowledge in a way comparable to the major theories in physics, surpassed only by discoveries such as fire or the number zero. However, it also created conceptual shortcuts, beliefs and misunderstandings that obscure the natural phenomena, hindering its better understanding. The deep conviction that no human knowledge is perfect, but only perfectible, should function as a fair safeguard against scientific dogmatism and enable open discussion. With this aim, this book will offer to its readers 30 chapters on current trends in the field of DNA replication. As several contributions in this book show, the study of DNA will continue for a while to be a leading front of scientific activities.

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