The Initiation Step of Eukaryotic DNA Replication

Helmut Pospiech, Frank Grosse, and Francesca M. Pisani

Abstract Eukaryotic initiation of DNA replication is a tightly regulated process. In the yeasts, S-phase-specific cyclin Cdk1 complex as well as Dfb4-Cdc7 kinase phosphorylate the initiation factors Sld2 and Sld3. These factors form a ternary complex with another initiation factor Dbp11 in their phosphorylated state, and associate with the origin of replication. This complex mediates the loading of Cdc45. A second complex called GINS and consisting of Sld5 and Psf1, 2 and 3 is also loaded onto the origin during the initiation process, in an interdependent manner with the Sld2/Sld3/Dpb11 complex. Both complexes cooperate in the recruitment of the replicative DNA polymerases, thus executing the initiation and subsequent establishment of the replication fork. Cdc45 and GINS are essential, well-conserved factors that are retained at the elongating replication fork. They form a stable helicase complex with MCM2-7 and mediate its contact to the replicative DNA polymerases. In contrast, the Sld2/Sld3/Dpb11 complex critical for the initiation is not retained by the elongating replication fork. Sld2 displays limited homology to the amino-terminal region of RecQL4 helicase, which may represent its metazoan orthologue, whereas Sld3 homologues have been identified only in fungi. Dbp11 and its fission yeast homologue Cut5 are members of a large family of BRCT-containing proteins including human TopBP1 and fruit fly Mus101. Similar principles of regulation apply also to human initiation of DNA replication, despite obvious differences in the detailed mechanisms. The regulatory initiation cascade is intimately intertwined with the cell cycle apparatus as well as the checkpoint control.

Keywords Cell cycle · Cdc45 · Checkpoint · Cut5 · DNA replication · Dpb11 · GINS · Initiation · Sld2 · Sld3 · TopBP1

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Abbreviations

Cdc	cell division cycle
Cdt	Cdc10-dependent transcription
CDK	cyclin-dependent kinase
Csm3	chromosome segregation in meiosis protein 3
Ctf4	chromosome transmission fidelity protein 4
DDK	Dbf4-dependent kinase
Dpb	DNA polymerase B subunit
Drc1	DNA replication and checkpoint protein 1
dsDNA	double-stranded DNA
GINS	Go, Ichi, Nii, and San
MCM	mini-chromosome maintenance
Mrc1	mediator of replication checkpoint protein 1
Mus	nitrogen mustard-sensitive
ORC	origin recognition complex
pre-RC	pre-replicative complex
pre-IC	pre-initition complex
Pob3	DNA polymerase α binding protein 3
Pol	DNA polymerase
Psf	Partner of Sld five
RPA	replication protein A
ssDNA	single-stranded DNA
Sld	synthetically lethal with Dpb11-1
Spt16	suppressor of protein Ty 16
TopBP1	DNA topoisomerase II binding protein
Tof1	topoisomerase 1-associated factor 1

Introduction

DNA replication is a highly controlled process. In eukaryotes, the "once per cell cycle" rule is enforced by global regulation of the formation of the pre-replication complex during the M and G1 phases of the cell cycle, also called replication licensing (Blow and Dutta, 2005; DePamphilis et al., 2006; Nasheuer et al., 2002). Since recent reviews give an excellent account of pre-replication complex formation and its regulation, this issue and the factors involved, including the MCM2-7 complex and Mcm10, will not be discussed here.

The entry into S phase is marked by the initiation of DNA synthesis. This process requires of S phase-specific cyclin-dependent kinase (S-CDK) and Dbf4-dependent kinase Cdc7 (DDK) activity (Sherr and Roberts, 2004; Masai et al., 2005; Oehlmann et al., 2007). S-phase-specific cyclins A and E CDK proteins are controlled at the expression level, and furthermore, S-CDK and DDK activities are tightly regulated by inhibitory as well as activating post-translational modifications. These control mechanisms prevent on the one hand a premature onset of the S phase in general,

and govern the timely firing of each origin on the other hand. During recent years, it became apparent that unwinding of the DNA at the replication origin, and probably even more important, loading of the replicative DNA polymerases (Pols), onto the origin are the critical steps of regulation of the individual origins. This review will concentrate on the factors and mechanisms involved in this process.

The Regulators of DNA Replication Initiation

Dpb11, Cut5 and TopBP1

Albeit many disciplines in biosciences have contributed to our current understanding of DNA replication and its initiation, yeast genetics has been instrumental for the identification of novel factors involved. Among these, the *DPB11* gene was identified as a multi-copy suppressor of temperature-sensitive mutations of the Pol ε genes *POL2* and *DPB2* in the search for novel factors that interact genetically with Pol ε in the budding yeast (Araki et al., 1995). Dpb11 protein also interacts physically with Pol ε, but this interaction appears to be transient and probably restricted to the initiation of replication (Masumoto et al., 2000). Dpb11 associates with early firing origins of replication at the same time as Pol ε at the onset of S phase. But in contrast to Pol ε and Cdc45, Dpb11 does not associate with chromosomal DNA distal to the origins when DNA replication progresses (Aparicio et al., 1997; Masumoto et al., 2000) indicating that Dpb11 does not migrate with the progressing DNA replication fork. This also argues for a function specific for the initiation, but not elongation, of Dpb11.

Dpb11 was found to be homologous to the fission yeast Cut5/Rad4 protein. As Dpb11, Cut5 is essential for cell viability and is required for DNA replication as well as cell cycle control (Araki et al., 1995; Saka and Yanagida, 1993; Saka et al., 1994; Reid et al., 1999). Both proteins share a repetitive structure containing two pairs of BRCT domains. BRCT domains constitute a phospho-peptide binding region that has been found in a variety of proteins from bacteria to men (reviewed in Rodriguez and Songyang, 2008). BRCT domains have been first identified in BRCA1 and are common among DNA damage response and repair proteins (Bork et al., 1997; Callebaut and Mornon, 1997). In addition to binding to phospho-proteins, BRCT domains have also been implicated in binding of unphosphorylated target proteins as well as various DNA structures (Yamane and Tsuruo, 1999; Glover et al., 2004). Despite the low conservation of Dpb11/Cut5 at the sequences level, these proteins represent probably a ubiquitous replication and DNA damage response factor in eukaryotes. Orthologues have also been identified in plants and several metazoans, including human TopBP1 and fruit fly Mus101 (see “Function of TopBP1 in Genome Stability” by Sokka et al., this book for a detailed review on metazoan TopBP1 homologues), reviewed in (Garcia et al., 2005). These homologues have a more complex structure with additional BRCT domains, which may be associated with additional functions. As their yeast counterparts, the metazoan proteins have

been implicated in DNA replication. The Dpb11 homologue in *Xenopus*, XCut5 is required for the transition from the pre-replication to the pre-initiation complex. In particular, the loading of Cdc45 and of Pol ε onto the origin depends on Dpb11/XCut5 (Masumoto et al., 2000; van Hatten et al., 2002; Hashimoto and Takisawa, 2003).

