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Molecular mechanisms of eukaryotic origin initiation, replication fork progression, and chromatin maintenance

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Abstract

Eukaryotic DNA replication is a highly dynamic and tightly regulated process. Replication involves several dozens of replication proteins, including the initiators ORC and Cdc6, replicative CMG helicase, DNA polymerase α -primase, leading-strand DNA polymerase ϵ , and lagging-strand DNA polymerase δ . These proteins work together in a spatially and temporally controlled manner to synthesize new DNA from the parental DNA templates. During DNA replication, epigenetic information imprinted on DNA and histone proteins is also copied to the daughter DNA to maintain the chromatin status. DNA methyltransferase 1 is primarily responsible for copying the parental DNA methylation pattern into the nascent DNA. Epigenetic information encoded in histones is transferred via a more complex and less well understood process termed replication-couple nucleosome assembly. Here, we summarize the most recent structural and biochemical insights into DNA replication initiation, replication fork elongation, chromatin assembly and maintenance, and related regulation mechanisms.

Keywords

Eukaryotic DNA replication; replication initiation; replication fork progression; chromosome maintenance; Replication origin; origin recognition complex; Mcm2–7 hexamer; replicative CMG helicase; replisome; DNA polymerase ϵ ; DNA polymerase δ ; polymerase α -primase; DNA methyltransferase 1; histone chaperone; structural biology; cryo-EM

INTRODUCTION

Although the double helix structure of DNA is elegantly simple, the molecular machinery that duplicates the DNA is remarkably complex. Seventy years since the discovery of the first DNA polymerase [1], we have gained an enormous amount of knowledge and mechanistic understanding of DNA replication. We have identified most (if not all) DNA polymerases, including five bacterial and sixteen archaeal/eukaryotic polymerases [2]. We now know that, in all domains of life, a minimal replisome should contain a helicase to unwind the duplex; a primase to synthesize the primer; a DNA polymerase to synthesize new DNA on a parental template strand extending from the primer; a sliding clamp that tethers the polymerase to DNA to increase activity and processivity; and a clamp loader that cracks

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open the clamp ring in order to topologically encircle the double-stranded DNA [1]. We have come to realize that the key factors involved in DNA replication are functionally conserved among bacteria and archaea/eukaryotes, despite their separate evolutionary origins [3–5]. The replication field has made exciting progress recently in both bacterial and eukaryotic species. This review is focused on the eukaryotic DNA replication; readers interested in bacterial/archaeal replication should consult several excellent reviews [6–10].

In the normal eukaryotic DNA replication, the central molecular players include the replication origin recognition complex [11–13], the helicase core component Mcm2–7 hexamer [14–16], the replicative helicase Cdc45-Mcm2–7-GINS (CMG) complex [17–20], the Pol α -primase, the leading-strand DNA polymerase Pol ϵ , and the lagging-strand DNA polymerase Pol δ [21–23]. These core players are further assisted or regulated by several dozen additional protein factors in order to accomplish the feat of genome duplication [23–25].

Advances in the *in vitro* reconstitution of replication origin activation [26, 27], in leading- and lagging-strand DNA replication [28], and in origin-dependent full DNA replication [29, 30] in the model system *Saccharomyces cerevisiae* have set the stage for structural studies of some of the most complex molecular machines in DNA replication. These exciting biochemical advances have coincided with the so-called “resolution revolution” in cryo-EM [31, 32]. The application of single-particle cryo-EM has significantly improved our understanding of the eukaryotic replication mechanism. There are several recent reviews of the regulation and molecular mechanism of eukaryotic DNA replication [20, 23, 33–38]. In this review, we highlight the structural and biochemical works on the yeast replication complexes published in the last two years that have helped us understand origin recognition and activation, the replisome architecture and progression, and the reestablishment of chromatin structure after replication.

1. Mechanism of Origin Initiation

a) Replication origin and origin recognition by ORC.—The initiation of DNA replication is a carefully regulated, multistep process (Fig. 1). Replication origins are specific DNA sequences where the origin recognition complex (ORC) binds [39, 40]. Replication initiation starts near the replication origins. In the budding yeast *S. cerevisiae*, there are several hundred replication origins; they are genetically defined, autonomously replicating sequences (ARS) about 100 to 150 bp long, and most origins are composed of A, B1, and B2 elements [41, 42]. The A element is more conserved in yeast than B1 and B2 and contains the AT-rich, 11-bp, ARS consensus sequence (ACS). In addition to DNA sequence, recent studies have shown that nucleosome positioning and occupancy also play a crucial role in defining replication origins and origin functions [43–45]. However, in the fission yeast *Saccharomyces pombe*, it is the AT content, but not the ARS-like consensus sequence, that largely defines the replication origins [46–48]. In fact, a simple AT-content-dependent and probability-based origin usage model can accurately predict the DNA replication timing and dynamics of *Saccharomyces pombe* [49].

