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Architecture of the *Saccharomyces cerevisiae* Replisome

Lin Bai,

Cryo-EM Structural Biology Laboratory, Van Andel Research Institute, Grand Rapids, MI, USA

Zuanning Yuan,

Biochemistry and Structural Biology Graduate Program, Stony Brook University, Stony Brook, NY, USA. Cryo-EM Structural Biology Laboratory, Van Andel Research Institute, Grand Rapids, MI, USA

Jingchuan Sun,

Cryo-EM Structural Biology Laboratory, Van Andel Research Institute, Grand Rapids, MI, USA

Roxana Georgescu,

Howard Hughes Medical Institute, The Rockefeller University, New York, NY, USA

Michael E. O'Donnell, and

Howard Hughes Medical Institute, The Rockefeller University, New York, NY, USA

Huilin Li

Biochemistry and Structural Biology Graduate Program, Stony Brook University, Stony Brook, NY, USA. Cryo-EM Structural Biology Laboratory, Van Andel Research Institute, Grand Rapids, MI, USA

Abstract

Eukaryotic replication proteins are highly conserved, and thus study of *Saccharomyces cerevisiae* replication can inform about this central process in higher eukaryotes including humans. The *S. cerevisiae* replisome is a large and dynamic assembly comprised of ~50 proteins. The core of the replisome is composed of 31 different proteins including the 11-subunit CMG helicase; RFC clamp loader pentamer; PCNA clamp; the heterologous DNA polymerases ϵ , δ , and α -primase; and the RPA heterotrimeric single strand binding protein. Many additional protein factors either travel with or transiently associate with these replisome proteins at particular times during replication. In this chapter, we summarize several recent structural studies on the *S. cerevisiae* replisome and its subassemblies using single particle electron microscopy and X-ray crystallography. These recent structural studies have outlined the overall architecture of a core replisome subassembly and shed new light on the mechanism of eukaryotic replication.

Keywords

Eukaryotic DNA replication; Replisome; Replicative helicase; Cryo-EM; Mcm; CMG; DNA polymerase; Structural biology

10.1 Introduction

The DNA genome is duplicated in semiconservative fashion wherein each strand serves as the template for synthesis of its complementary strand (Meselson and Stahl 1958; Watson and Crick 1953a, b). This process may seem simple, but nothing could be further from the truth; it requires >50 different proteins in eukaryotes, and many of their individual functions remain unknown, much less how they work together to accomplish high-fidelity genome duplication (Costa et al. 2013; Li and Araki 2013; MacNeill 2012). Unlike protein synthesis and mRNA synthesis that are well conserved across the three domains of life, DNA synthesis is not well conserved. Many key replication proteins, such as the replicative helicase, DNA polymerases, primase, and single strand binding protein, are evolutionarily unrelated between bacteria and archaea/eukarya (Forterre et al. 2004; Leipe et al. 1999). Thus one cannot rely on the wealth of bacterial studies to understand eukaryotic replication, and detailed studies of eukaryotic replication mechanisms are warranted. Fortunately, the replication machinery within eukaryotes is highly conserved, and thus the relatively simple *Saccharomyces cerevisiae* can be used as a reliable model for replication in higher eukaryotes (Leman and Noguchi 2013).

The “core replisome” encompasses proteins that are necessary to propagate the replication fork (Zhang and O'Donnell 2016). The eukaryotic replisome core is composed of CMG helicase, the leading strand DNA polymerase (Pol ϵ), lagging strand Pol δ , Pol α -primase, along with the RFC clamp loader, PCNA clamp, and RPA single strand (ss) DNA-binding protein. Many other components, such as Ctf4, Mcm10, Topo I, the checkpoint response factors Mrc1, Tof1, Csm3, and the FACT nucleosome mobility factor, either travel with the replisome or hop on and off at different points during replication (Bell and Labib 2016).

The eukaryotic helicase is an 11-protein machine composed of Cdc45, the Mcm2-7 heterohexamer, and the four-protein GINS complex (Psf1-3, and Sld5) referred to as CMG (Bochman et al. 2008; Ilves et al. 2010; Moyer et al. 2006). Eukaryotic CMG helicase is assembled through a series of replication initiation events (reviewed in (Costa et al. 2013; O'Donnell et al. 2013; Tanaka and Araki 2013; Tognetti et al. 2015)). Briefly, the origin recognition complex (ORC) along with Cdc6 loads onto replication origins in the G1 phase of the cell cycle (Bell and Stillman 1992) and then recruits the helicase core Mcm2-7 heterohexamer with the help of Cdt1 (Cocker et al. 1996; Liang et al. 1995; Mimura et al. 2004; Nishitani et al. 2000; Santocanale and Diffley 1996; Sun et al. 2013; Tanaka and Diffley 2002). Subsequently, another Mcm2-7 is recruited onto double-stranded (ds) DNA forming a double hexamer of Mcm2-7 that is inactive (Duzdevich et al. 2015; Evrin et al. 2009; Remus et al. 2009; Sun et al. 2014; Tica et al. 2015). Finally, at the G1-to-S transition, the double hexamers are converted to two active helicases comprised of Cdc45-Mcm2-7-GINS (CMG) that each encircle opposite strands of single-stranded (ss) DNA to carry out bidirectional separation of the DNA duplex (Bell and Botchan 2013; Duzdevich et al. 2015).