Hiroyuki Araki and his co-worker continued their search for new factors involved in the initiation of DNA replication by seeking for genetic interaction partners of *DPB11*. In a screen for genes synthetically lethal with *DPB11*, the group of *SLD* mutants was discovered (Kamimura et al., 1998). The mutants fell into six complementation groups, thus representing six different genes. *SLD1* encodes Dpb3, which is the third largest subunit of Pol ε. *SLD4* is identical to *CDC45*, and *SLD6* is the same as the checkpoint kinase $RAD53^{CHK2}$. The three other *SLD* genes were found to be novel and not yet characterized.

Sld2 – A New Player in the Initiation of DNA Replication

SLD2 has been the first of the new *SLD* genes to be characterized (Kamimura et al., 1998 reviewed in Nasheuer et al., 2007). The same gene has been independently identified as a dosage suppressor of the *dpb11-1* mutation named *DRC1* (DNA replication and checkpoint 1) (Wang and Elledge, 1999). Sld2 is a protein of 453 amino acids in budding yeast and 337 amino acids in fission yeast without similarity to any characterized structural domain. Sld2 is a substrate of S-CDK activity (Masumoto et al., 2002; Noguchi et al., 2002). During S phase, the Sld2 protein becomes phosphorylated at least at six different serine and threonine residues both in fission and in budding yeast, and this modification facilitates an interaction with Dpb11/Cut5. Phosphorylation and apparently the Sld2-Dpb11 interaction is required for DNA replication, since a mutant deficient for all six phosphorylation residues is defective in chromosomal DNA replication, as are *SLD2* gene deletions (Masumoto et al., 2002; Noguchi et al., 2002). Surprisingly, these multiple phosphorylations at canonical motifs do not play a direct role in complex formation, but are barely the prerequisite for the phosphorylation of a further, non-canonical site within a 28 amino acid sequence that is responsible for binding of the carboxy-terminal BRCT pair of Dpb11 (Tak et al., 2006).

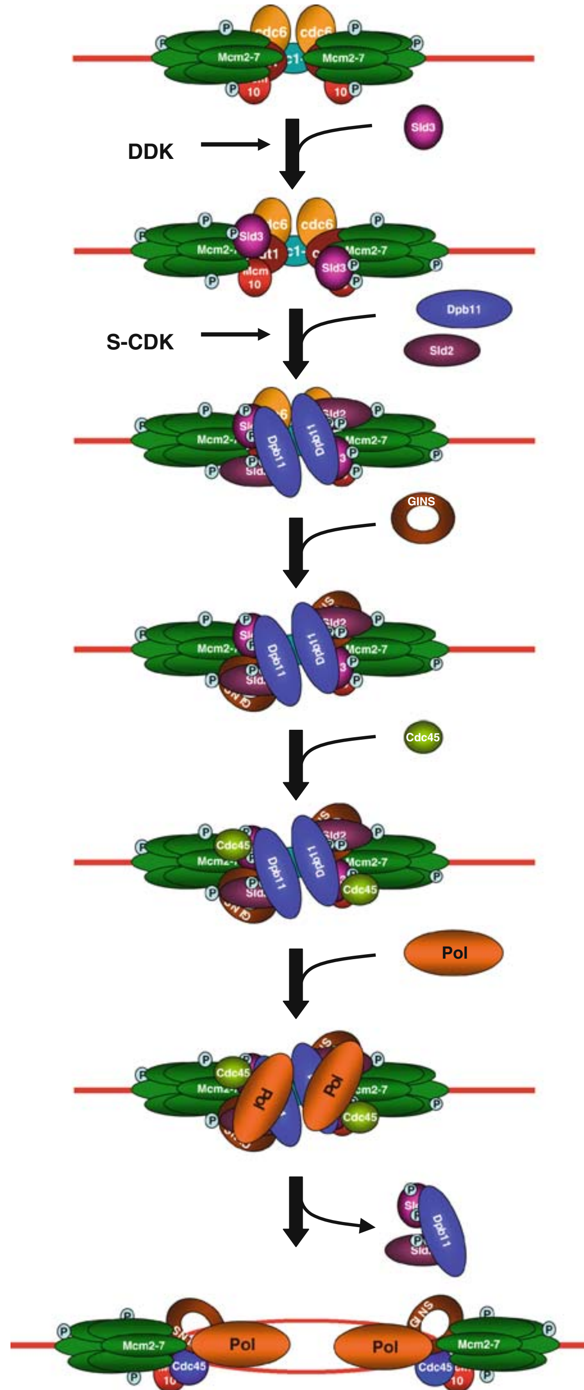
Recent reports suggest that RecQL4 protein may be the functional orthologue of Sld2 in animals. RecQL4 is one of five RecQ-like helicases identified in humans (reviewed in Bachrati and Hickson, 2008). Mutations in the *RecQL4* gene have been associated with Rothmund-Thompson, RAPADILINO and Baller-Gerold syndromes. Patients with these syndromes exhibit various physical and mental developmental abnormalities, increased risk of osteosarcoma, and features of premature aging. Disease-causing mutations lead to truncations or amino acid substitutions in the RecQ helicase domain. Sequences amino-proximal to the RecQ domain that are unique to metazoan RecQL4 are generally not affected in the patients. It is this amino-terminal region that shows limited homology to yeast

Sld2 (Sangrithi et al., 2005; Matsuno et al., 2006). Mice with a disruption in the helicase domain are viable, but exhibit growth retardation and genomic instability (Hoki et al., 2003; Mann et al., 2005). In contrast, mice with a homozygous disruption of exons 5–8 coding for part of the amino-terminal region die very early during embryogenesis (Ichikawa et al., 2002), emphasizing the importance of these sequences. RecQL4 appears essential for DNA replication, since depletion of the protein from *Xenopus* egg extracts inhibited initiation of DNA replication (Sangrithi et al., 2005; Matsuno et al., 2006). The amino-terminal fragment of RecQL4 lacking the helicase domain is sufficient to rescue the replication activity (Matsuno et al., 2006). This fragment included the Sld2 homology region and is able to bind XCut5. In RecQL4 depleted extracts, the pre-replication complex assembled normally, and Cut5, GINS and Cdc45 were loaded onto the chromatin. Instead, RecQL4 depletion suppresses the loading of DNA polymerases and RPA (Sangrithi et al., 2005; Matsuno et al., 2006). Recently results suggest that the amino-terminal, Sld2-like domain of RecQL4 promotes ATP-dependent DNA unwinding independently of the RecQ domain (Xu and Liu, 2009), despite the absence of conserved helicase motifs. Therefore, Sld2 and its metazoan homologue may have a direct role in the DNA unwinding at the origin of DNA replication.

