Helicase Activation and Establishment of Replication Forks at Chromosomal Origins of Replication

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Many replication proteins assemble on the pre-RC-formed replication origins and constitute the pre-initiation complex (pre-IC). This complex formation facilitates the conversion of Mcm2–7 in the pre-RC to an active DNA helicase, the Cdc45–Mcm–GINS (CMG) complex. Two protein kinases, cyclin-dependent kinase (CDK) and Dbf4-dependent kinase (DDK), work to complete the formation of the pre-IC. Each kinase is responsible for a distinct step of the process in yeast; Cdc45 associates with origins in a DDK-dependent manner, whereas the association of GINS with origins depends on CDK. These associations with origins also require specific initiation proteins: Sld3 for Cdc45; and Dpb11, Sld2, and Sld3 for GINS. Functional homologs of these proteins exist in metazoa, although pre-IC formation cannot be separated by requirement of DDK and CDK because of experimental limitations. Once the replicative helicase is activated, the origin DNA is unwound, and bidirectional replication forks are established.

The main events at the initiation step of DNA replication are the unwinding of double-stranded DNA and subsequent recruitment of DNA polymerases, to start DNA synthesis. Eukaryotic cells require an active DNA helicase to unwind the origin DNA. The core components of the replicative helicase, Mcm2–7, are loaded as a head-to-head double hexamer connected via their amino-terminal rings (Evrin et al. 2009; Remus et al. 2009; Gambus et al. 2011) onto Orc-associated origins, to form the pre-RC in late M and G₁ phases (see Bell and Kaguni 2013). However, Mcm2–7 alone does not show DNA helicase activity at replication origins.

After the formation of the pre-RC, other replication factors assemble on origins, and the pre-initiation complex (pre-IC) is formed. The pre-IC is defined as a complex formed just before the initiation of DNA replication (Zou and Stillman 1998); in yeast, it contains at least seven additional factors: Cdc45, GINS, Dpb11, Sld2, Sld3, Cdc45, and DNA polymerase ϵ (Pol ϵ) (Muramatsu et al. 2010). The formation of the pre-IC is a prerequisite for the activation of the Mcm2–7 helicase; two additional factors, Cdc45 and GINS, associate with Mcm2–7 and form a tight complex, the Cdc45–Mcm–GINS (CMG) complex (Gambus et al. 2006; Moyer

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S. Tanaka and H. Araki

et al. 2006). This reaction requires components of the pre-IC and two protein kinases, cyclin-dependent kinase (CDK) and Dbf4-dependent kinase (DDK) (for reviews, see Labib 2010; Masai et al. 2010; Tanaka and Araki 2010). In this article, we summarize and discuss the manner via which the pre-IC is formed in yeasts and metazoa. Although there are some discrepancies, the process of formation of the pre-IC is conserved fairly well in these organisms.

OVERVIEW OF THE FORMATION OF THE PRE-IC IN THE BUDDING YEAST *Saccharomyces cerevisiae*

The initiation reaction is best understood in a unicellular model eukaryote, the budding yeast *Saccharomyces cerevisiae*. The outline of the reaction is shown schematically in Figure 1. The reactions involved in the formation of the pre-IC can be further separated into two steps in yeast cells according to the requirements of the

two protein kinases, CDK and DDK, which are essential for the initiation of chromosomal DNA replication. In the first step, Sld3–Sld7–Cdc45 associates with the pre-RC-formed origins in a DDK-dependent manner (Heller et al. 2011; Tanaka et al. 2011a). In the second step, Sld2, Dpb11, Pol ϵ , and GINS associate with the origins that bind to Sld3 and Cdc45, in a CDK-dependent manner (Masumoto et al. 2002; Tak et al. 2006; Tanaka et al. 2007b; Zegerman and Diffley 2007; Muramatsu et al. 2010; Heller et al. 2011). After assembly, the CMG complex, which is a holo-replicative helicase, is formed (Gambus et al. 2006; Moyer et al. 2006). Because the Mcm2–7 complex embraces the double-stranded origin DNA in the pre-RC (Evrin et al. 2009; Remus et al. 2009) and the single-stranded template of the leading strand at the replication forks (Fu et al. 2011), the CMG complex somehow encircles the single-stranded DNA to unwind the double-stranded DNA. Then, DNA polymerase α (Pol α), which associates tightly

Figure 1. Schematic drawings of the formation of the pre-IC and the initiation of DNA replication. See text for details.

with primase, is recruited to the unwound origins, to start DNA synthesis.