Eukaryotic replication requires at least three DNA polymerases to propagate fork movement (Burgers 2009). These include Pol α -primase, Pol ϵ , and Pol δ . Pols α , ϵ , and δ are all members of the B family of DNA polymerases (Steitz 1999). Pol α initiates DNA synthesis

on both the leading and lagging strands by synthesizing a RNA/DNA hybrid primer (Conaway and Lehman 1982). Numerous genetic, cell biology, and biochemical studies concur that Pol ϵ and Pol δ extend primers on the leading and lagging strands, respectively (Burgers et al. 2016; Clausen et al. 2015; Kunkel and Burgers 2008; Miyabe et al. 2011; Nick McElhinny et al. 2008; Pursell et al. 2007), although one report suggests that Pol δ functions on both strands leaving some uncertainty for future studies to resolve (Johnson et al. 2015).

The antiparallel structure of dsDNA, combined with unidirectional synthesis by DNA polymerases, results in continuous synthesis on the leading strand that travels in the direction of DNA unwinding and discontinuous synthesis on the lagging strand that is extended as multiple Okazaki fragments in the direction opposite DNA unwinding. A preformed primed site is required for DNA polymerase function, and both strands are primed by Pol α -primase. However, Pol α -primase is needed repeatedly on the lagging strand, once for each 100–200 bp Okazaki fragment. Pol α -primase is a four-subunit enzyme; the largest subunit is a DNA polymerase that lacks a proofreading 3'–5' exonuclease, the second largest is the B subunit (unknown function), and the two small subunits, PriL and PriS, contain the priming activity (Banks et al. 1979; Conaway and Lehman 1982; Kaguni et al. 1983a, b).

There have been numerous important advances on the structure of components of the eukaryotic replisome in the last decade. In this chapter, we focus mainly on structures that have been solved during the past 3 years, specifically of proteins directly involved in moving replication forks. We first review the cryo-EM structure of the CMG helicase and compare the structures of CMG components GINS and Cdc45 of yeast and human. We then review the relative positions of Pol ϵ , Ctf4, and Pol α in the context of the CMG helicase and the possible replisome architecture at the replication fork. We end our chapter by providing a brief perspective on key missing information and what to expect in the coming years.

10.2 The CMG Structure

The first structure of eukaryotic CMG was determined in the *Drosophila melanogaster* system by negative stain EM at low resolution (Costa et al. 2011). The structure revealed a Mcm2-7 ring braced on the side by Cdc45 and GINS that interacted mainly with Mcm2, Mcm3, and Mcm5, apparently forming a secondary pore. However, this secondary pore is essentially filled in by side chains in the higher resolution cryo-EM map of the *S. cerevisiae* CMG at a resolution of 3.7–4.8 Å (Fig. 10.1a–c) (Yuan et al. 2016). The secondary pore also disappears in a medium resolution cryo-EM map of *Drosophila* CMG at 7–10 Å resolution (Abid Ali et al. 2016). The Mcm2-7 core in the CMG forms a two-tiered ring structure: an N-terminal domain (NTD) tier composed of a helical subdomain, a Zn-binding motif, and an oligonucleotide/oligosaccharide-binding (OB) motif, while the C-terminal domain (CTD) tier contains the AAA+ motors. This domain architecture is similar to that of other hexameric helicases including the archaeal MCM hexamer and the inactive yeast Mcm2-7 in the double hexamer (Brewster et al. 2008; Enemark and Joshua-Tor 2006; Li et al. 2015; Miller et al. 2014; Singleton et al. 2000; Slaymaker and Chen 2012). Cdc45 and GINS mainly bind the NTD-tier ring of Mcm2-7, forming a rigid unit of Cdc45-GINS-Mcm2-7

NTD. More precisely, Cdc45 contacts only the NTDs of Mcm2 and Mcm5, and GINS contacts only the NTDs of Mcm3 and Mcm5 (GINS and Cdc45 also contact one another). It is the tight interaction between the NTD of Mcm5 and Cdc45-GINS that occludes the secondary pore.