Sld3– The Initiator of Initiation

Sld3 is another essential *SLD* gene identified in yeast by genetic screening (Kamimura et al., 2001; Nakajima and Masukata, 2002). Sld3 is a protein of 668 amino acids in fission and budding yeast that forms a complex with Cdc45. As for Sld2, no functional information can be extracted from the primary structure of Sld3. It is required for the association of yeast Cdc45 with the MCM2-7 complex, recruitment of Cdc45 to origins of DNA replication, and subsequent loading of RPA to the origin (Kamimura et al., 2001; Nakajima and Masukata, 2002; Pollok et al., 2003). In the budding yeast, Sld3 and Cdc45 associate simultaneously to origins at the time of origin firing, and their origin association is mutually dependent. Instead, in the fission yeast, Sld3 associates to origins without Cdc45 in the *nda4-108/mcm5* mutant (Yamada et al., 2004; Kanemaki and Labib, 2006). These studies indicate that loading of Sld3 and formation of an unstable complex with MCM2-7 is the initial step in the assembly of the initiation complex (Fig. 1). This is followed by the loading of Dpb11/Cut5, GINS and finally Cdc45 followed by unwinding of DNA at the origins (Nakajima and Masukata, 2002; Yabuuchi et al., 2006). However, differences in the loading order can be observed between early and late firing origins in fission yeast. In fact, association of *S. pombe* Sld3 with Cut5 and Cdc45 may occur before their loading at late firing origins (Yabuuchi et al., 2006). Sld3 loading requires DDK, but not S-CDK activity, whereas the later steps during initiation depend on S-CDK phosphorylation of Sld3 (Nakajima and Masukata, 2002; Yabuuchi et al., 2006; Tanaka et al., 2007b). Sld3 phosphorylated by S-CDK binds to the amino-terminal BRCT repeats of Dpb11 (Zegerman and Diffley, 2007).

Fig. 1 A model for the ordered assembly of the pre-initiation complex. The names of the proteins follows the nomenclature of the budding yeast *Saccharomyces cerevisiae*, where this process has been studied in most detail. A similar order of events is likely to take place also in other eukaryotes despite some variation in the factors involved. See text for details



After establishment of DNA replication forks, Sld3 is no longer required for the completion of DNA replication. This is in line with Sld2 and Dpb11, but in contrast to Cdc45 and GINS that are components of the elongation machinery, too. And in difference to the other factors involved, no structural or functional homologue of Sld3 could be identified thus far in organisms other than fungi, suggesting some diversity in the regulation of initiation complex formation in different groups of eukaryotes.

The Replication Factor Cdc45

Discovery and Characterization of Cdc45

CDC45 was first described as a cold-sensitive cell division cycle mutant in *Saccharomyces cerevisiae* (Moir et al., 1982 for review Nasheuer et al., 2007). Subsequently it has been shown to be essential for the initiation (Aparicio et al., 1997; Hardy, 1997; Owens et al., 1997; Uchiyama et al., 2001a) and elongation of DNA replication (Bauerschmidt et al., 2007; Pacek and Walter, 2004; Tercero et al., 2000) in a variety of eukaryotic organisms. The importance as replication factor is underlined by the fact that Cdc45 is highly conserved from yeast to man and that conservation between men and mice amounts to 92% identical amino acids. On the other hand, Cdc45, which is called Sna41 in *Schizosaccharomyces pombe*, and Tsd2 in *Ustilago maydis*, is apparently not present in archeabacteria (Grabowski and Kelman, 2003; Matsunaga et al., 2001), the third kingdom of life that shares many replication and transcription factors with the eukaryotes (Sclafani and Holzen, 2007). Sequence comparisons revealed only a conserved domain with acidic amino acids and a bipartite nuclear localization sequence (NLS) (Hopwood and Dalton, 1996; Loebel et al., 2000; Miyake and Yamashita, 1998; Shaikh et al., 1999; Zou et al., 1997). In yeast, Cdc45 is transported into the nucleus through the classical NLS transporter importin α (Pulliam et al., 2009).

Expression of Cdc45 and Its Control

Like many other genes involved in DNA replication, Cdc45 expression is regulated by the E2F family of transcription factors (Arata et al., 2000; Loebel et al., 2000; Stevens et al., 2004). There are several splice variants of its mRNA described. The main form, known as Cdc45L, lacks both the complete exon 7 as well as 36 base pairs from exon 18 (Kukimoto et al., 1999; Saha et al., 1998). Obviously, there exist some other forms of Cdc45 in various human tissues as detected by Northern blot analyses (Shaikh et al., 1999). Cdc45 mRNA expression in *S. cerevisiae* is maximal at the G1/S transition, whereas the protein level remains nearly constant over the cell cycle (Hardy, 1997; Owens et al., 1997). Similar results were also observed for Cdc45 mRNA and protein from fission yeast and humans (Saha et al., 1998;

Uchiyama et al., 2001b), the latter being in accordance with regulation by E2F/Rb. Cell cycle dependent degradation of Cdc45 is most likely achieved by an anaphase promoting complex (APC/C)-mediated ubiquitylation and subsequent degradation by the 26S proteasome. This was inferred from the presence of various APC/C-specific destruction boxes in the primary sequence and the enrichment of Cdc45 in the presence of proteasome inhibitors (Pollok and Grosse, 2007). This argues for a dynamic equilibrium in the protein levels supported by a half life of approximately 10 h (Pollok et al., 2007). In accordance with these regulatory circuits, differentiated or quiescent cells do not express Cdc45, but may turn on transcription and translation after the addition of growth factors or heavy metal ions (Arata et al., 2000). Since Cdc45 expression is tightly associated with proliferation this protein may be a promising candidate for a novel proliferation marker in cancer cell biology (Pollok et al., 2007). Highest protein levels were found in 0–4 h old embryos of the fruit fly *Drosophila melanogaster* (Loebel et al., 2000), which apparently stockpile this protein to allow several rounds of rapid cell proliferation without ongoing transcription. The highest amounts of human Cdc45 mRNA were found by Northern blots in proliferating tissues such as testis, placenta, thymus, thyroid glands, and colon epithelia, whereas non- or slowly proliferating tissue such as liver, brain and kidney were practically devoid of this mRNA (Shaikh et al., 1999).

As already mentioned thermo-sensitive yeast mutants of Cdc45 displayed a growth-arrest phenotype at the G1/S transition of the cell cycle at the restrictive temperature (Hennessy et al., 1991; Hopwood and Dalton, 1996; Miyake and Yamashita, 1998; Uchiyama et al., 2001a; Zou et al., 1997). Comparably, depletion of Cdc45 from *Xenopus* oocyte extract resulted in the inhibition of replication initiation and elongation (Mimura and Takisawa, 1998; Pacek and Walter, 2004). Interestingly, an RNA interference (RNAi) mediated Cdc45 knockdown caused chromosome-condensation in *D. melanogaster* (Christensen and Tye, 2003) and fragmented chromosomes in *Arabidopsis thaliana* (Stevens et al., 2004), whereas RNAi knockdown of Cdc45 in human tumor cells induced apoptosis (Feng et al., 2003). Heterozygous CDC45$^{+/-}$ mice did not display any anatomical abnormalities whereas homozygous CDC45$^{-/-}$ mice died during the peri-implantation state of the blastocyste about 7 days after conception (Yoshida et al., 2001). In humans the CDC45 gene is localized to chromosomal region 22q11, a region where micro-deletions are associated with the DiGeorge syndrome, a developmental defect that is thought to be due to a haploinsufficient expression of this part of the human chromosome. DiGeorge patients typically develop heart failures, insufficient aortas, craniofacial abnormalities, and underdeveloped thymus and parathyroid glands, which in turn give rise to immunodeficiencies and hypocalcemiae, respectively (Shaikh et al., 1999). Fluorescence in situ hybridizations indicate that at least 90% of all patients with DiGeorge syndrome display mono-allelic micro-deletions of the chromosomal region 22q11 including the coding region of CDC45. The resulting hypomorphic expression of Cdc45 together with its essential role in replication and proliferation may be one reason for the underdevelopment of the thymus and the parathyroid glands. On the other hand, since heterozygous mutant mice develop into adulthood without any apparent abnormalities, it is unlikely that hemizygosity

of CDC45 alone is responsible for the cardiac and craniofacial defects of this congenital syndrome (Yoshida et al., 2001).