DDK-Dependent Association of Cdc45 with Origins

DDK consists of Cdc7 catalytic and Dbf4 regulatory subunits (Sclafani 2000). Its activity is regulated by the level of the Dbf4 protein, which increases at G₁/S boundary; is kept high in S phase; and decreases from late M phase (Cheng et al. 1999; Oshiro et al. 1999; Ferreira et al. 2000). DDK facilitates the association of Sld3, Sld7, and Cdc45 with the pre-RC-formed origins, in vivo as well as in vitro (Heller et al. 2011; Tanaka et al. 2011a). Sld3 and Sld7 are the subunits of the tight complex (the Sld3–Sld7 complex), which loosely associates with Cdc45 (Tanaka et al. 2011b). Sld3 and Cdc45 associate with origins in a mutually dependent manner (Kamimura et al. 2001), whereas Sld7 is dispensable but is required for efficient initiation of DNA replication (Tanaka et al. 2011b). The DDK-dependent association of Sld3–Sld7–Cdc45 with origins seems to be weak, because this association is detected only via chromatin-immunoprecipitation assay using cross-linking reagents (Kamimura et al. 2001; Kanemaki and Labib 2006). When CDK is activated at the G₁/S boundary, association of Cdc45 with chromatin, which is usually examined in metazoa, is detected in the absence of cross-linking reagents

(Zou and Stillman 1998); however, this association requires Dpb11, Sld2, and GINS.

Accumulating evidence strongly suggests that DDK phosphorylates the Mcm2–7 complex and that this phosphorylation step enhances the association between Sld3–Sld7–Cdc45 and origins. Eukaryotic Mcm2, Mcm4, and Mcm6 have extended unstructured serine/threonine-rich domains (NSD) at their amino termini (Fig. 2), which are extensively phosphorylated by DDK in vitro (Randell et al. 2010). Consistent with the in vitro phosphorylation, the combination of mutant Mcm4 and Mcm6, both of which have multiple alanine substitutions at DDK phosphorylation sites, does not support cell growth (Fig. 2) (Randell et al. 2010). Moreover, mutations of Mcm4 and Mcm5 are reported to bypass the requirement of DDK (Hardy et al. 1997; Sheu and Stillman 2010). Furthermore, the results of a recently reported in vitro replication assay suggest that DDK-catalyzed phosphorylation of the pre-RC is required for the association of the pre-RC with Sld3–Sld7–Cdc45 (Heller et al. 2011). This assay consists of three consecutive steps: incubation of immobilized template DNA with a G₁-phase extract (formation of the pre-RC), treatment of the pre-RC with DDK after separation from the extract, and incubation with an S-phase extract (DNA synthesis). The S-phase extract is prepared from cells that bear the *cdc7-4* temperature-sensitive mutation and are arrested at a

Figure 2. Schematic drawings of the structure of budding yeast Mcm2, Mcm4, and Mcm6. (Orange) Unstructured amino-terminal portion, (light green) conserved OB-fold like domain, (light blue) conserved AAA⁺-type ATPase domain. The numbers on top indicate the positions of amino acid residues. (White arrow) The amino-terminal portion of Mcm4 corresponding to the $\Delta 74-174$ mutation. The positions of alanine substitutions in the phosphorylation site mutants (Randell et al. 2010) are shown by arrowheads; (green) AD/E + ASP/Q, (blue) AP + AQ.

S. Tanaka and H. Araki

nonpermissive temperature. The step of treatment of the pre-RC with DDK is indispensable for the initiation of DNA replication and for association of Sld3 and Cdc45 with the template DNA in this assay (Heller et al. 2011).

The manner via which phosphorylation of the Mcm2–7 complex promotes the interaction between this complex and Sld3–Sld7–Cdc45 remains obscure. However, it is likely that the phosphorylation prevents unknown inhibitory effects during the interaction. In budding yeast, this inhibitory effect is intrinsic, at least in the case of Mcm4 (Sheu and Stillman 2010). The NSD (1–174) of Mcm4 contains redundant phosphorylation sites for DDK, followed by a “docking region” for DDK in the structured amino-terminal domain (Fig. 2) (Sheu and Stillman 2006). Furthermore, the region located between residues 74 and 174 has inhibitory activity for the initiation (Fig. 2), because Mcm4 lacking this region (*mcm4*^{Δ74–174}) bypasses the requirement of DDK (Sheu and Stillman 2010). Thus, phosphorylation of the Mcm4 NSD by DDK promotes the initiation of DNA replication by alleviating inhibitory activity in Mcm4.