In the medium resolution cryo-EM structures of the *Drosophila* CMG, density corresponding to six nucleotides of ssDNA was observed either inside the CTD-tier motor ring or inside the NTD-tier ring (Abid Ali et al. 2016). This observation is consistent with CMG acting as an ssDNA translocase. Interestingly, in an archaeal MCM hexamer, ssDNA was found to bind the NTD-tier ring as a circle perpendicular to the MCM channel axis and was proposed to be an intermediate in origin initiation (Froelich et al. 2014). How a CMG engages DNA at a replication fork remains an important issue in the field.

10.3 The Mcm2-7 Hexamer Undergoes Large Conformational Changes from the Inactive Double Hexamer to the Active CMG Helicase

Recent studies have provided two yeast Mcm2-7 hexamer structures: one determined in the context of the inactive Mcm2-7 double hexamer and the other in the context of active CMG as described above (Li et al. 2015; Yuan et al. 2016). This allows a comparison of the structures to gain insight into the mechanism by which the helicase is activated. To transition from the inactive Mcm2-7 double hexamer to the single Mcm2-7 in CMG requires large domain movements of up to 20 Å and rotations of up to 30° (Yuan et al. 2016). Movements in the CTD-tier motor domains are more extensive than the NTD-tier ring. As an example, we compare Mcm2-Mcm5 in the two structures (Fig. 10.2a–c). Notably, the interface between the CTDs of Mcm2 and Mcm5 is looser in the CMG structure than in the double hexamer. Activation of CMG requires it to encircle the leading strand and exclude the lagging strand. The looser Mcm2/5 interface at the C-tier ring may have allowed the extrusion of the lagging strand to the outside surface during activation of the CMG helicase. Another major change is the insertion of the Mcm5 C-terminal winged helix domain (WHD) into the interior channel in the active helicase, while the Mcm5 WHD of the inactive Mcm2-7 hexamer is visualized as weak density outside the central channel (Li et al. 2015; Yuan et al. 2016). The Mcm6 C-terminal WHD in the active and inactive Mcm2-7 was also reconfigured. Considering that the WHD is usually involved in DNA binding or interaction with other proteins, the observed movement of these WHDs may have important consequences for helicase activation and replisome function during fork progression.

10.4 GINS Is a Protein-Binding Hub of the Replisome but Does Not Contain a Central Channel for ssDNA

The yeast CMG structure reveals for the first time the complete structure of the GINS heterotetramer (Yuan et al. 2016). The GINS subunits are related in evolution and are composed of two domains each (A and B) (Chang et al. 2007; Choi et al. 2007; Kamada et al. 2007). The B domain of the Psf1 subunit was missing in all previously reported GINS structures (Chang et al. 2007; Choi et al. 2007; Kamada et al. 2007). The Psf1 B domain interacts with Cdc45 by forming an interfacial cross-molecule β -sheet (Yuan et al. 2016).

Perhaps the Psf1 B domain is disordered in the absence of Cdc45, explaining why it was not resolved in the crystal structures of the GINS alone.

Earlier low-resolution EM observations as well as the crystal structure of the human GINS suggested a potential hole in the middle of GINS (Boskovic et al. 2007; Chang et al. 2007; Kubota et al. 2003). It was further suggested that ssDNA might thread through the central hole of the GINS. The structure of the full-length yeast GINS contains only a small hole of ~ 7 Å diameter, too small to thread ssDNA (Fig. 10.3) (Yuan et al. 2016). Importantly, although the GINS structures are very similar between human and yeast, structural features surrounding the central region vary (Chang et al. 2007; Choi et al. 2007; Kamada et al. 2007; Yuan et al. 2016). The yeast GINS has two insertions in Psf1 and Psf2 that are absent in human GINS, and the human GINS has a Psf3 insertion loop that is absent in the yeast GINS. The structural variation, together with the small size, argues against ssDNA threading through the central hole. Instead, the GINS functions mainly to scaffold the replisome by recruiting other proteins. The CMG structure appears to bear this out. Thus GINS interacts extensively with Mcm3/5 and Cdc45 (Yuan et al. 2016). The Psf1 B domain forms the main connection to Cdc45, and the Sld5 subunit binds the Ctf4 trimer scaffold protein that in turn binds to Pol α -primase (Simon et al. 2014). An interaction between Psf1 and the B subunit of Pol ϵ has also been documented by biochemical studies (Gambus et al. 2009), and recently, the direct interaction between GINS and Pol ϵ was visualized by negative stain EM (Sun et al. 2015).