Dynamics of Cdc45 in the Cell

In the cell, Cdc45 is mainly localized within the nucleus of yeast cells, as detected by live cell imaging with fusions between Cdc45 and the green fluorescent protein (Hopwood and Dalton, 1996). Confirmatory results were obtained with hemaggluttinin-tagged Cdc45 (Owens et al., 1997). Similarly, immunofluorescence studies with *Drospophila* embryos revealed chromatin-bound Cdc45 during interphase and dissociation from chromatin and a corresponding cytoplasmic localization during mitosis, when the cell nucleus is dissolved. Biochemical fractionations with human U2OS cells displayed a cytoplasmic and a nuclear distribution of Cdc45 during S phase (Saha et al., 1998), whereas immunoprecipitation and immunoflourescence experiments with HeLa S3 cells revealed a nuclear localization during interphase and chromatin-bound Cdc45 during S and G2 phase (Bauerschmidt et al., 2007).

Interaction Partners of Cdc45

Cdc45 interacts with a variety of other proteins, as determined by genetic interactions, two-hybrid screens and pull-down techniques. There is plentiful data describing the binding of Cdc45 to the MCM2-7 helicase of eukaryotic cells (Bauerschmidt et al., 2007; Dalton and Hopwood, 1997; Dolan et al., 2004; Gambus et al., 2006; Hennessy et al., 1991; Hopwood and Dalton, 1996; Kneissl et al., 2003; Kubota et al., 2003; Kukimoto et al., 1999; Loebel et al., 2000; Masai et al., 2006; Masuda et al., 2003; Mimura and Takisawa, 1998; Moyer et al., 2006; Pacek et al., 2006; Pacek and Walter, 2004; Uchiyama et al., 2001b; Zou and Stillman, 2000) as well as to the GINS complex (Bauerschmidt et al., 2007; Gambus et al., 2006; Kubota et al., 2003; Moyer et al., 2006; Pacek et al., 2006). Similar to Cdc45, the GINS complex is also recruited to the activated origins of replication, where it assembles with Cdc45 and the MCM2-7 proteins to form the Cdc45/MCM2-7/GINS (CMG) complex (see below), the presumed replicative helicase (Moyer et al., 2006; Boskovic et al., 2007). Cdc45 has also been shown to associate with Dpb11/Cut5/Mus101/TopBP1 either directly (Hashimoto and Takisawa, 2003; Wollmann et al., 2007) or indirectly via Sld3 (Kamimura et al., 1998; Nakajima and Masukata, 2002; Yamada et al., 2004). Cdc45 has been described to interact with all three replicative DNA polymerases of eukaryotes, i.e. Pol α (Aparicio et al., 1999; Hashimoto and Takisawa, 2003; Kubota et al., 2003; Kukimoto et al., 1999; Mimura et al., 2000; Mimura and Takisawa, 1998; Uchiyama et al., 2001a, b), Pol δ (Bauerschmidt et al., 2007) and Pol ε (Bauerschmidt et al., 2007; Kubota et al., 2003; Zou et al., 1997). In this respect it came as no surprise that Cdc45 also interacted

with the presumptive Pol α chromatin loader Mcm10 (Christensen and Tye, 2003; Gregan et al., 2003; Homesley et al., 2000; Loebel et al., 2000; Ramachandran et al., 2004; Sawyer et al., 2004) and the single-strand DNA binding protein RPA (Bauerschmidt et al., 2007; Dalton and Hopwood, 1997; Zou and Stillman, 2000). Thus, Cdc45 may act as a tether that bridges the MCM2-7-GINS helicase complex (see below) with the elongating DNA polymerases (Bauerschmidt et al., 2007; Kim et al., 1996).

The Role of Cdc45 During DNA Replication

DNA replication starts during S phase of the cell cycle at distinct origins of replication. Bidirectional DNA synthesis from each of the about 25,000 origins (in humans) follows a still ill-defined program with many origins firing early in S phase, fewer origins starting later and only a couple of origins starting very late the DNA synthesis phase of the cell cycle (Vogelauer et al., 2002). Apparently, at least in yeast Cdc45 is loaded into the early firing origins rather early and in the late firing origins late (Aparicio et al., 1999; Zou and Stillman, 2000), which taken together with its relative low abundance observed in the human cell (Pollok and Grosse, 2007) suggests a regulatory function, mainly since origin recruitment of Cdc45 is tightly associated with an activation of these origins. Until now there is only limited evidence for a phosphorylation of Cdc45 by any of the S phase kinases, though Cdc45 from *S. cerevisiae* is phosphorylated at least in vitro by Dbf4-Cdc7 (Weinreich and Stillman, 1999). Since in higher eukaryotes and particularly in mammals there is no known homologue of the yeast Sld3 protein, the question arose of how mammalian Cdc45 may become recruited to the initiation complex. One suggestion is that Cdc45 binds directly to the Dpb11 homologue TopBP1 (Schmidt et al., 2008). This might require phosphorylation of human Cdc45 by S phase kinases. Interestingly, a proteomic approach revealed a cluster of phosphorylation sites at position 130–151 of human Cdc45 (Dephoure et al., 2008), which however has not yet been shown to become phosphorylated by S phase kinases or being a binding module for TopBP1's BRCT domains. Another possibility is that TopBP1 binds unphosphorylated Cdc45 but may nevertheless be regulated by internal phosphorylation events. In this regard it is noteworthy to mention that depletion of protein phosphatase 2A from *Xenopus* egg extracts abolish loading of Cdc45 into the pre-replicative complex and that the initiation of eukaryotic DNA replication is regulated at the level of Cdc45 loading by a combination of stimulatory and inhibitory phosphorylation events (Chou et al., 2002).