Currently, the mechanisms underlying this inhibitory effect are not understood. The study of DDK in fission yeast provides clues regarding this inhibition. In fission yeast, DDK activity is not required for the initiation of DNA replication at high temperature or in the absence of Mrc1 or Rif1 (Matsumoto et al. 2011; Hayano et al. 2012) (see below). Although the same is not true in budding yeast, it is conceivable that the obstacles to the interaction between the pre-RC and Sld3–Sld7–Cdc45, such as chromatin structure, are removed by DDK-dependent phosphorylation.

Interestingly, the association of DDK with Mcm2–7 and the phosphorylation of Mcm2–7 in the pre-RC require prior (priming) phosphorylation of the pre-RC (Francis et al. 2009). DDK is an acidophilic kinase; thus, priming phosphorylation at the +1 position of the DDK phosphorylation site will be favored (Masai et al. 2006; Montagnoli et al. 2006; Sasanuma et al. 2008; Wan et al. 2008). Recently, the DDK phosphorylation sites on Mcm2, Mcm4, and Mcm6 in the pre-RC were determined in vitro,

as were the “priming” phosphorylation sites required for DDK phosphorylation in the same proteins (Randell et al. 2010). Two types of priming sites were identified: S/T-P and S/T-Q (Randell et al. 2010). A member of the phosphoinositide 3-kinase family, Mec1 (ATR kinase in budding yeast), is responsible for SQ phosphorylation in S phase. However, the identity of the SP kinase remains unclear (Randell et al. 2010). CDK may be a candidate priming kinase for S/T-P sites because this motif matches the CDK recognition site (S/T-P), and a role for CDKs in modifying Mcm2–7 has been proposed in eukaryotes (Masai et al. 2000; Montagnoli et al. 2006; Devault et al. 2008; Sheu and Stillman 2010).

CDK-Dependent Association of GINS with Origins

GINS is recruited in a CDK-dependent manner to the Sld3–Sld7–Cdc45-associated pre-RC to form the CMG complex. CDK phosphorylates two essential replication proteins, Sld2 and Sld3, and promotes the formation of the Sld2–Dpb11–Sld3 complex (Fig. 1).

At the onset of S phase, S-phase CDK (S-CDK; Clb5–Cdc28 and Clb6–Cdc28 in the budding yeast) is activated. Phosphorylation of Sld2 and Sld3 by S-CDK promotes the interaction with Dpb11 (Masumoto et al. 2002; Tak et al. 2006; Tanaka et al. 2007b; Zegerman and Diffley 2007). Dpb11 has four BRCA1 carboxy-terminal (BRCT) domains. A tandem pair of BRCT domains constitutes a phosphopeptide-binding domain (Glover et al. 2004), and the amino-terminal BRCT pair of Dpb11 interacts with phosphorylated Sld3 and the carboxy-terminal BRCT pair interacts with phosphorylated Sld2 (Tak et al. 2006; Tanaka et al. 2007b; Zegerman and Diffley 2007). The phosphorylation-dependent Dpb11–Sld2 interaction leads to the formation of the pre-loading complex (pre-LC) (Muramatsu et al. 2010). The pre-LC contains phosphorylated Sld2, Dpb11, GINS, and Pol ε. Importantly, formation of the pre-LC requires CDK, whereas it does not require the pre-RC. Once S-CDK is activated, association of pre-LC components with replication origins

is observed. Therefore, the pre-LC seems to assemble first via Sld2 phosphorylation, followed by association with replication origins.

On the other hand, Sld3, together with Sld7 and Cdc45, associates with the pre-RC-formed origins in a DDK-dependent manner, as described above (Heller et al. 2011; Tanaka et al. 2011a). This association requires neither CDK activity nor components of the pre-LC; rather, the association of Sld3 and Cdc45 with origins is a prerequisite for that of the pre-LC (Muramatsu et al. 2010). The copy numbers of Sld3, Sld7, and Cdc45 (especially Sld3) are limited in comparison with the number of origins (Mantiero et al. 2011; Tanaka et al. 2011a); thus, most of these proteins associate with origins in G₁ phase. The origins associating with these proteins in G₁ fire early in S phase (Tanaka et al. 2011a). Therefore, it is conceivable that Sld3 on origins is phosphorylated by CDK, associates with the amino-terminal BRCT domains of Dpb11 in the pre-LC, and eventually recruits GINS to origins. After pre-LC association, the origin-DNA-protein complex might be remodeled, and the replicative helicase, the CMG complex, is formed and activated, to establish bi-directional replication forks. Sld3–Sld7, Dpb11, and Sld2 do not move with the replication forks (Fig. 1).