10.5 Cdc45 Has Two RecJ-Like Domains and a Protruding Helical Motif

Cdc45 has been predicted to have a RecJ-like fold (Krastanova et al. 2012; Onesti and MacNeill 2013; Sanchez-Pulido and Ponting 2011). The structure of yeast and human Cdc45 confirmed this prediction and further revealed that there are actually two RecJ-like α -/ β -domains that are separated by a small helical inter-domain (ID) (Fig. 10.4a–c) (Simon et al. 2016; Yuan et al. 2016). Human and yeast Cdc45 are highly conserved at the structure level and superimpose with a root-mean-square deviation of <2 Å (Fig. 10.4c). Cdc45 has a protruding helical motif (PHM) that contains a highly negatively charged and disordered loop (D166 – R217 in yeast). The protrusion may interact and support the largely flexible N-terminal catalytic domain of Pol2 (see below), because a lysine at the tip of the PHM helix (K222) cross-links to the N-terminal catalytic domain of Pol2 (Yuan et al. 2016). The helical ID contacts and stabilizes the NTDs of Mcm2 and Mcm5 (Fig. 10.1a, c).

The fact that Cdc45 faces the neighboring Mcm2 and Mcm5 subunits in the CMG helicase is suggested to poise Cdc45 to capture DNA if the Mcm2/5 gate breaches open (Petojevic et al. 2015). Although Cdc45 does not have a nuclease activity, there is evidence that Cdc45 binds DNA weakly and nonspecifically (Krastanova et al. 2012; Simon et al. 2016; Bruck and Kaplan 2013). RecJ forms an O-like structure with a pore in the middle to serve as a ssDNA-binding groove, but unlike RecJ the yeast and human Cdc45 do not have a central pore. Therefore, Cdc45 does not contain an internal DNA-binding groove. It is possible that the exterior surface of Cdc45 may help to coordinate DNA in the context of CMG, but the nature of the DNA contact is currently unknown.

Meier-Gorlin syndrome (MGS) is a rare autosomal recessive disorder characterized by short stature (dwarfism) (Klingseisen and Jackson 2011). Previous work has found that mutations in genes involved in establishing the pre-replication complex, such as ORC1, ORC4, ORC6, CDT1, and CDC6, can cause MGS (Hossain and Stillman 2016). More recently, eight point mutations in *CDC45* have been identified in MGS patients (Fenwick et al. 2016). The mutations are scattered across the protein structure (Fig. 10.4d). Because Cdc45 is involved in both replication initiation and elongation, it is unclear whether initiation or ongoing fork movement, or both, is the underlying cause of the disease.

10.6 CMG Helicase May Function as an Oil Rig Pump Jack to Inchworm Along DNA

Cryo-EM analysis of CMG particles embedded in vitreous ice revealed two conformations, an extended structure (conformer I) and a compact structure (conformer II) at 4.7 Å and 4.8 Å resolution, respectively (Fig. 10.5a, b) (Yuan et al. 2016). In conformer I, when viewed from the Cdc45-GINS side of the structure, the CTD ring of Mcm2-7 containing the AAA+ motors is tilted by ~10° with respect to the NTD ring, leading to an approximate spiral arrangement of motor domains comprising the CTD ring. In conformer II, the CTD motor ring is approximately parallel to the NTD ring, and CMG is more compact than conformer I. In both conformers, the Mcm2-7 NTD ring-GINS-Cdc45 unit appears to be a rigid platform upon which the CTD AAA+ motor domains switch between extended and compact states during cycles of ATP hydrolysis. These structures suggest that CMG may function like an oil rig pump jack attached to a stable platform, nodding up and down to inchworm along ssDNA and unwind dsDNA at a forked junction (Fig. 10.5c) (Yuan et al. 2016). This linear inchworm ratchet mechanism is distinct from the sequential rotary ATP hydrolysis unwinding mechanism proposed for the homo-hexameric helicases such as *E. coli* DnaB, *E. coli* Rho transcription terminator, and bovine papillomavirus (BPV) E1 (Enemark and Joshua-Tor 2006; Itsathitphaisarn et al. 2012; Thomsen and Berger 2009). Additionally, single point mutants of the ATP sites in the Mcm2-7 subunits of *Drosophila* CMG show major helicase defects in only two of the six mutants, inconsistent with a sequential rotary hydrolysis mechanism that predicts each ATP site would be important to helicase activity (Ilves et al. 2010). Similar conclusions of nonequivalent ATP sites have been made by mutational studies of yeast Mcm2-7 (Schwacha and Bell 2001).