Outside the unicellular eukaryotes, a clear consensus sequence has not been found and may not exist. Higher organisms have tens of thousands of replication origins that are usually AT-

rich, located in certain G-quadruplex sites and/or nucleosome-free regions that may also function as transcription start sites [50]. Origin selection and activation are context-dependent and are influenced by multiple factors including the DNA sequence and the chromatin environment and architecture [51].

ORC is a six-subunit complex composed of Orc1–6 and is conserved in all eukaryotes [40, 52]. Early low-resolution structural studies established the overall architecture of the ORC and showed that Cdc6 fills a gap in ORC to form a replication factor C (RFC)-like spiral that serves as a landing pad for the Mcm2–7 hexamer [53–55]. These were followed by crystal structure determinations of the *Drosophila* ORC and truncated human ORC [56, 57].

The capture of the replication intermediate composed of ORC–Cdc6–Cdt1–Mcm2–7 (OCCM) on origin DNA and the subsequent near-atomic resolution structure provided the first insight into how the origin is recognized by ORC and Cdc6 [58, 59]. Orc4 was found to have a unique insertion helix that interacts with the DNA major groove. Because this helix is absent in other eukaryotic species, which lack origin sequence specificity, the Orc4 insertion helix was proposed to be a major determinant of origin specification in yeast, but the 3.9-Å resolution of the OCCM structure was insufficient to resolve the DNA sequence.

The more recent 3.0-Å cryo-EM structure of *S. cerevisiae* ORC bound to the *ARS305* origin DNA was a breakthrough in which the detailed DNA–ORC interaction was clearly resolved [60] (Fig. 2). The structure revealed that ORC encircles the A element of the origin DNA and interacts extensively with both the DNA backbone and the bases. The Orc1 basic patch (Orc1-BP) and the initiator-specific motifs (ISMs) in the AAA+ domains of Orc1, Orc4, Orc5, Orc3 are arranged into a spiral to grip onto the origin DNA. Three β-hairpin motifs of the winged helix domains of Orc2, Orc4, and Orc5 contact the DNA major groove and span the ACS sequence. Origin recognition is achieved by sequence-specific interactions of the Orc4 insertion helix in the major groove and the Orc1-BP and Orc2-ISM in the minor groove of the ACS site. Lys-362, Arg-367, and Phe-372 of Orc1 and Trp-396 of Orc2 interact with the bases in the T-rich part of the ACS site (Fig. 2). The base-specific interactions with the methyl and the double-bond oxygens of thymine bases explain ORC's recognition of the T-rich origin ACS sequences of *S. cerevisiae*. At the B1 element downstream of the ACS site, Orc2-BP, Orc5-BP, and Orc6 interact with DNA bases and further contribute to origin specification. This work pinpoints a conserved role of ORC in modulating DNA structure to facilitate origin selection.

Mutations in key origin sequence determinants, including the major-groove-interacting Orc4 insertion helix and the minor-groove-interacting Orc2 ISM, induce *de novo* replication origins and changes of the origin firing pattern in *S. cerevisiae* [61]. It was noted that the conservation of the Orc4 α-helix and the Orc2 ISM is restricted to a small clade of *Saccharomyces*-related species having defined replication origin sequences and that the acquisition of the sequence-specific origins coincided with the acquisition of ORC–Sir4-mediated transcriptional silencing. It also coincided with the loss of the RNA interference pathway in those yeast species.

b) How ORC loads the first Mcm2–7.—Although yeast ORC binds replication origins throughout the cell cycle [24], origins are “licensed” (become active) only in G1 phase [62]. In the first step of origin activation, Cdc6 is recruited to the origin by binding to ORC and thereby closing the gap between Orc1 and Orc2. ORC–Cdc6 then forms a ring-shaped AAA+ complex to encircle the origin DNA [53]. In this way, Cdc6 converts ORC from an inactive origin DNA binder to an active Mcm2–7 loader and drives the ordered assembly of the pre-replicative complex [63]. However, ORC–Cdc6 does not directly recruit the Mcm2–7 hexamer; instead, ORC–Cdc6 recruits a Cdt1-bound Mcm2–7 [64, 65]. Cdt1 is an essential licensing factor, and its role is to stabilize the Mcm2–7 hexamer for loading onto DNA.

In the presence of ATP γ S, ORC–Cdc6 on origin DNA recruits the first Cdt1-bound Mcm2–7 to form the loading intermediate OCCM [58, 59] (Fig. 3). The loading requires the binding of ATP but not its hydrolysis, so the intermediate was captured in the presence of ATP γ S. A total of eight ATP γ S molecules are trapped in the OCCM complex: four in ORC–Cdc6 and four in Mcm2–7. When alone in solution, the Mcm2–7 hexamer is a left-handed, open but flexible spiral, with the Mcm2–Mcm5 gate propped open. Cdt1 binding stabilizes the open configuration for the origin DNA to enter into the central channel [66, 67]. In the OCCM structure, Cdt1 is in a highly extended three-domain configuration that binds on the Mcm2–Mcm4–Mcm6 half-ring to keep open the DNA entry gate between Mcm2 and Mcm5. Cdt1 seems to pull the Mcm6 winged helix domain (WHD) to the edge, thereby clearing out the C-tier AAA+ face of Mcm2–7 for docking with ORC–Cdc6, as well as for a subsequent specific interaction with the Orc4 WHD.