A Phosphorylation Switch for the Initiation of DNA Replication

Recent experiments have shown that S-CDK-dependent phosphorylation of Sld2 and Sld3 initiates DNA replication in budding yeast (Zegerman and Diffley, 2007; Tanaka et al., 2007b). Dpb11 forms a ternary complex with the replication initiation

factors Sld2 and Sld3 when these become phosphorylated (Fig. 1). This complex then controls the association of Cdc45 and the replicative DNA polymerases with the origins of DNA replication (Masumoto et al., 2000). Tanaka and co-workers (Tanaka et al., 2007b) were able to demonstrate that a phospho-mimetic form of Sld2 (Sld2-11D) confers S-CDK-independent DNA replication when combined with either the *JET1* mutation of Cdc45, or overexpression of Dpb11. Both *JET1* and Dpb11 over-expression overcomes the requirement for Sld3 phosphorylation for initiation of DNA replication. Zegerman and Diffley (2007) fused an *Sld3* mutant that cannot become phosphorylated by S-CDK and that is deficient for DNA replication with the sequence for the amino-terminal BRCT domain pair of Dpb11. In a strain where S-CDK activity was inhibited at the same time, almost no DNA replication occurs. But when wild-type *SLD2* is in addition replaced by the phospho-mimetic *SLD2-T84D* variant, extensive DNA replication occurs, bypassing the requirement for S-CDK activity. Therefore, the phosphorylation of Sld2 and Sld3, and their subsequent binding by Dpb11 represents the minimal requirement for CDK-dependent activation of replication initiation in yeast (Tanaka et al., 2007a). The subsequent recruitment of Cdc45 into this complex (via Sld3) might be the rate-limiting step for the formation of an active replicative DNA helicase, i.e. the Cdc45-MCM2-7-GINS complex (Moyer et al., 2006; Boskovic et al., 2007; Aparicio et al., 2009).

As discussed above, the corresponding regulatory mechanisms in higher eukaryotes are poorly understood. Considering the roles of TopBP1/XCut5, RecQL4 and Cdc45 for the loading of the replicative DNA polymerases and establishment of the replication fork both in *Xenopus* and in human cells, a similar regulatory network as in yeast can be assumed for vertebrates. The targets for S-CDK and DDK among these proteins have not yet been identified. What is more, it remains unclear, which vertebrate factor takes over the role of yeast Sld3. Since human Cdc45 interacts directly with TopBP1, it is conceivable that human TopBP1 abrogates a requirement of Sld3. But in yeast, DDK-dependent loading of Sld3 appears to be the most upstream event in the initiation cascade, and a comparable regulatory step is not yet in sight in higher eukaryotes.

GINS: An Evolutionarily Conserved Key Player in DNA Replication

Identification of the GINS Complex

The GINS complex consists of four paralogous proteins (Sld5, Psf1, Psf2 and Psf3), whose encoding genes are present in all sequenced eukaryotic genomes. The name GINS is the acronym of the Japanese words “Go-Ichi-Ni-San” which mean “five-one-two-three”. The genes coding for the subunits of the GINS complex were identified by independent research groups in 2003. In their genetic analyses in *S. cerevisiae* aimed at discovering novel interaction partners of *DPB11* (Kamimura et al., 1998) Araki and co-workers identified mutations in the *SLD5* gene, which

were lethal in a *DPB11* temperature-sensitive mutant yeast strain (Takayama et al., 2003). In the same study, *PSF1*, *PSF22* and *PSF3* were identified as Partners of Sld Five by a combination of multi-copy suppression analysis and two-hybrid screens. Cell-cycle studies on yeast strains bearing thermo-sensitive mutants of the *SLD5* and *PSF1* genes revealed a defect of DNA replication under non-permissive conditions suggesting that GINS could be involved in DNA replication. Consistently, Ch-IP (Chromatin-Immuno-Precipitation) assays indicated that budding yeast GINS first associates with replication origins and then with proximal sequences during S phase (Takayama et al., 2003).

In a contemporaneous study Kubota and colleagues reported the identification and biochemical characterization of the GINS complex from *Xenopus laevis* (Kubota et al., 2003). Antibodies raised against each subunit of the *Xenopus* GINS complex were used to demonstrate that the four proteins form stable complexes in frog egg extracts. Furthermore, the four proteins were co-expressed in insect cells and found to co-purify. Sedimentation through glycerol gradients of the recombinant as well as endogenous complex indicated an apparent molecular weight of about 100 kDa consistent with an equimolar stoichiometric ratio of the four subunits. *Xenopus* GINS was found to be required for DNA replication, because immunodepletion of GINS abolished the incorporation of dNTPs into sperm chromatin. This effect was rescued by addition to the depleted egg extract of the full complex but not by an Sld5-Psf1-Psf2 sub-complex (Kubota et al., 2003). In *S. cerevisiae* as well as in *Xenopus* egg extracts, the GINS complex was found to be tightly associated with replicating chromatin fractions. Here it interacted with Cdc45 and the MCM2-7 complex and was loaded onto replicating chromatin together with Cdc45 in a mutually dependent fashion (Kubota et al., 2003; Takayama et al., 2003).

In a subsequent study in budding yeast, the GINS subunits were shown to be essential for the establishment of the DNA replication fork at firing origins as well as for normal progression of the replisome away from these origins (Kanemaki et al., 2003). *S. cerevisiae* strains were produced in which the gene of interest was fused to a "heat-inducible degron" cassette. The latter targeted the protein for rapid proteolysis at 37°C so that the immediate effects of bulk protein depletion could be evaluated. Using this strategy, three genes were identified (named *CDC101*, *CDC102* and *CDC105*) and found to be essential for the initiation and elongation phases of DNA replication (Kanemaki and Labib, 2006; Kanemaki et al., 2003). By immunoprecipitation experiments and mass spectrometry the corresponding proteins were found to be part of a complex together with a fourth component referred to as Yol146w. These four budding yeast genes corresponded to *SLD5* (*CDC105*), *PSF1* (*CDC101*), *PSF2* (*CDC102*) and *PSF3* (Yol146w).

The essential physiological role of GINS was also demonstrated in higher eukaryotes. Homozygous *PSF1* knockout mice died in uterus at around the time of implantation due to a defect in proliferation of the inner cell mass (Ueno et al., 2005). A subsequent two-hybrid screen confirmed that murine Sld5 was an interaction partner of murine Psf1 (Kong et al., 2006). Interestingly, both alleles of *PSF1* are required for the maintenance of the pool size of immature hematopoietic cells and acute bone marrow regeneration (Ueno et al., 2009).

A bioinformatic analysis revealed that the subunits of the GINS complex belong to a family of paralogous proteins, despite the low level of primary structure conservation (Makarova et al., 2005). In this report proteins orthologous to the eukaryotic GINS subunits were also identified in Archaea, as described below.

The Archaeal GINS Complex

The four subunits of the GINS complex display similar peptide chain lengths (around 220 amino acid residues) but very limited sequence identity. A bioinformatics analysis indicated particular similarities between Sld5 and Psf1 on the one hand and between Psf2 and Psf3 on the other hand (Makarova et al., 2005). This study also revealed that Archaea possess GINS homologues. Some archaeal species (such as *Sulfolobus solfataricus* and *Pyrococcus furiosus*) contain two members of the GINS family, one similar to Sld5/Psf1 and the other more close to Psf2/Psf3 (Marinsek et al., 2006; Yoshimochi et al., 2008). Other archaeal species (such as *Methanothermobacter thermoautotrophicus* and *Archeoglobus fulgidus*) apparently possess a single GINS protein which is homologous to Sld5/Psf1 (Makarova et al., 2005; Yoshimochi et al., 2008). Biochemical studies showed that the Sld5/Psf1 (GINS15) and the Psf2/Psf3 (GINS23) homologues of *Sulfolobus solfataricus* form a 2:2 tetrameric complex (Marinsek et al., 2006). The *Sulfolobus* GINS complex co-immunoprecipitated from cell extracts together with the MCM-like protein and the heterodimeric eukaryotic-like DNA primase. Two-hybrid analyses confirmed that the GINS23 subunit interacts with MCM and DNA primase (Marinsek et al., 2006). However, the *Sulfolobus* GINS complex did not exert any effect on the catalytic functions of these interaction partners in vitro.