Aside from its main function in unwinding parental DNA, CMG also functions as a scaffold for assembly of the replisome. Indeed, the CMG helicase interacts with many other replicative factors, chief among them are the leading strand Pol ϵ (Langston et al. 2014) and the Pol α -primase that is required repeatedly on the lagging strand (Gambus et al. 2006).

10.7 Pol ϵ Binds to the CTD Motor Side of CMG

In 1990 Pol ϵ was identified as a third DNA polymerase in budding yeast that is essential for cellular replication (Morrison et al. 1990). Subsequent studies revealed that Pol ϵ is also involved in many other pathways such as the DNA damage checkpoint response, epigenetic silencing, sister chromatid cohesion, and possibly DNA recombination during repair of DNA lesions (Pursell and Kunkel 2008). Nearly two decades after its discovery, Pol ϵ was

assigned as the major leading strand polymerase (Clausen et al. 2015; Kunkel and Burgers 2008; Miyabe et al. 2011; Nick McElhinny et al. 2008; Pursell et al. 2007). In all eukaryotes studied to date, Pol ϵ consists of four proteins; the largest is the DNA polymerase, followed by the B subunit and two small histone-fold subunits (Pursell and Kunkel 2008). The Pol ϵ of *Saccharomyces cerevisiae* consists of Pol2, Dpb2 (DNA polymerase-binding protein 2), Dpb3, and Dpb4. Pol2 is composed of two subdomains: a N-terminal catalytic domain that contains the polymerase and exonuclease active sites and an inactive C-terminal domain that shares homology to B-family DNA polymerases (Fig. 10.6a) (Tahirov et al. 2009). The ternary crystal structure of the N-terminal catalytic subdomain of Pol2 in complex with primer-template DNA and incoming dNTP was recently determined, and it shares many features of other B-family DNA polymerases such as Pol δ (Hogg et al. 2014). However, a P domain, new to the B-family polymerases, enables Pol ϵ to encircle the nascent dsDNA and enhances the processivity of DNA synthesis. This may explain why Pol ϵ has the highest fidelity among B-family polymerases despite the absence of an extended β -hairpin loop that is required for high-fidelity replication by other B-family polymerases (Fortune et al. 2005; Hogg and Johansson 2012; Hogg et al. 2014).

Cryo-EM analysis of yeast Pol ϵ revealed a bilobed overall architecture (Asturias et al. 2006). Pol ϵ has also recently been shown to bind directly to CMG helicase, independent of DNA, and the 15 protein complex is referred to as CMGE (Langston et al. 2014). Negative stain EM analysis of CMGE provided a 16 Å 3D map (Fig. 10.6b) (Sun et al. 2015). This low-resolution structure revealed that Pol ϵ is positioned on the CTD-tier side of CMG, sitting atop the CTDs of Mcm2 and Mcm5, and GINS and Cdc45. This Pol ϵ position was confirmed by extensive cross-linking mass spectrometry analysis (Fig. 10.6c). The visible density of Pol ϵ could only account for about 70 % the mass of Pol ϵ , and one suggestion for this missing density was that the catalytic Pol ϵ N-terminal subdomain was too mobile to visualize (Fig. S6 in ref. (Sun et al. 2015)). However, cross-linking mass spectrometry analysis of CMGE demonstrated that the Pol ϵ N-terminal subdomain was in proximity to the other subunits of Pol ϵ and thus located nearby.

10.8 Pol α and Ctf4 Are Located on the NTD-Tier Side of CMG, Opposite from Pol ϵ

DNA synthesis activity of Pol ϵ and Pol δ requires the primed site synthesized by the four-subunit Pol α -primase, which synthesizes a hybrid RNA/DNA primer (Conaway and Lehman 1982). A 7-9mer RNA is synthesized de novo by the Pri1/2 subunits followed by an internal transfer of the RNA primer to the DNA polymerase which adds a further 10–20 deoxyribonucleotides (Nethanel et al. 1992). Thus Pol α -primase “counts” the length of RNA and DNA and stops synthesis. The underlying mechanism of how this enzyme functions has been revealed by a series of elegant crystal structures and biochemical studies (Coloma et al. 2016; Kilkenny et al. 2013; Klinge et al. 2009; Perera et al. 2013).

Crystal structures of the yeast Pol α catalytic core (349–1258; 910 amino acids) in the apo form, bound to an RNA/DNA primer template (the binary complex), and in a ternary complex with RNA/DNA primer template and incoming dGTP show that Pol α specifically

recognizes the A-form RNA/DNA helix for extension with dNTPs (Perera et al. 2013). But once a full turn of double-helix DNA has been synthesized, Pol α is no longer in direct contact with the A-form RNA/DNA helix, which causes its release from DNA. A recent crystal structure of human Pol α -polymerase subunit in complex with a DNA/DNA helix supports this view, but interestingly, the DNA/DNA helix in contact with the polymerase is in a hybrid A-B form (Coloma et al. 2016). It was suggested that the free energy cost of distorting DNA from B- to the A-B hybrid, rather than the loss of specific contacts, drives the termination of primer synthesis.