There are a total of six WHDs in the ORC–Cdc6 complex, one in each of the six ATPase subunits (Orc1–5, Cdc6), and five in Mcm2–7; Mcm2 lacks such a domain. The recruitment of Cdt1-bound Mcm2–7 by ORC–Cdc6 is largely mediated by these two sets of WHDs, forming an extensive interface [59]. Inside the Mcm2–7 chamber, the DNA-translocating hairpin loops of Mcm2, Mcm4, Mcm6, and Mcm7 are arranged like a spiral and grip on the double-stranded DNA. The hairpin loops of Mcm3 and Mcm5 do not interact with DNA at this stage. The OCCM structure represents a near completion of the recruitment of the first Mcm2–7, because the hexamer is already encircling the origin DNA [68].

The removal of the Mcm6 WHD slows the loading reaction [64]. Cryo-EM analysis of ORC–Cdc6 loading of the Mcm6 WHD-truncated Cdt1–Mcm2–7 revealed that the recruitment of the first Mcm2–7 is itself a multiple-step process [69]. At the first encounter, the two WHDs of Mcm3 and Mcm7 latch onto the Orc2–Cdc6–Orc1 half-ring, while the main body of the Mcm2–7 hexamer remains largely free, leading to the formation of a “semi-attached OCCM” intermediate. Next, the remaining 3 Mcm WHDs engage ORC–Cdc6 such that the main body of Mcm2–7 docks onto it, forming the “pre-insertion OCCM”. In this intermediate, the origin DNA is bent and positioned adjacent to the open DNA entry gate, poised for insertion at the Mcm2–Mcm5 interface. Then, the DNA disengages Orc6 to straighten up and pass through the Mcm2–Mcm5 gate to form the OCCM complex; this loaded intermediate was also observed with the mutant Mcm2–7. Eventually, the Mcm2–5 gate completely closes and the first Mcm2–7 becomes fully loaded on the origin DNA. Because the actual DNA insertion process is dynamic and could not be visualized by

structural approaches, molecular dynamics simulation was used to identify a 10-substep transition process from the “pre-insertion OCCM” to the final, DNA-inserted and gate-closed, OCCM structure [69].

c) How ORC–Cdc6 loads the second Mcm2–7 hexamer.—As mentioned above, the recruitment of the first Mcm2–7 is an ATP-hydrolysis-independent process. ATP hydrolysis in OCCM seems to lead to three sequential disassembly steps: 1) Cdt1 is released, leading to the formation of ORC–Cdc6–Mcm2–7 (OCM) and the Mcm2–7 ring closure [70–72]; 2) Cdc6 is released, forming ORC–Mcm2–7 (OM) [73]; and 3) ORC is released from the first Mcm2–7 hexamer, leaving the first Mcm2–7 hexamer alone encircling the origin DNA. The last step is deduced from a recent study capturing the Mcm2–7–ORC–Cdc6 complex (MO), in which the ORC has moved from the C-tier motor ring of the Mcm2–7 hexamer to the opposing N-tier side of the DNA-loaded Mcm2–7 hexamer, while encircling the B2 element of the *ARS1* origin DNA in an opposing orientation [74] (Fig. 4).

The formation of MO prepares ORC–Cdc6 on the N-tier to recruit the second Cdt1-bound Mcm2–7 hexamer and to form the M–OCCM intermediate. Conceivably, another round of the ATP hydrolysis and release of Cdt1, Cdc6, and ORC would lead to the assembly of the stable Mcm2–7 double hexamer [26, 70, 72, 74–77] (Fig. 4). This mechanism of the second Mcm2–7 recruitment is highly satisfactory for three reasons.

1. It explains the extended origin DNA sequence, which is much longer than a single ORC or ORC–Cdc6 could bind.
2. The B2 element is a much weaker ORC binding site, so the presence of the secondary ORC binding site at the N-tier of Mcm2–7 explains why the ORC–Cdc6 can locate to this weak DNA site to load the second Mcm2–7 in an opposing direction.
3. It reconciles the apparently contradictory observations in which two ORC complexes at two different and opposing origin DNA sites assemble the double hexamer [78], yet the same C-terminal surface of ORC–Cdc6 is used to recruit the first Mcm2–7 hexamer as well as the second [79].