Pol ϵ in the pre-LC is important for the retention of GINS in this complex (Muramatsu et al. 2010). Pol ϵ consists of four subunits: Pol2, Dpb2, Dpb3, and Dpb4. Pol2 is a catalytic subunit, Dpb2 is an essential subunit of unknown function, and Dpb3 and Dpb4 are nonessential subunits that contain a histone fold (Hamatake et al. 1990; Morrison et al. 1990; Araki et al. 1991; Ohya et al. 2000). The amino-terminal polymerase domain of Pol2 is dispensable for DNA replication (Dua et al. 1999; Kesti et al. 1999), probably because DNA polymerase δ (Pol δ) compensates for the lack of catalytic activity of Pol ϵ . However, the carboxy-terminal half of Pol2, which interacts with other subunits, is essential for DNA replication and cell growth (Dua et al. 1999; Kesti et al. 1999). Thus, it is likely that the formation of the pre-LC at the initiation step of DNA replication is an essential function of Pol ϵ .

Subsequent Events

After origin unwinding, the ssDNA-binding protein RPA associates with origin DNA and the Pol α /primase complex is recruited. Then primase synthesizes primer RNA, and Pol α synthesizes short DNA strands using the primers. Pol ϵ and Pol δ elongate the leading and lagging strands, respectively (Burgers 2009). The Mcm10 protein is evolutionarily conserved in the eukaryotic domain of life and is required for DNA replication (Tye 1999). Noticeably, it was identified as a component of replication forks (Gambus et al. 2006) and is required for the stability of the large subunit of Pol α in budding yeast (Ricke and Bielinsky 2004). Studies in yeasts and vertebrates show that Mcm10 is required for the chromatin loading of Cdc45, which suggests a role in the formation of CMG (Wohlschlegel et al. 2002; Gregan et al. 2003; Ricke and Bielinsky 2004; Sawyer et al. 2004). However, a recent in vitro study showed that Mcm10 is loaded to origins after GINS loading, which might indicate that the loading of Mcm10 occurs after CMG formation and is required for the further loading of Pol α and Pol δ (Heller et al. 2011). Mcm10 is not required for the loading of Pol ϵ in vitro, because Pol ϵ is loaded to origins as a component of the pre-LC (Muramatsu et al. 2010; Heller et al. 2011). Recent in vivo studies indicate that Mcm10 is required for the unwinding of origin DNA and further loading of Pol α to origins after Cdc45, Mcm2–7, and GINS form a complex (Kanke et al. 2012; van Deursen et al. 2012; Watase et al. 2012). Because CDK and DDK are required for the activation of the replicative helicase, the question of whether CMG formation is sufficient for the loading of Mcm10 is intriguing.

FORMATION OF THE PRE-IC IN THE FISSION YEAST *Schizosaccharomyces pombe*

The orthologs of Dpb11, Sld2, and Sld3 in the fission yeast *Schizosaccharomyces pombe* are Cut5 (also called Rad4), Drc1, and Sld3, respectively (Saka et al. 1994; Nakajima and Masukata 2002; Noguchi et al. 2002). These proteins are well

S. Tanaka and H. Araki

conserved and, thus, easily identified by sequence similarity. The CDK-catalyzed phosphorylation of Drc1 and Sld3 facilitates the interaction between Cut5 and Drc1 and Sld3, as observed in budding yeast (Saka et al. 1994; Nakajima and Masukata 2002; Noguchi et al. 2002; Fukuura et al. 2011). However, phosphorylation of Sld3 is not essential, although it is important, for the association of Cut5 with origins; the disruption of phosphorylation sites reduces the interaction between Cut5 and Sld3 and renders cells sensitive to low temperature (Nakajima and Masukata 2002; Fukuura et al. 2011). Interestingly, the interaction between Cut5 and Drc1 further enhances the interaction with Sld3 (Fukuura et al. 2011). Sld3 associates with replication origins before initiation in a DDK-dependent manner (Yabuuchi et al. 2006), and its association with origins is a prerequisite for the subsequent recruitment of Cut5, GINS, Drc1, and Cdc45 (Yabuuchi et al. 2006). Pol ϵ plays an essential role in the assembly of the CMG complex (Handa et al. 2012). Moreover, the DNA polymerase domain of the catalytic subunit of Pol ϵ is dispensable for replication, whereas the carboxy-terminal half of the catalytic subunit, which functions in the recruitment of GINS as a component of the pre-LC in budding yeast, is essential, as observed in budding yeast (Feng and D'Urso 2001). Therefore, the mechanism of formation of the pre-IC is conserved well in fission and budding yeasts, although the manner via which Cdc45 and GINS are recruited onto origins in fission yeast remains obscure.