Pol α -primase is a bilobed structure in which Pol1 binds to the B subunit and Pri1-Pri2 dimer through its CTD, and the CTD of Pol1 is connected to the polymerase region by a flexible stalk (Nunez-Ramirez et al. 2011). Pol α -primase requires CMG for priming activity on both the leading and lagging strands during unwinding of the forked DNA in the presence of RPA (Georgescu et al. 2015b). Pol α -primase is anchored to CMG helicase by the Ctf4 homotrimer (Gambus et al. 2009; Simon et al. 2014; Tanaka et al. 2009). The C-terminal domain of Ctf4 self-associates into a disk-shaped trimer that binds to a peptide sequence in Sld5, a subunit of the GINS complex within CMG (Simon et al. 2014). The same region of Ctf4 also binds to a homologous peptide sequence within the catalytic subunit of Pol α (Simon et al. 2014). Therefore, Ctf4 acts as a scaffold that cross-links CMG and Pol α -primase, underlying the mechanism by which Ctf4 recruits Pol α -primase to the replisome (Gambus et al. 2009). These physical interactions were visualized in a recent EM analysis of combinations of CMG with Pol ϵ , Pol α -primase, and Ctf4 (Fig. 10.7) (Sun et al. 2015). Unexpectedly, Pol α -primase and Ctf4 were found to bind to the NTD-tier of CMG, opposite from the CTD-tier where Pol ϵ binds. This organization was novel because all previous illustrations of the replisome placed the polymerases and primase on the same side of the helicase, after DNA unwinding.

10.9 Future Perspectives

We have reviewed here just some of the numerous recent structural advances in the field of replication that have revealed the structures of replicative polymerases, the 11 subunit CMG helicase, and the overall architecture of the eukaryotic replisome core. There are numerous important questions that remain to be answered, in fact too many to list. One important question for future studies is to understand the DNA path through the replisome and how CMG translocates along DNA. Available biochemical data indicate that CMG functions by steric exclusion in which both the CTD and NTD rings of Mcm2-7 encircle and translocate along one strand of ssDNA, excluding the other strand to the outside of the ring and thus separating the strands (Fu et al. 2011). However, the atomic structure of the Mcm2-7 double hexamer reveals a possible side channel for one DNA strand that lies between the CTD and NTD rings, suggesting the DNA split point may be internal to CMG (Li et al. 2015). Also, the proposed inchworm pump jack mechanism of CMG translocation (Yuan et al. 2016) is a new alternative to the rotary sequential ATP hydrolysis mechanism of hexameric helicase translocation (Enemark and Joshua-Tor 2006; Itsathitphaisarn et al. 2012; Thomsen and Berger 2009). However, there is still very little evidence to support either model. Given the knowledge that CMG helicase moves on the leading strand in a 3'-5' direction (Bochman and Schwacha 2008; Kang et al. 2012; Moyer et al. 2006), that the 3' terminus of ssDNA

enters CMG through the CTD of Mcm2-7 (Costa et al. 2014), and that Pol ϵ binds to the CTD side of CMG (Sun et al. 2015), the leading strand DNA may need to travel a long and winding path before reaching Pol ϵ . These facts, taken together, suggest that the leading strand ssDNA threads through the CTD ring of Mcm2-7, exits the NTD ring of Mcm2-7, and then needs to make a U-turn to reach Pol ϵ at the CTD side of CMG (Fig. 10.8a). This DNA path assumes that the Mcm2-7 CTD motor ring translocates ahead of the NTD ring and thus pushes against the dsDNA/forked junction, as indicated by EM studies using a biotinylated DNA-streptavidin bound to *Drosophila* CMG (Costa et al. 2014). Furthermore, in the steric exclusion model of unwinding, the DNA split point would be just before DNA entry into CMG, and thus the newly unwound lagging strand ssDNA would need to traverse the outside surface of the Mcm2-7 ring to reach Pol α -primase on the NTD-tier side of CMG. Why nature would thread DNA through the replisome in this fashion is not understood, considering the extensive ssDNA exposed in this orientation. However, one may propose that this orientation enables Pol ϵ to be the first to interact with nucleosomes, as Pol ϵ is known to interact with nucleosomes (Foltman et al. 2013; Tackett et al. 2005) and to function in heterochromatin maintenance (Iida and Araki 2004; Tackett et al. 2005). On the other hand, it is interesting to note that BPV E1 helicase translocates on DNA such that the NTD ring faces the forked junction (Enemark and Joshua-Tor 2008; Lee et al. 2014). If the NTD ring of Mcm2-7 within CMG were to push against the forked junction, this orientation would place Pol α -primase at the top of the fork adjacent to the DNA split point, where Pol α -primase could prime the DNA template immediately after strand separation, and Pol ϵ would be behind CMG to extend the leading strand immediately after the DNA exits from the Mcm2-7 channel (Fig. 10.8b). We note that CMG helicase and the replisome architectures determined so far are based on in vitro assembled complexes on artificial substrates. It remains to be investigated if the in vivo assembled structures starting from origin DNA are the same especially in the threading of the DNA.