Taken together, ORC–Cdc6 bound to the A and B1 elements of the *ARS1* origin loads the first Mcm2–7 and dissociates from DNA, then the first loaded Mcm2–7 encircling DNA recruits a second ORC–Cdc6 to the B2 element of the *ARS1* origin. That in turn loads the second Cdt1-bound Mcm2–7, eventually assembling the head-to-head Mcm2–7 double hexamer. It may not matter whether the same ORC–Cdc6 that recruited the first Mcm2–7 or a different one is responsible for loading the second Mcm2–7, because the first ORC–Cdc6 is released to solution before an ORC–Cdc6 binds to the opposite side to form the MO complex.

d) The Mcm2–7 double hexamer bends but does not melt dsDNA.—*In vitro* reconstitution of the Mcm2–7 double hexamer from purified proteins and plasmid DNA containing the *ARS1* origin was accomplished by two groups at nearly the same time [26, 27]. The head-to-head (N-tier to N-tier) architecture of the Mcm2–7 double hexamer is

similar to that of the SV40 large T antigen double hexamer [80], the bovine papillomavirus E1 double hexamer [81], and the archaeal MCM double hexamer [82, 83]. The Mcm2–7 double hexamer can be purified endogenously in S phase yeast cells. Because the double hexamer is able to freely diffuse along dsDNA, the use of DNase I to digest DNA during protein purification resulted in the loss of DNA inside the double hexamer chamber and an apo double hexamer structure [66]. Despite the lack of DNA, the apo structure is important because it provided the first atomic model of the eukaryotic Mcm2–7. The structure revealed tightly interlocked N-tiers of the two Mcm2–7 hexamers. Further effort on *in vitro* assembling of the Mcm2–7 double-hexamer on multiple *ARS1*-containing plasmids having specific nuclease cleavage sites led to the isolation of the double hexamer on a long stretch of dsDNA, long enough to prevent the double hexamer from diffusing off. Those efforts led to the Mcm2–7 double hexamer structure on dsDNA at 3.9 Å resolution [77], which revealed 60 base pairs of DNA in the central channel and the involvement of the Mcm2–7 N-terminal peptides, in particular, the well-structured N-terminal peptides of Mcm5 and Mcm7 in assembling the interlocked double hexamer (Fig. 5). The extensive and interlocked interface explains the remarkable stability of the double hexamer. Interestingly, the disordered (and thus invisible) N-terminal peptides of Mcm2 and Mcm4—but not of Mcm6—are crucial for Mcm2–7 loading and double-hexamer assembly; deletions of those peptides prevented double-hexamer assembly [84]. Another effort that computationally separated the double-hexamer particles with bound dsDNA from the majority of particles that have lost the DNA led to a dsDNA-bound cryo-EM structure at a lower resolution of 7 Å [74]. These structures revealed that the staggered association of the two head-to-head Mcm2–7 hexamers bends the dsDNA inside the channel in a zig-zag manner, yet no unwinding or formation of an initial DNA bubble has occurred, consistent with the fact that the Mcm2–7 double hexamer on dsDNA is inactive.

e) How Mcm2–7 double hexamer is transformed into two CMG helicases is not well understood.—The Mcm2–7 double hexamer encircling the dsDNA is inactive. A dozen or so initiation factors, including the Dbf4-dependent kinase (DDK), cyclin-dependent kinase (CDK), Sld2, Sld3-Sld7, Dpb11, Mcm10, and Pol ϵ , along with the helicase components Cdc45 and the four-subunit GINS complex, are required to separate the two origin DNA strands and convert the double-hexamer into two activated replicative helicases, the Cdc45–Mcm2–7–GINS (CMG) complexes [85, 86]. This activation process has been achieved *in vitro* with purified proteins on an *ARS1* origin-containing plasmid DNA [29, 87], although the detailed molecular mechanism of such a dramatic transformation is still unclear. Mcm2 and Mcm4 each contain a N-terminal serine-rich domain, but they are separated by Mcm6 in the Mcm2–7 hexamer. Mcm2 and Mcm4 become neighbors across the double-hexamer interface and form an integrated site for docking by DDK, a serine/threonine protein kinase that extensively phosphorylates Mcm2 and Mcm4 [88–91]. EM studies showed that DDK phosphorylation neither separates the two Mcm2–7 hexamers nor drastically alters the overall structure of the double hexamer [92]. However, phosphorylation of Mcm2 and Mcm4 leads to the binding of Cdc45 and Sld3–Sld7 to the double hexamer. CDK phosphorylates ORC to prevent it from further loading the Mcm2–7 hexamer. CDK also phosphorylates Sld2 and Sld3 to facilitate the assembly of the pre-loading complex (pre-LC), which is composed of Dpb11, GINS, Pol ϵ , and Sld2. The

recruitment of the pre-LC to the Mcm2–7 double hexamer leads to the assembly of two CMG complexes that still encircle the double-stranded origin DNA. Finally, Mcm10 binding and the ATP hydrolysis by CMG would exert enough force to untwist the dsDNA, leading to the extrusion of the lagging strand from the central channel [93–96]. A single-molecule study showed that the CMG harbors an ssDNA gate for lagging strand extrusion, and this gate may be distinct from the Mcm2–Mcm5 gate used during Mcm2–7 hexamer loading to the double-stranded origin DNA [97]. The two CMG helicases can leave the origin only when they have extruded the lagging strand and are encircling only their respective leading strand DNAs.