More recently, Ishino and colleagues reported the biochemical characterization of the GINS complex of *Pyrococcus furiosus* (Yoshimochi et al., 2008). Similarly to the *S. solfataricus* GINS complex, it consists of two subunits (GINS15 and GINS23) forming a 2:2 tetramer. Nonetheless, differently from the *Sulfolobus* GINS, the ATPase and strand displacement activities of the *Pyrococcus* MCM complex were stimulated by GINS in vitro (Yoshimochi et al., 2008). However, the molecular mechanism for this activation is not clear because the *Pyrococcus* GINS was unable to bind nucleic acids in band shift assays and did not form a stable complex with MCM under gel filtration conditions. In addition, Ch-IP assays revealed that the *Pyrococcus* GINS preferentially associated with the chromosomal replication origin during the exponential growth phase but not in non-replicating cells. Moreover, a two-hybrid analysis showed that the *Pyrococcus* GINS interacts with the Cdc6/Orc1 homolog (Yoshimochi et al., 2008). These results suggest that, as observed for the eukaryotic GINS complex, the archaeal GINS may play a role in the initiation and/or the elongation phase of DNA replication.

The archaeal species, whose genome does not contain a Psf2/Psf3 homologue, might possess a very simplified version of the GINS complex consisting of only one Sld5/Psf1-like subunit. Alternatively, it is plausible that for these species the

in silico analyses failed because of the high sequence divergence of the Psf2/Psf3 homologue. The characterization of the archaeal GINS complex suggests that the four eukaryotic GINS subunits may have evolved from a common evolutionary origin by subsequent events of gene duplications and permutations (Makarova et al., 2005). This hypothesis has been found to be consistent with the recent structural analyses of the human GINS complex (Chang et al., 2007; Choi et al., 2007; Kamada et al., 2007).

Structural Studies on the GINS Complex

The first structural observations of the GINS complex were carried out by transmission Electron Microscopy (EM) (Kubota et al., 2003). This analysis revealed for the first time that the *Xenopus* GINS adopts a ring-like (or C-shaped) structure with an average diameter of 95 Å and a central pore of about 40 Å. This molecular shape was considered reminiscent of PCNA (Proliferating Cell Nuclear Antigen), the homotrimeric sliding clamp of Pol δ. Accordingly, it was proposed that GINS might act as a processivity factor for Pol ε (Kubota et al., 2003). Based on a gene multi-copy suppression analysis and two-hybrid assays, the four subunits of the GINS complex were proposed to be arranged in the order Psf2:Sld5:Psf1:Psf3 (Takayama et al., 2003). This subunit arrangement was recently confirmed for the human GINS complex by a combination of mass spectrometry and monoclonal antibody mapping using EM (Boskovic et al., 2007). In this study a three-dimensional reconstruction of the GINS complex has revealed a horseshoe-like shape with a central hole of 30–35 Å in diameter, large enough to encircle dsDNA (double-stranded DNA) and ssDNA (single-stranded DNA). According to this study, the central hole has the shape of a funnel because its diameter on one side is 70 Å wide, whereas it is narrower (about 25 Å) on the opposite site indicating the possibility of different functions for each side of the complex. In this report, the human GINS complex was found to bind nucleic acids with a clear preference for ssDNA. Based on the report that the *Drosophila* GINS complex is stably associated with MCM2-7 and Cdc45, the authors propose a model where GINS acts as a co-factor for the MCM2-7 replicative helicase by encircling ssDNA in its central hole (Boskovic et al., 2007).

However, the crystal structure of the human GINS complex as solved by three groups seems not to be consistent with the proposal that the central hole encircles ssDNA. The overall structure as well as the fold of the individual subunits and their interactions were essentially the same in all three publications (Chang et al., 2007; Choi et al., 2007; Kamada et al., 2007), although two groups used crystals of the complex with deletion of the last 50 amino acid residues of Psf1 (Choi et al., 2007; Kamada et al., 2007). The resulting Psf1-truncated form of GINS was as stable as the complex containing full-sized Psf1, indicating that the missing Psf1 fragment is not essential for tetramer formation and complex stability. This agrees well with the full-length structure where the C-terminal 51 residues of Psf1 were not visible suggesting that this portion of the polypeptide chain is intrinsically disordered (Chang

et al., 2007). The hetero-tetrameric GINS complex resembles a trapezoid with Sld5 and Psf1 forming the top layer and Psf2 and Psf3 associated at the bottom. Few contacts are observed between Sld5 and Psf3 and between Psf1 and Psf2. This subunit arrangement is fully consistent with the results of the genetic analyses and two-hybrid assays carried out in *S. cerevisiae* (Takayama et al., 2003). One important finding was that the fold of Sld5 and Psf2 is similar to that of Psf1 and Psf3, respectively, despite the limited sequence identity within each couple of subunits. In addition, each subunit is composed of two structural domains: an α-helix-rich (A) domain and a β-strand-rich (B) domain. These two domains are found in the order A-B in Sld5 and Psf1, whereas they are inverted (B-A) in Psf2 and Psf3. The A domain consists of four α-helices (with the exception of Psf2 whose A domain contains 2 α-helices and one β-strand) forming an arc; the B domain consists of two small anti-parallel β-sheets forming a jelly-roll structure. The linker region connecting the A and B domains is only 6 residues long in Psf2 and Psf3 but 21 residues in Sld5 and possibly also in Psf1. The B domain of Sld5, Psf2 and Psf3 is stably anchored to the respective A domain whereas in Psf1 the B domain is loosely associated to the A domain. The unstructured B domain of Psf1 is likely involved in physical interaction with other DNA replication factors. To test this hypothesis, the *Xenopus* DNA replication system was used (Kamada et al., 2007). Frog egg extracts, immuno-depleted of the GINS complex, were unable to replicate DNA while DNA replication activity was restored by adding the recombinant full-length human GINS complex. In contrast, addition of a complex containing a deletion of the Psf1 B domain or addition of the Psf1 B domain alone ($Psf1_{140–196}$) or a combination of these two were not sufficient to support DNA replication in depleted extracts. Analysis of the chromatin-bound fraction from frog egg extracts by western blots revealed that the ORC and MCM2-7 complexes were associated with chromatin in the immuno-depleted extracts supplemented with either the intact or the mutant human GINS complex. Conversely, chromatin-association of Cdc45 and Pol ε varied according to the replication activity of each GINS mutant used to complement the egg extracts.