In fission yeast, DDK is also required for the initiation of replication. As in budding yeast, Mcm4 and Mcm6 are phosphorylated in a DDK-dependent manner, and alanine-substitution mutations in their DDK phosphorylation sites cannot be combined (Masai et al. 2006). Although several mutations in Mcm4 and Mcm5 can bypass the requirement of DDK in budding yeast, a similar rescue has not been reported in fission yeast. Noticeably, high temperature or deletion of *mrc1* or *rif1* can bypass the requirement of DDK in fission yeast (Matsumoto et al. 2011; Hayano et al. 2012). High temperature may ease the unwinding of double-

stranded DNA. Mrc1 functions at checkpoints and stabilization of paused replication forks (Alcasabas et al. 2001; Tanaka and Russell 2001; Katou et al. 2003; Osborn and Elledge 2003). The bypass of the DDK requirement in *mrc1 Δ* is independent of its checkpoint function (Matsumoto et al. 2011). Mrc1 associates with early-firing origins and is likely to mark these origins, although *mrc1 Δ* early-firing origins fire earlier than do those of the wild type (Hayano et al. 2011). In the budding yeast, Mrc1 facilitates the phosphorylation of the priming sites of chromatin-bound Mcm2–7 by Mec1 in S phase, although this might be part of the checkpoint function of Mrc1 (Randell et al. 2010). Rif1 binds to many chromosome-arm loci as well as telomeres and affects the timing of origin firing (Hayano et al. 2012). In summary, although DDK is required for the association of Sld3 with origins, the requirement of DDK in fission yeast may be less than that observed in budding yeast. Further analyses are needed to understand the DDK-dependent process in this organism.

FORMATION OF THE PRE-IC IN METAZOA

The replication machinery, such as DNA polymerases and DNA helicase components (Mcm2–7, Cdc45, and GINS), is well conserved in eukaryotic organisms. In contrast, the regulatory components have diverged during evolution. Dpb11, Sld2, and Sld3 are examples of this divergence. These factors sense CDK activity and promote the initiation of chromosomal DNA replication, as described above. Because the cellular environment differs among organisms, the sensing mechanism of CDK activity may have diverged. Nonetheless, TopBP1 (also denoted Cut5 and Mus101), RecQ4 (also denoted RecQL4), and Treslin/Ticrr are reported as functional homologs of Dpb11, Sld2, and Sld3, respectively. Each of them shows limited sequence similarity to their yeast counterparts, respectively, and all of them are much larger in size than their yeast counterparts (Fig. 3) (Makiniemi et al. 2001; Van Hatten et al. 2002; Hashimoto and Takisawa 2003; Garcia et al. 2005; Sangrithi et al. 2005; Matsuno et al. 2006;

Figure 3. Schematic drawings of Dpb11/TopBP1 and Sld3/Treslin/Ticrr proteins. (A) Dpb11/TopBP1 proteins. (Light blue) BRCT domains. (B) Sld3/Treslin/Ticrr proteins. (Magenta) The Sld3/Treslin domain; (red) the conserved short patch that contains conserved CDK phosphorylation sites; (orange) the amino-terminal region that is conserved in animal and plant Treslin. (C) Multiple alignments of amino acid sequences that contain conserved CDK phosphorylation sites. (Red asterisks in the top row) Conserved serine and threonine.

Kumagai et al. 2010; Sansam et al. 2010). Although these proteins are required for the initiation of DNA replication, the requirement for, and order of, association with chromatin vary slightly among them.

TopBP1

TopBP1 is involved in DNA replication and checkpoints, as observed for its yeast counterparts (Garcia et al. 2005; Walter and Araki 2006; Tanaka et al. 2007a; Labib 2010; Tanaka and Araki 2010). Although yeast Dpb11/Cut5 has four BRCT domains, metazoan TopBP1s have multiple BRCTs (more than six). Moreover, ver-

tebrate TopBP1 has eight BRCTs (BRCT1–8), and its amino-terminal portion, which contains five BRCTs (BRCT1–5), shows sequence similarity to the yeast counterparts, with the exception of BRCT3 (Fig. 3A) (Garcia et al. 2005). A recent structural analysis of human TopBP1 showed that vertebrate TopBP1 has one additional BRCT domain at the very end of its amino terminus; this BRCT domain is called BRCT0, to maintain consistency with the existing nomenclature (Rappas et al. 2011).