f) The two CMGs have to cross each other in order to leave the origin.—The head-to-head (N terminus-to-N terminus) orientation of the two Mcm2–7 hexamers in the double hexamer intuitively suggests that once activated, the two Mcm2–7 would move away from each other, with their respective C-tier motor ring leading the way and pushing on the DNA fork. In fact, such a scenario had prevailed in the field for some time. In a systematic experimental investigation with streptavidin-bound biotinylated DNA substrates, including single-stranded DNA, tailed DNA, and a forked DNA, it was unambiguously demonstrated that the CMG helicase translocates on DNA with its N-tier ring ahead and with its C-tier motor ring pushing from behind [98]. Such CMG translocation directionality was later confirmed by *in vitro* CMG activation and EM studies [95, 99]. Because the two replication forks initiated from the same origin migrate away from each other in the bi-directional replication mechanism, the N-tier first translocation directionality predicts that the two CMG helicases activated from the same Mcm2–7 double hexamer have to pass each other in order to leave the origin. This CMG activation mechanism is hypothesized to encode a quality control mechanism to establish the bidirectional replication forks [37, 98] (Fig. 6). Both CMG helicases have to be encircling the leading ssDNA for them to cross each other and leave the origin.

g) A potential role of liquid-liquid phase separation in replication initiation.—Liquid-liquid phase separation (LLPS) refers to the condensation of biological molecules such as proteins, DNA, and RNA. LLPS is driven by multivalent interactions of intrinsically disordered regions (IDRs) in biomolecules and occurs in both the nucleus and the cytoplasm to form membraneless pseudo-organelles and co-localized bodies. The condensates are dynamic, regulated, and involved in diverse cellular functions [100, 101]. Interestingly, the IDRs of Orc1, Cdc6, and Cdt1 of metazoans—but not of yeast—mediate LLPS in the presence of DNA *in vitro* and *in vivo*, and the condensates regulate multiple aspects of chromatin biology, including chromosome recruitment, initiation co-assembly, and Mcm2–7 loading on origin DNA [102]. The IDRs of Orc1, Cdc6, and Cdt1 are positively charged and form multivalent interactions with negatively charged DNA to phase-separate. The Mcm2–7 hexamer itself does not form phase-separated condensates but is recruited by the Orc1-, Cdc6-, and Cdt1-mediated condensates for loading and helicase assembly. This is the first report of replication initiator-mediated and DNA-dependent LLPS [102]. A more detailed understanding of the role of LLPS in DNA replication requires further investigation.

2. Molecular Mechanism of Replication Fork Progression

a) How the CMG helicase unwinds the DNA duplex.—Structural and functional studies of the simpler homo-hexameric replicative helicases—such as the bacterial DnaB hexamer [103], the papillomavirus E1 hexamer [104], and the archeal MCM helicase [105, 106]—have led the way in understanding the DNA unwinding mechanism. They have revealed that only one strand passes through the central channel, and the ATP-hydrolysis-powered unwinding is accomplished by the spirally arranged hairpin loops binding on the phosphate backbone. The Mcm proteins are dumbbell-shaped and they assemble a two-tiered hexameric ring with an N-tier ring (NTD) and a C-tier ring (CTD) that are connected by flexible loops [20, 107]. All Mcm proteins have a similar architecture and can be divided into four domains: N-terminal helical domain (HD), oligosaccharide/oligonucleotide (OB) domain, ATPases associated with various cellular activities (AAA+) domain, and C-terminal winged-helix domain (WHD). A zinc finger domain is embedded in the OB domain. Several structural features interact with DNA and contribute to DNA unwinding: the N-tier ring zinc finger (ZF) domain, the hairpin loop of the OB domain, and the presensor 1 (PS1) and helix-2 insertion loops of the C-terminal AAA+ domains. In eukaryotes, the six Mcm proteins (Mcm2, 3, 4, 5, 6, and 7) maintain the same architecture, but each Mcm protein has acquired unique features and plays distinct roles in replication initiation and elongation [108].

Mcm2–7 had long been expected to be the eukaryotic replicative DNA helicase [109, 110]. The true identity of the eukaryotic helicase, the Cdc45–Mcm2–7–GINS (CMG) complex, was only discovered in 2006 in *Drosophila* by the Botchan lab [17]. The CMG was shown to hydrolyze ATP and translocate on the leading strand DNA in a 3′–5′ direction to unwind the DNA [17, 111]. In the apo CMG structure, Cdc45 and GINS brace the N-tier half-ring of Mcm2, 3, and 5 and lock shut the Mcm2–Mcm5 DNA gate [112, 113]. An intermediate 6.7-Å resolution cryo-EM structure of the yeast CMG helicase provided the first picture on how the eukaryotic helicase is situated on a forked DNA [98]. This intermediate was captured in the presence of ATP, which allowed strand unwinding but was subsequently stalled by a double streptavidin block in front of the fork. ATPγS was found to be insufficient to support the assembly of the helicase-forked DNA complex, because the helicase bound the single-stranded DNA tail but was unable to advance to the fork junction. The structure revealed that parental dsDNA enters the N-tier ring formed by six Zinc finger domains of Mcm2–7, demonstrating that the C-tier motor ring pushes from behind to unwind the dsDNA. A subsequent cryo-EM study of the *Drosophila* CMG bound to a forked DNA at 5.0-Å resolution confirmed the parental DNA entering the N-tier of Mcm2–7 ring, and it captured four conformations that suggested a rotary DNA translocation mechanism [99]. However, the resolutions of these studies were insufficient to specify what structural elements are responsible for strand separation and where the two DNA strands separated.