By modelling of the Psf1 B domain on the structure of the corresponding Sld5 B domain Kamada et al. identified the linker region and residues in Psf1 that could be exposed and form the binding interface for other proteins (Kamada et al., 2007). The corresponding residues in Sld5 are involved in binding Psf2. Substitution of these residues of Psf1 with alanine reduced (although did not completely abolish) the ability of the GINS complex to support the DNA replication activity in immunodepleted *Xenopus* egg extracts. Similar effects were observed by mutating specific residues that are likely to be located in the Psf1 linker region. Therefore, the Psf1 B domain located on the surface of the GINS complex plays a critical role in the initiation process where it mediates chromatin-association of other replication factors, such as Cdc45 and Pol ε. In addition to the Psf1 B domain, other unstructured regions were observed in both Psf3 and Sld5 in the X-ray structure. In particular, the Psf1 B domain is close to the unstructured C-terminal tail of Psf3 (residues 194–216) and to the disordered fragment of Sld5 (residues 65–71). On the same side, but on the other end of the hetero-tetramer, lies an unstructured region within the Psf3 B

domain, which may also serve as a protein-binding site. This putative wide distribution of the protein interaction surfaces may allow simultaneous interactions of more than one binding partner to the GINS complex.

An important issue regarding the function of the GINS complex is whether its central pore exists and is accessible. One high-resolution crystallographic study showed that the central cleft that is almost closed at the bottom (Kamada et al., 2007). Choi and co-workers reported that the human GINS complex contains a central channel, but only with an internal diameter of about 5 Å that is not large enough to accommodate nucleic acids (Choi et al., 2007). In contrast, in the X-ray structure of human GINS by the Chen group the diameter of the central pore is 10 Å and a mechanism was suggested by which the opening of the central pore may be regulated (Chang et al., 2007). In fact, a careful inspection of the crystal structure revealed that a 16-residues loop from the N-terminus of Psf3 is not tightly bonded to the pore surface and therefore may regulate its accessibility by moving outside and inside this central cavity. Upon the removal of this N-terminal 16-residues loop of Psf3 the diameter of the central pore is increased from 10 to 18 Å. Multiple sequence alignments indicate that the first 16 N-terminal residues of Psf3 are only present in human and higher eukaryotes. In addition, the human GINS complex, in which Psf3 bears a truncation of the first 10 or 18 residues from the N-terminus, was found to be as stable as the hetero-tetramer containing full-sized Psf3 (Chang et al., 2007). The latter proposed that the central pore may be involved in holding a domain of MCM2-7 complex, Cdc45 or a DNA polymerase at the replication fork or, alternatively, that it can bind DNA in its open state.

GINS in the Initiation and Elongation Phases of DNA Replication

The abundance and composition of GINS were found to be constant during the cell cycle in *S. cerevisiae* (Takayama et al., 2003). Association of GINS to chromatin at the replication origins takes place at the onset of S phase and requires the activity of both S-CDK and DDK (Kanemaki and Labib, 2006; Yabuuchi et al., 2006). The ordered assembly of various initiation factors to the pre-RC and their regulation by the cell-cycle kinases has already been discussed above in detail. Importantly, both in *S. cerevisiae* and *S. pombe* Sld3 is required for the association of GINS to replication origins and two-hybrid assays revealed that fission yeast Sld3 directly interacts with GINS (Yabuuchi et al., 2006). Also Dpb11-Sld2 are loaded in an interdependent way with GINS in both budding and fission yeast and a direct association among these factors was suggested on the basis of two-hybrid studies (Takayama et al., 2003; Yabuuchi et al., 2006).

It has become clear that GINS is required not only for the establishment but also for the progression of the DNA replication fork in budding yeast (Takayama et al., 2003; Kanemaki et al., 2003). Several studies revealed that GINS is a stable component of the eukaryotic replisome. Calzada and co-workers described a method for inducing pausing of the replisome at natural Replication Fork Barriers

(RFBs) in specially-engineered *S. cerevisiae* strains where a RFB is located in the vicinity of specific early replication origins (Calzada et al., 2005). Ch-IP assays revealed that paused replisomes contain MCM2-7, Cdc45, GINS, the proteins forming the fork protection complex (Mrc1, Tof1 and Csm3) and Pols α and ε. A similar approach was employed to dissect the molecular anatomy of the replisome in *Xenopus* egg extracts where sequence-specific replication fork pausing was induced with biotin-streptavidin-modified plasmids. Here, the replicative Pols α, ε and δ, GINS, MCM2-7, Cdc45 and MCM10 were identified as components of the vertebrate replisome (Pacek et al., 2006). In the presence of aphidicolin, a DNA polymerase inhibitor, MCM2-7, Cdc45 and GINS were still found to be associated to the pause sites, whereas the replicative DNA polymerases showed a more dispersed distribution along the plasmid DNA. This result suggested that MCM2-7, Cdc45 and GINS form a stable heterologous complex with DNA unwinding activity (the so-called "unwindosome") that could be "uncoupled" from the machinery responsible for DNA synthesis. Association of GINS and MCM2-7 to the replication fork was also demonstrated by a proteomic study carried out in *S. cerevisiae* (Gambus et al., 2006). A yeast strain, which expressed differently tagged versions of Sld5 and MCM4, was created to purify complexes containing both MCM2-7 and GINS from cell extracts. After digesting the chromosomal DNA, the so-called Replisome Progression Complexes (RPCs), large protein assemblies (>1,400 kDa), were purified. Mass spectrometry identified the components of the RPCs, which included (in addition to GINS and MCM2-7) Cdc45, MCM10, DNA topoisomerase I, factors involved in the stabilization of stalled forks (Mrc1, Tof1 and Csm3), in sister chromatid cohesion (Ctf4), and in chromatin-remodelling (Spt16 and Pob3). The RPCs are formed only during S phase and disappear at the end of chromosomal replication, but are not disassembled when forks from early-firing origins are stalled by inhibitors of DNA synthesis (Gambus et al., 2006).

A complex containing Cdc45, MCM2-7 and GINS was also isolated by Moyer and colleagues from extracts of *Drosophila* embryos (Moyer et al., 2006). Biochemical and immunological analyses identified the six proteins of the MCM2-7 complex and four subunits of GINS as components of these purified samples. This protein assembly was called CMG (Cdc45/MCM2-7/GINS). Enzymatic assays revealed that the CMG complex possessed an ATPase-dependent DNA helicase activity with 3′–5′ directionality (Moyer et al., 2006).

The structural analyses of the human GINS suggest that this complex mediates the interaction with other replication factors. Two-hybrid analyses revealed that *S. cerevisiae* Psf1 and Dpb2 (the 60-kDa subunit of Pol ε) interact physically and, thus, a direct contact between GINS and Pol ε at the replication fork in budding yeast can be postulated (Takayama et al., 2003). In addition, a direct interaction between fission yeast Psf3 and subunits of the MCM2-7 complex has been detected by the two-hybrid system (unpublished data in Yabuuchi et al., 2006). A direct physical interaction between the N-terminal portion of the *Sulfolobus* MCM complex and the Psf2/Psf3 homolog has also been detected in a two-hybrid screening (Marinsek et al., 2006). Therefore, it is plausible that Psf3 is responsible for directly contacting the MCM complex within the replisome. However, also Psf1 could be

involved in the formation of a complex with Cdc45 and the MCM2-7 proteins, according to the analysis performed using the *Xenopus* egg extracts (Kamada et al., 2007). Furthermore, the *Sulfolobus* GINS complex was found to stably interact with the eukaryotic-like heterodimeric DNA primase in vitro and in vivo through the Psf2/Psf3 (GINS23) subunit (Marinsek et al., 2006). This finding is consistent with a recent report that the recombinant human GINS complex physically interacts in vitro with the Pol α-primase complex and stimulates its DNA synthesis activity (De Falco et al., 2007). This interaction is likely to take place through the Psf2 subunit, as recently proposed (Chang et al., 2007) and to involve other replication factors, such as MCM10 and Ctf4 (Zhu et al., 2007).