The DNA replication machinery must perform many additional tasks beyond DNA synthesis. For example, the replisome must deal with DNA lesions, transcribing RNA polymerase, DNA-bound proteins, recombination intermediates, nucleosomes in both euchromatin and heterochromatin, and cohesion rings that hold the sister chromosomes together after replication. Thus it may come as no surprise that there are numerous additional proteins that travel with or transiently interact with the replisome, and it is very likely that many new replisome interactive proteins will be identified in future studies. A first glimpse at the multitude of proteins that bind the replisome is provided by pull-outs using antibodies directed against CMG components followed by mass spectrometry to identify CMG-binding proteins (Gambus et al. 2006). This methodology identified a group of proteins referred to as the replisome progression complex (RPC) that contains CMG along with Ctf4, Mcm10, Tof1, Csm3, checkpoint mediator Mrc1, histone chaperone FACT (Spt16 and Pob3), and topoisomerase I (Gambus et al. 2006). The function of FACT in binding H2A/H2B and stimulating transcription through chromatin and of Topo I in relief of supercoiling is understood (Belotserkovskaya et al. 2003; Brill et al. 1987; Kim and Wang 1989; Orphanides et al. 1998; Orphanides et al. 1999; Schlesinger and Formosa 2000). However, most proteins of the RPC have nearly unknown functions. The Ctf4 trimer recognizes a peptide sequence to bind Pol α and Sld5 suggesting that Ctf4 acts as a platform

for recruitment and exchange of different replication factors (Villa et al. 2016), in loose analogy to the many proteins that traffic on and off PCNA (Georgescu et al. 2015a). The Mrc1 checkpoint mediator is a stably attached replisome component that appears to aid normal replisome progression in addition to its checkpoint function (Lou et al. 2008), but how Mrc1 facilitates these activities and the spatial orientation of Mrc1 within the replisome are unknown. Tof1 and Csm3 are two proteins that ride with the replisome and appear to pause replication forks at protein-DNA barriers, but how they carry out this function is not understood (Calzada et al. 2005; Mohanty et al. 2006; Tourriere et al. 2005). Mcm10 is an essential replication factor that is reported to interact with ssDNA, dsDNA, Pol α -primase, the CMG helicase, and other factors (reviewed in Bielinsky 2016; Thu and Bielinsky 2013). Mcm10 appears to be the last initiation protein to act at an origin, because two complete CMGs form at an origin yet cannot produce ssDNA until Mcm10 is present (Heller et al. 2011; Kanke et al. 2012; Watase et al. 2012; Yeeles et al. 2015). How Mcm10 fulfills this initiation role and whether Mcm10 moves with the replisome are presently unknown. Besides the RPC proteins, the flap endonuclease 1 (FEN1) and DNA ligase both interact with PCNA and function with Pol δ /PCNA on the lagging strand to remove the RNA primer and join the Okazaki fragments, respectively. How the Pol δ lagging strand polymerase and the RFC clamp loader associate with the replisome, if at all, is another ongoing mystery. Finally, the replisome must replicate through every nucleosome in the genome, including tightly packed heterochromatin without disrupting the epigenetic state of the cell. The CMG, Pol ϵ , and Pol α all bind nucleosomes or histone subassemblies (Foltman et al. 2013; Huang et al. 2015; Tackett et al. 2005; Wang et al. 2015), yet how the replisome handles nucleosomes and passes them onto the daughter strands is largely unexplored territory. Answers to these many important questions will require a multitude of experimental approaches, but one can be quite certain that cryo-EM structure determination will be essential to understanding the complete picture of these very large and complicated multiprotein assemblies.

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Fig. 10.1.

The CMG structure. The CMG structure is shown in cartoon view with each subunit a different color (PDB 3JC7). The surface representation of the structure is superimposed as a dim gray surface view. The structure is shown from the following angles: **(a)** top CTD ring view, **(b)** a side view, and **(c)** a bottom NTD view

Fig. 10.2.