The recent 3.9-Å resolution cryo-EM structure of the *S. cerevisiae* CMG on a forked DNA has partially addressed these questions [114]. This structure showed that DNA unwinding is achieved via a “dam-and-diversion tunnel” mechanism. The strand separation occurs at the bottom of the Mcm2–7 N-tier OB sub-ring, where the leading strand is pulled down by the C-tier motor ring below, but the lagging strand is blocked and diverted sideways by the OB

hairpin loops of Mcm3, Mcm4, Mcm6, and Mcm7 (Fig. 7). The lagging strand is disordered after strand separation, so its exit path from Mcm2–7 is unclear, but is likely through a gap between the Zn finger domains of Mcm3 and Mcm5. In the lower C-tier motor ring, the leading strand contacts eight spirally arranged DNA-translocation loops, 4 PS1 loops and 4 H2I loops of Mcm2, 3, 5, and 6. Each PS1 loop interacts with two phosphates and each H2I loop interacts with one base, thereby pulling the leading strand DNA downward (Fig. 7). Another recent cryo-EM study of the CMG–forked DNA complex reached a resolution of 3.0–3.5 Å, due to the fork stabilization by Tof1–Csm3 [115]. This study confirms the strand separation inside the N-tier ring of Mcm2–7 and the involvement of the OB hairpin loops of Mcm3, 4, 6, and 7 in strand separation. The improved resolution further revealed that the highly conserved Phe-363 of the Mcm7 OB hairpin loop makes a π - π interaction with DNA, potentially functioning as a strand separation pin.

b) Pol ϵ and CMG form the core of the leading-strand replisome.—Pol ϵ has the dual function of copying the leading-strand DNA in the S phase and participating in replisome assembly in the G1-to-S transition [23, 116]. Pol ϵ has a two-lobed architecture and is composed of four subunits: the catalytic Pol2, and the accessory molecules Dpb2, Dpb3, and Dpb4. The Pol2 N-terminal catalytic domain (NTD) accounts for one lobe, and the C-terminal noncatalytic domain, together with the Dpb2, accounts for the other lobe [117]. The Pol2 NTD can be further divided into an N-terminal subdomain, a proofreading 3'–5' exonuclease, a palm, a finger, and a thumb that are organized into a toroid to surround the DNA substrate [118]. Unique among the replicative DNA polymerases, Pol ϵ has a conserved Pol2-family-specific catalytic core peripheral subdomain (POPS) preceding the palm subdomain that is important for the enzyme's functions in both replisome assembly and DNA synthesis [119]. Dpb3 and Dpb4 are histone-fold proteins and they form a heterodimer [120]. The location of Dpb3–4 in the holoenzyme had been unclear due to the lack of a high-resolution structure [121]. The overall architecture has recently been established by a 3.5-Å cryo-EM structure of yeast Pol ϵ [122] showing that the Dpb3–Dpb4 subunits bridge the two lobes of Pol ϵ , holding them rigid. Further, the nearly 100-residue loop between the Pol2 NTD and CTD, long expected to be a flexible linker, was found to fold into an L-shaped mooring helix that anchors Dpb3–4 in the middle of Pol2.

Pol ϵ is recruited onto the Mcm2–7 double hexamer during origin activation and helicase assembly [123]. Pol ϵ physically associates with the helicase by binding to the C-tier motor ring of the CMG, forming the leading strand replisome [124, 125]. The interaction between Pol ϵ and CMG is very tight, with a dissociation constant of 12 nM [126]. Pol ϵ appears to slow down CMG, particularly to facilitate pausing at protein-bound replication barriers such as transcription sites [127]. In the cryo-EM map of CMG–Pol ϵ , only the Pol2 CTD and Dpb2 were visible and bound on the Mcm2–3-5 half ring and GINS complex; the Pol2 NTD, Dpb3, and Dpb4 were invisible and likely flexible [117] (Fig. 8a–b). The Pol2 CTD contacts the Mcm2 AAA+ domain, and Dpb2 binds over the Mcm5 and Mcm3 AAA+ domains while also making contacts with the GINS complex. Together, the Pol2 CTD and Dpb2 brace and stabilize the Mcm2–5-3 C-tier AAA+ half-ring in a manner similar to that of GINS–Cdc45 latching across the N-tier region of Mcm2–5-3 half ring. *In vitro*, Pol ϵ , Pol ϵ -cat, or Pol2 CTD–Dpb2 (but not the isolated Pol2, Pol2 CTD, or Dpb2) was able to

convert the inactive Mcm2–7 double hexamer into the active CMG complex, indicating that Dpb3, Dpb4, and Pol2 NTD are not required for assembly [30, 117]. The solution of the Pol ϵ holoenzyme—along with the medium-resolution structure of CMG-fork in complex with Pol2 CTD–Dpb2 and the near-atomic structure of CMG-forked DNA—has led to a plausible model for the core of the leading-strand replisome [122] (Fig. 8c). The structural model places the OB fold of Dpb2 in the midpoint between where the leading ssDNA exits the CMG and where the leading strand enters the Pol ϵ active site, thereby suggesting a role of the Dpb2 OB in directing the leading-strand DNA path within the replisome.