Initiation and Checkpoint

A Role for Initiation Factors During Checkpoint Response

When an ongoing replication fork encounters an obstacle or experiences a diminution of nucleotide building blocks it stops and triggers an intra S phase checkpoint mechanism that in turn prevents loading of initiation factors onto distal origins and subsequent firing of the later ones (Machida et al., 2005). This mechanism is conserved from yeast to man (Aparicio et al., 1999; Costanzo et al., 2003; Falck et al., 2002; Liu et al., 2006; Petersen et al., 2006). Signalling is typically initiated by the phosphatidyl inositol kinase-like kinases (PIKK) ataxia telangiectasia mutated (ATM) or ataxia telangiectasia and Rad-3 related (ATR) that phosphorylate the effector kinases Chk2 and Chk1, respectively. Inhibition of ATM or ATR by caffeine leads to an accumulation of Cdc45 on chromatin (Costanzo et al., 2003; Falck et al., 2002; Shechter et al., 2004). There is an intimate interplay between the checkpoint apparatus and DNA initiation factors that regulate initiation in response to DNA damage or physiological stress, but also during normal DNA replication. Most critical for the activation of replication are the S phase kinases Cdk2 (S-CDK) and Cdc7 (DDK). These proteins are regarded as being the most important targets for checkpoint control during S phase. ATM initiated signalling targets Cdk2 (Costanzo et al., 2000), while ATR mediated signalling targets also Cdc7 (Costanzo et al., 2003; Shechter et al., 2004). Several replication and initiation factors, including Sld2 have been implicated in checkpoint control (Wang and Elledge, 1999; Tourrière and Pasero, 2007). But without doubt, the initiation factor Dpb11/Cut5 and its metazoan TopBP1 homologues have emerged as the key player for the checkpoint regulation of initiation of replication. Budding yeast Dpb11 is required for S phase checkpoint control, as has Cut5 in fission yeast (Saka et al., 1994; Araki et al., 1995), reviewed in (Garcia et al., 2005). This role appears to be universal in eukaryotes (reviewed in “Function of TopBP1 in Genome Stability” by Sokka et al., this issue). It has been best studied in vertebrates, where TopBP1 and its homologues have been shown to represent a general activator of ATR (see “Function of TopBP1 in Genome Stability” by Sokka et al., this issue for a detailed review on this topic).

This function has also been confirmed for the yeast orthologues (Navadgi-Patil and Burgers, 2008; Mordes et al., 2008). There exists apparently a negative feedback loop, where TopBP1 is recruited by the checkpoint apparatus involved in ATR activation. This in turn leads to inhibition of the S phase kinases S-CDK and DDK, preventing initiation-activating phosphorylation of Sld2 and Sld3. It is therefore not surprising that ATM and ATR and their mediator kinase Chk1 affect also initiation of DNA replication in undamaged cells. They are believed to inhibit the firing of distal origins by inactivating phosphorylation of the S phase kinases and the MCM2-7 complex (Fisher and Méchali, 2004; Shechter et al., 2004; Shechter and Gautier, 2005).

DNA Initiation Factors and Stalled DNA Replication Forks

The DNA initiation factors discussed in the review also appear to have a role in the stabilization and re-activation of stalled replication forks. Cdc45 interacts with the mediator of the replication checkpoint 1 (Mrc1, known as claspin in mammals) and the topoisomerase 1-associated factor 1 (Tof1, known as Tim1 or *timeless* in mammals) (Katou et al., 2003). Mrc1/claspin is necessary for the activation of the intra S phase checkpoint whenever a replication fork stalls (Kumagai and Dunphy, 2000; Alcasabas et al., 2001). After the loading of Cdc45 both Mrc1/claspin and Tof1/Tim1 are recruited into the replication origin and all three proteins co-migrate with the ongoing replication fork (Katou et al., 2003; Osborn and Elledge, 2003). When such a fork hits a replication blockade Mrc1, Tof1 and other factors prevent disassembly of the replication machinery (Calzada et al., 2005; Katou et al., 2003; Nitani et al., 2006). Moreover, both proteins are important for the recovery of DNA synthesis at stalled forks after the block has been removed (Tourrière et al., 2005). The resulting stabilization of stalled replication forks is fundamental for preventing genomic instability in eukaryotes (Branzei and Foiani, 2006). This function primarily depends on the ATR pathway, including Mrc1/claspin. Stalled replication forks are also stabilized in a checkpoint-independent manner where the key players are Tof1/Tim1 plus Mrc1 (Katou et al., 2003; Tourrière et al., 2005). Although TopBP1 and its orthologues are not part of the progressing DNA replication fork, they have been shown to re-localize to the sites of stalled replication and they are involved in the restart of the stalled replication fork (Mäkiniemi et al., 2001). It is conceivable that a regulatory switch comparable to the replication initiation reaction is also required for the replication restart.

Conclusions

Eukaryotes have restricted replication to a specific phase of the cell cycle dedicated to extensive DNA synthesis. This has led to the invention of a sophisticated regulatory mechanism that ensures correct timing of origin firing. It is probably for the

sake of an efficient regulation that the critical targets of S-phase kinase-dependent promotion of replication, Sld2 and Sld3, are proteins that are dedicated to the assembly of the replication fork, but are not required for fork progression. Dpb11 (and its metazoan TopBP1 orthologues) likely represents a master controller that integrates mitogenic cell cycle and checkpoint signalling to regulate the initiation at the level of the individual origin of replication.

The role of the Sld2-Sld3-Dpb11 phosphorylation switch lies in the modification of the pre-replication complex that leads to the loading of Cdc45, GINS and eventually the replicative DNA polymerases.

Since no enzymatic activities have been assigned to Cdc45 and GINS, it is likely that these factors play rather structural roles at the replication fork. Cdc45 and GINS have been on the one hand proposed as MCM2-7 helicase cofactors that form together the CMG unwindosome, and on the other hand as bridging factors between the replicative helicase and DNA polymerases. They not only seem to coordinate helicase and DNA polymerases action but may also retrieve and integrate signals from several pathways to uncouple the CMG helicase from the Pols δ and ε.

Despite the picture emerging for the initiation and its regulation, major challenges are left for the future: the mechanisms of the regulatory switch in metazoan initiation are still to be elucidated, the structural understanding of the initiation complex is still rudimentary, and the interplay between initiation and checkpoint control awaits to be defined in detail. These and many other unresolved issues will provide topics for many years of DNA replication research.

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