TopBP1 is required for the loading of Cdc45, RecQ4, and Pol ϵ onto chromatin, but not for pre-RC assembly, chromatin loading of Treslin/Ticrr, or the elongation stage of DNA

S. Tanaka and H. Araki

replication (Van Hatten et al. 2002; Hashimoto and Takisawa 2003; Kumagai et al. 2010). Intriguingly, TopBP1 associates with chromatin in two distinctive modes: CDK-independent low-level and CDK-dependent high-level associations. The CDK-independent association of TopBP1 with chromatin depends on ORC but not on the pre-RC, whereas CDK-dependent association depends on the pre-RC. The CDK-independent low-level association of TopBP1 with chromatin fully supports DNA replication (Van Hatten et al. 2002; Hashimoto and Takisawa 2003). A recent study showed that the amino-terminal portion of TopBP1, which contains BRCT0–3 (originally denoted as BRCT I–III), is sufficient to fully support DNA replication in *Xenopus* egg extracts, and that BRCT4–5, which shows similarity with the carboxy-terminal BRCT pair of the yeast counterparts, are dispensable for DNA replication (Kumagai et al. 2010). Such dispensability of BRCT4–5 of TopBP1 might reflect the fact that vertebrate RecQ4 does not require CDK for its association with TopBP1 and chromatin (see below). Moreover, Cdc45 binds to BRCT0–2 and BRCT6 of TopBP1 (Schmidt et al. 2008), whereas such bindings have not been reported in yeast.

Treslin/Ticrr

Treslin/Ticrr was identified via screening for TopBP1-binding proteins (Treslin) in *Xenopus* (Kumagai et al. 2010) and for G₂/M checkpoint regulators in zebrafish (Ticrr) (Sansam et al. 2010). Subsequently, the sequence homology between Sld3 and Treslin/Ticrr was shown using a sophisticated method, and the Sld3–Treslin/Ticrr domain (STD), which is conserved across eukaryotic species, including animals, fungi, and plants, was identified (Fig. 3B) (Sanchez-Pulido et al. 2010). Interestingly, Treslin/Ticrr family members in animals and plants have an additional conserved region in their amino terminus (Sanchez-Pulido et al. 2010), although its function is unknown (see below).

Treslin/Ticrr shows many functional similarities to Sld3. A central region of Treslin, located proximal to the carboxy-terminal side of the STD domain, binds to BRCT0–2 of TopBP1,

which is similar to BRCT1–2 of Dpb11, a binding domain for phosphorylated Sld3 in budding yeast (Boos et al. 2011; Kumagai et al. 2011). This binding requires CDK-catalyzed phosphorylation of specific serine and threonine. The short stretches of amino acid sequences around these phosphorylation sites are well conserved in animals and fungi (Fig. 3C). Moreover, CDK phosphorylation of these sites is required for DNA replication in vivo (Boos et al. 2011; Kumagai et al. 2011). Treslin also interacts with Cdc45 (Kumagai et al. 2011) and is required for the association of Cdc45 with chromatin. Moreover, the Treslin–TopBP1 interaction is reduced in cells treated with hydroxyurea in a checkpoint kinase 1 (Chk1)-dependent manner (Boos et al. 2011). This is reminiscent of the situation in which the Rad53 checkpoint kinase phosphorylates Sld3 to inhibit initiation by disrupting the Sld3–Dpb11 interaction in budding yeast (Lopez-Mosqueda et al. 2010; Zegerman and Diffley 2010).

The association of Treslin/Ticrr with chromatin, however, is slightly different from that observed for Sld3, because it depends mostly on the pre-RC and not on TopBP1 or CDK (Kumagai et al. 2010). TopBP1 associates with chromatin in a Treslin-independent manner. Thus, it is proposed that the CDK phosphorylation-dependent interaction between TopBP1 and Treslin/Ticrr occurs on chromatin, which renders the complex competent for recruiting Cdc45 (Kumagai et al. 2010). Moreover, the amino terminus of Treslin, which is specific in animal and plant orthologs (Fig. 3B) (Sanchez-Pulido et al. 2010), shows a weak interaction with TopBP1 (Kumagai et al. 2011).