The physical association between CMG and Pol ϵ does not guarantee a functional coupling between DNA unwinding and DNA synthesis. In fact, an inactive mutant Pol ϵ attached to the CMG helicase can lead to excessive DNA unwinding *in vitro*, demonstrating a lack of coupling of these two activities in budding yeast [128]. However, the checkpoint kinase Rad53 can slow the CMG helicase to reduce excessive DNA unwinding. It is known that cells with Pol ϵ that lack the entire catalytic Pol2 NTD are viable, but cells with the full-length Pol ϵ carrying catalytic mutations are not. The observed helicase–polymerase uncoupling in the presence of an inactive Pol2 NTD may provide an explanation. Pol δ can access the leading-strand primer template for compensatory DNA synthesis in the case of the truncated Pol2 NTD, while the presence of the catalytically dead Pol2 NTD in Pol ϵ would prevent Pol δ from binding to the leading-strand primer template, producing its lethality.

c) Mcm10, Mrc1–Tof1–Csm3, and Ctf18–RFC are integral parts of the leading-strand replisome.—Mcm10 assembles an oligomer *in vitro* and plays essential roles in both replication initiation and replication elongation [129, 130]. Mcm10 interacts with the inactive Mcm2–7 double hexamer and helps to recruit Cdc45 and GINS [93, 131], and it is also required to activate the CMG helicase [95, 132–134]. The CMG helicase is first assembled on dsDNA; Mcm10 facilitates dsDNA melting by CMG for lagging-strand exclusion and origin unwinding [95, 96]. A recent single-molecule study demonstrated that Mcm10 promotes ssDNA passage through a unique gate distinct from the Mcm2–Mcm5 gate, giving CMG the capacity to transition between encircling ssDNA and encircling dsDNA [97]. This novel capacity may help the helicase to stay on DNA during fork repair. Interestingly, Mcm10 was able to remove RPA from ssDNA and to reanneal complementary DNA strands, an activity that may prevent fork regression during normal DNA replication [135]. Mcm10 is also required for the recruitment of Pol α -primase to the replisome [136]. Mcm10 interacts with Mcm2–7 [94], and specifically with the N-tier of Mcm2, riding ahead of the replication fork [133] (Fig. 9). However, a cross-linking mass spectrometry study recently showed that Mcm10 interacts with multiple subunits across the CMG helicase, including Mcm2, Mcm6, Cdc45, and GINS [135], suggesting that Mcm10 may have a highly extended structure. The multiple interfaces with the CMG helicase are consistent with Mcm10 functioning as the glue that stabilizes the replisome for fork progression [137, 138], and this agrees with the fact that no Mcm10 density has been observed so far by cryo-EM of the Mcm10–CMG complex.

Mrc1, Tof1, and Csm3 together form the fork protection complex that functions in normal DNA replication as well as in stabilizing a stalled fork. *In vitro* reconstitution has revealed

that all three proteins are required to function synergistically with PCNA to achieve the maximal rate of replisome progression [30]. A single-molecule study demonstrated a threefold speed-up of the leading-strand replisome by the Mrc1–Tof1–Csm3 complex [134]. Tof1 and Csm3 form a crescent-shaped heterodimer, with each subunit having an extended helical repeat structure [139]. The Tof1–Csm3 dimer binds to the parent dsDNA in front of the replisome [99]. The cryo-EM structure of the Mrc1–Tof1–Csm3 complex bound to the CMG–forked DNA showed that the Tof1–Csm3 dimer sits on the zinc finger domains of the Mcm4–6–7 half ring at the Mcm2–7 N-tier ring to “grip” onto the parental DNA duplex [115] (Fig. 9). The heterodimer embraces 3/4 of a turn of the parental DNA duplex, making extensive contact with the phosphate backbone as well as the major and minor grooves. The Tof1–Csm3 gripping of the parental DNA ahead of the fork may be the underlying mechanism for its ability to efficiently pause replication to avoid head-on collisions with barriers such as an RNA polymerase. However, Tof1–Csm3 also recruits topoisomerase I in front of the replisome to help slow down the fork progression [140]. Interestingly, the 125-kDa Mrc1 protein was largely invisible in the cryo-EM structure, despite its nearly stoichiometric binding to the CMG complex [115]. Cross-linking mass spectrometry revealed that Mrc1 interacts with multiple subunits in the replisome, including Tof1, Mcm2, Mcm6, Cdc45, and Ctf4. Therefore, Mrc1 may be a highly extended protein that functions like Mcm10 to glue the replisome subunits together.