Conformation changes proceeding from the inactive Mcm2-7 at an origin to Mcm2-7 in the active CMG helicase. The figure focuses on a side view of the Mcm2 and Mcm5 subunits in the Mcm2-7 hexamer in which one Mcm2-7 hexamer has been extracted from the inactive double hexamer (PDB 3JA8) (left side) and the Mcm2-7 hexamer has been extracted from the active CMG structure (PDB 3JC7) (middle). The two Mcm2-7 structures are then superimposed (right side). Structures are in cartoon view wrapped in a dim gray surface view. Mcm5 is gold and Mcm2 is blue. The Mcm2/5 interface is nearly opened in Mcm2-7 within CMG

Fig. 10.3.

Comparison of the human and the yeast GINS structures. **(a)** Human GINS with each subunit shown in a different color (PDB 2Q9Q). An N-terminal insertion loop in Psf3 is highlighted by a semitransparent circle near the central pore region. **(b)** The yeast GINS structure, extracted from the CMG structure (PDB 3JC6), is shown in the same color scheme as in **(a)**. **(c)** Superimposition of the human and the yeast GINS structures, revealing that the structural variation is focused in the central region. **(d)** Surface representation of the yeast GINS structure. The central pore is too small to thread ssDNA

Fig. 10.4.

Comparison of the yeast and human Cdc45 structures. **(a)** The domain structure of Cdc45. **(b)** Cartoon view of the yeast Cdc45 structure extracted from the CMG structure (PDB 3JC6), with each domain colored according to the domain sketch in **(a)**. PHM refers to protruding helical motif. **(c)** Superimposition of the yeast and human Cdc45 structures. Human Cdc45 is shown in light purple (PDB 5DGO). **(d)** Human Cdc45 point mutations identified in patients with Meier-Gorlin syndrome are highlighted as spheres

Fig. 10.5.

CMG helicase alternates between tilted (extended) and untilted (compact) conformations. (a) Side view of CMG conformer I (extended) in which the CTD motor ring is tilted relative to the NTD ring. (b) Side view of the CMG in conformer II (compact) with an untilted CTD ring. In panels (a, b), the cryo-EM density map is shown as a semitransparent surface rendering, and the atomic model is shown in cartoon (EMD-6535, EMD-6536, PDB 3JC5, and 3JC7). (c) An oil rig-like pump jack DNA unwinding model. Panel (c) is reproduced in part from Figure 7 in Yuan et al. (2016) with permission. Note that the CTD-tier ring pushing on the dsDNA is only for the purpose of illustration. The pump jack model would still function as a translocase if CMG were oriented with the NTD-tier ring pushing on the dsDNA

Fig. 10.6.

Architecture of the CMGE complex. **(a)** The Pol2 (P2) subunit of Pol ϵ is comprised of a catalytic N-terminal subdomain and an inactive B-family Pol in the C-terminal subdomain. **(b)** Negative stain 3D EM map of the CMGE in four different views (EMD-6465). The EM density is segmented and colored according to individual subunits except for the Pol ϵ where the complex is shown in green. Part of the Pol ϵ density is not visible in the EM and may be the N-terminal subdomain of Pol2 as proposed in Sun et al. (2015). **(c)** Cross-linking mass spectrometry identified multiple contacts between Pol2 and GINS, Cdc45, and the CTD regions of Mcm2, Mcm5, and Mcm6 (This figure is modified with permission from Figure 6 of Yuan et al. 2016)

Fig. 10.7.

Selected 2D class averages of several replisome complexes. **(a)** The Ctf4 trimer binds to the GINS from the bottom NTD side of CMG. **(b)** Pol α -primase appears as a fuzzy density suggesting a flexible association with the Ctf4 just under the NTD ring of Mcm2-7. **(c)** Simultaneous binding of Pol ϵ to the CTD side of Mcm2-7 on top and the Ctf4 trimer to the NTD side of CMG. **(d)** Pol ϵ binds to the CTD side of CMG in the presence of a primed forked DNA. **(e)** Pol ϵ binds to the CTD side of CMG, while Ctf4 and Pol α bind to the NTD side of CMG (This figure is modified with permission from Figure 5 of Sun et al. 2015)

Fig. 10.8.

The eukaryotic replisome positions Pools ϵ and α on opposite sides of CMG. Depending on whether the CTD motor ring or the NTD ring pushes against the forked junction, the polymerases are positioned either: (a) with Pol ϵ in front and Pol α -primase in back of CMG or (b) with Pol α -primase in front and Pol ϵ in back of CMG