RecQ4

Whether RecQ4 is a functional homolog of Sld2 remains controversial because it seems to work at a subsequent stage of the initiation step in a CDK-independent manner, unlike yeast Sld2/Drc1 (Fig. 1). RecQ4 belongs to the RecQ helicase family of vertebrates, which plays various roles in DNA metabolism (Bachrati and Hickson 2008). Mutations in the carboxy-terminal RecQ helicase domain are responsible for the

subset of Rothmund–Thomson, Baller–Gerold, and Rapadilino syndromes, which show increased genetic instability and share key symptoms, such as growth deficiency (Capp et al. 2010). However, the RecQ helicase domain is dispensable for DNA replication, whereas the amino-terminal portion, which has a weak similarity to Sld2, is essential for cell growth and DNA replication (Sangrithi et al. 2005; Matsuno et al. 2006). RecQ4 associates with chromatin in a TopBP1- and pre-RC-dependent manner. Pol α and RPA require RecQ4 for association with chromatin, whereas TopBP1, Cdc45, GINS, and Pol ϵ are recruited to chromatin in the absence of RecQ4, unlike what is observed in yeast. Moreover, the association of RecQ4 with chromatin and the association between TopBP1 and the RecQ4 amino-terminal domain do not require CDK (Sangrithi et al. 2005; Matsuno et al. 2006). Furthermore, the formation of the CMG complex in HeLa cells requires RecQ4 and Mcm10 (Im et al. 2009), and RecQ4 expressed in human 293 cells binds to Mcm10 and associates with the CMG complex in an Mcm10-dependent manner (Xu et al. 2009). These results suggest that RecQ4 functions later in the initiation step compared with Sld2.

Other Factors

Two additional proteins, GEMC1 (Balestrini et al. 2010) and DUE-B (Chowdhury et al. 2010), that bind to both TopBP1 and Cdc45 are required for the recruitment of Cdc45 to chromatin. However, their actual functions have not been elucidated. The function of metazoan Mcm10 is also controversial; it may function in the formation of the pre-IC or collaborate with AND-1 (the counterpart of yeast Ctf4/Mcl1) in recruiting Pol α to chromatin (Wohlschlegel et al. 2002; Zhu et al. 2007). The function of metazoan Pol ϵ in the initiation step is obscure. Depletion of Pol ϵ in *Xenopus* egg extracts reduces DNA replication, but not the association of Cdc45 with chromatin (Waga et al. 2001; Fukui et al. 2004). Knockdown of the catalytic subunit of Pol ϵ in *Drosophila* inhibits DNA replication in mitotic and endocycling cells (Suyari et al. 2012). Moreover, deletion of the DNA polymerase domain in

the catalytic subunit of Pol ϵ abolishes endoreplication in the salivary gland but does not affect DNA replication in the mitotic cycle (Suyari et al. 2012). Thus, the DNA polymerase activity of Pol ϵ is dispensable for replication in the *Drosophila* mitotic cycle, similar to what is observed in yeast. This suggests an essential function for the carboxy-terminal half of the catalytic subunit of Pol ϵ , similar to that of yeast.

In budding yeast, Sld7 was recently identified as an Sld3-interacting protein that is not essential for DNA replication; however, *sld7* deletion slows DNA replication (Tanaka et al. 2011b). Although Sld7 does not show a clear sequence similarity to metazoan proteins, it will be intriguing to determine whether Treslin/Ticrr associates with the Sld7-like protein in vertebrate cells, as do Sld3 and Sld7 in budding yeast.

In addition to CDK, DDK is required for the initiation of DNA replication in metazoa. Studies using *Xenopus* egg extracts showed that DDK could complete its function in the absence of CDK (Jares and Blow 2000; Walter 2000). This is consistent with the recent finding in an in vitro replication assay of budding yeast: The requirement of DDK activity precedes that of CDK activity. As in budding yeast, MCM2–7 is a target of DDK, and their phosphorylation facilitates the loading of Cdc45 onto chromatin in mammalian cells (Masai et al. 2006). An in vitro study showed that DDK phosphorylates mainly the amino-terminal tail domain of MCM2, MCM4, and MCM6 and that this phosphorylation is stimulated by prior phosphorylation by CDK (Masai et al. 2000). As observed in budding yeast, *Xenopus* Mcm2–7 assemble as a head-to-head double hexamer in the pre-RC (Gambus et al. 2011). These results suggest that DDK phosphorylates the NTD of Mcm2/4/6 commonly in eukaryotes to activate the replicative helicase, although the exact role of phosphorylation requires further investigation.

REGULATORY ASPECTS OF THE FORMATION OF THE PRE-IC

DNA replication in eukaryotes initiates from multiple origins of replication (see Leonard

S. Tanaka and H. Araki

and Méchali 2013). It is widely known that the timing of the activation of individual origins in the S phase differs among origins, with the exception of some embryonic cells: Some origins fire early in the S phase (early-firing origins), whereas others fire late in S phase (late-firing origins). The pre-RCs are assembled at all potential origins in G₁ phase, and formation of the pre-ICs determines the timing of origin firing in budding yeast because the replication proteins that assemble at this step are limited. Origins with a high affinity for such factors fire earlier in S phase (Patel et al. 2008; Wu and Nurse 2009; Mantiero et al. 2011; Tanaka et al. 2011a). For details regarding the temporal regulation of replication, see Rhind and Gilbert (2013).

The formation of the pre-IC is also the target of checkpoint kinases in yeast and humans. When the replication fork is stalled by DNA damage or dNTP depletion after hydroxyurea treatment, the DNA damage- or intra-S-phase-checkpoint pathways are activated. Under such conditions, further activation of unfired origins is inhibited (Painter and Young 1980; Santocanale and Diffley 1998; Shirahige et al. 1998; Larner et al. 1999). Studies using budding yeast showed that the checkpoint kinase Rad53 inhibits both the CDK- and DDK-dependent pathways, which are essential for the initiation of replication, by phosphorylating Sld3 and Dbf4, respectively (Lopez-Mosqueda et al. 2010; Zegerman and Diffley 2010). Human Treslin/Ticrr, a functional homolog of Sld3, is also inhibited by the phosphorylation checkpoint kinase Chk1 when cells are exposed to DNA-replication stress (Boos et al. 2011).

Because DNA replication in eukaryotes occurs as a two-step reaction (origin licensing and initiation), these reactions are temporally separated in the cell cycle, to limit the activation of replication origins to once per cell cycle. Failures in this regulation cause rereplication, which is genotoxic (Nguyen et al. 2001; Mimura et al. 2004; Tanaka and Araki 2011). It is well established that multiple regulatory mechanisms prevent relicensing at activated origins after S phase (Arias and Walter 2007) (also see Siddiqui et al. 2013). Because budding yeast G₁ cells have DDK activity (although weak), the bypass of

CDK activity can induce DNA replication and cause a loss in cell viability (Tanaka and Araki 2011). To prevent the premature initiation of licensed origins in G₁ phase, budding yeast cells have multiple regulatory mechanisms: The targets of S-CDK, Sld2, and Sld3 are not phosphorylated, and the protein levels of pre-LC components Dpb11 and Sld2 are low. These mechanisms contribute to the prevention of the reaction promoted by S-CDK (Tanaka and Araki 2011). As in the case of prevention of relicensing, the presence of multiple inhibitory mechanisms is important for the prevention of the untimely initiation of chromosomal replication, to preserve the stability of the genome over generations. Although much less is known regarding how premature initiation is prevented during the G₁ phase in other eukaryotes, similar multiple mechanisms might contribute to the prevention of premature initiation because the molecular mechanisms involved in replication initiation seem to be largely conserved.

GENERAL VIEW OF THE FORMATION OF THE PRE-IC

Several replication proteins assemble on replication origins during the formation of the pre-IC to form the active helicase (the CMG complex). This reaction requires two protein kinases: DDK and CDK. DDK facilitates the association of Cdc45 with origins, and CDK promotes the association of GINS with origins. However, the DDK-dependent and CDK-independent association of Cdc45 with origins is detected only via a chromatin-immunoprecipitation assay using cross-linking reagents. Thus, this association of Cdc45 with origins has not been reported in metazoa. Rather, the CDK-dependent association of GINS with origins is well conserved in eukaryotic cells. The interaction between BRCT-containing proteins and CDK-phosphorylated initiation proteins seems to recruit GINS to origins, although the CDK-dependent association of GINS with origins has not been well elucidated in metazoa.

The association of Cdc45 and GINS with origins requires initiation-specific proteins, such as Sld3 (Treslin/Ticrr), Sld2, and BRCT-con-

taining proteins (Dpb11, Cut5, and TopBP1). These proteins are important for connecting DNA replication to the cell cycle via DDK- and CDK-dependent phosphorylation. Moreover, it is conceivable that these proteins are also required for the conversion of the assembly of Mcm2–7, Cdc45, and GINS to the tight CMG complex. Furthermore, the manner via which the CMG complex encircles single-stranded DNA, rather than the interaction with double-stranded DNA by Mcm2–7, in the pre-RC remains unknown.

The order of association of the initiation proteins varies slightly among different eukaryotic cells. However, we must be aware of the execution points of these proteins. The apparent association may vary according to the assay method used. For example, TopBP1 associates with chromatin before CDK activation; however, the execution point seems to occur after the time of association. Future studies will clarify these points.

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S. Tanaka and H. Araki

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