Ctf18–RFC is an alternative PCNA clamp loader that is a heptameric complex composed of Ctf18, Ctf8, Dcc1, and Rfc2–5. The complex functions in chromosome cohesion [141], replication stress checkpoint activation [142–144], and DNA damage repair [145, 146]. Ctf18–RFC has an extra module beyond a canonical RFC that is referred to as Ctf18–1–8 and is composed of the C-terminus of Ctf18, Dcc1, and Ctf8. The Ctf18–1–8 module binds to the leading-strand Pol ϵ [147–149]. Recent biochemical and structural studies have shown that Ctf18–RFC forms a stable 1:1 complex with Pol ϵ with a large yet flexible interface [150, 151]. The Ctf18–1–8 module is a hook-like structure that binds to either dsDNA or the Pol ϵ catalytic domain, suggesting that Ctf18–RFC may be an integral part of the replisome and functions as the leading-strand PCNA clamp loader (Fig. 9). By maintaining a constitutive presence in the replisome, Ctf18–RFC is well positioned to protect stalled forks, activate S-phase checkpoints, and facilitate DNA damage repair.

d) Ctf4 trimer tethers Pol α -primase to the CMG helicase to initiate DNA synthesis for both the leading and lagging strands.—Unlike Pol ϵ , which directly binds the CMG helicase, Pol α -primase is tethered to the CMG via a scaffolding protein called Ctf4 [152, 153]. Ctf4 contains an N-terminal WD40 domain that is flexibly linked to the middle β -propeller domain and the C-terminal six-helix bundle, and it assembles a homo-trimer via the three β -propellers [154, 155]. Pol α -primase is a four-subunit complex, with its two primase subunits PriL and PriS forming a subcomplex that is flexibly tethered to polymerase subunit Pol I [156–159]. Pol α -primase is recruited to the replisome by Ctf4 via an interaction between a short N-terminal α -helix of Pol I and the peripheral C-terminal helical bundle of Ctf4 [154]. EM studies have revealed that Pol α -primase rides in the front of the CMG helicase by binding to the N-tier of CMG, opposite of Pol ϵ that is on the C-tier side of the CMG [125].

Studies of the interaction between Ctf4 and the CMG helicase initially focused on GINS [152], particularly the short N-terminal α -helix of Sld5 that stacks on the C-terminal helical bundle of Ctf4 [154]. However, Cdc45 of the CMG helicase was also reported to interact with Ctf4 [160]. Recent cryo-EM studies of the yeast and human CMG-Ctf4 complexes have shown that the interface between the Ctf4 helical bundle and Sld5 is relatively minor. Two more-extensive interfaces are formed between the Ctf4 β -propeller and the Cdc45 and Psf2 subunits of the CMG helicase [115, 161, 162]. The Ctf4 interface with the three CMG subunits amounts to a buried area of 1700 Å², a surface much larger than the stable contact between Cdc45 and the Mcm2–7 hexamer or between GINS and the Mcm2–7 hexamer. Therefore, the interaction between Ctf4 and the CMG helicase is very tight, forming a stable platform to scaffold the replisome [33] and to recruit additional replication factors such as the helicase Chl1, the multifunctional nuclease/helicase Dna2, the rDNA-associated protein Tof2, and the transcription termination helicase Sen1 [163–165].

While the leading strand going through the central channel of the helicase, the lagging strand is deflected off the top of the Mcm2–7 N-tier ring. Because Pol α -primase is tethered by Ctf4 to the N-tier side of the helicase, it has access to the lagging-strand template in order to synthesize a 7–12 ribonucleotide RNA segment using its primase, followed by extending a short DNA segment using its DNA polymerase activity [158, 159]. This primer is then extended by the lagging-strand Pol δ to generate Okazaki fragments. However, the leading strand emerging from the C-tier motoring of the helicase has already passed the Pol α -primase, and it has been unclear how the leading strand is primed. This apparent conundrum was recently resolved by an elegant *in vitro* reconstitution study [166]. It was shown that the Pol ϵ action is mostly outside the origin sequence. Within the initiation zone, the leading-strand DNA synthesis of the left fork is actually started from the lagging-strand primer of the right fork, and vice versa. Pol δ first extends the lagging-strand primer of the right fork until it catches up with the advancing CMG helicase on the left replication fork; it then transfers the 3' end of the nascent strand to the CMG–Pol ϵ complex. This mechanism is further supported by a genomic mapping study demonstrating that Pol δ is universally responsible for initiating leading-strand synthesis [167]. The mapping study also revealed that leading-strand synthesis is switched again from Pol ϵ back to Pol δ during replication termination.

e) The PCNA toolbelt model for lagging-strand DNA synthesis by Pol δ .—Due to the anti-parallel nature of DNA, the leading strand is copied continuously by Pol ϵ , while the lagging strand is copied by Pol δ discontinuously [168]. Therefore, the lagging strand needs repeated cycles of priming, looping, and Okazaki fragment synthesis, and the lagging strand DNA polymerase Pol δ is recycled from one Okazaki fragment to the next [23]. Pol δ is composed of the catalytic Pol3 subunit and the regulatory Pol31 and Pol32 subunits [169]. Significant progress has been made recently in understanding lagging-strand DNA replication at the molecular level. A recent cryo-EM structure of the Pol δ holoenzyme at 3.2 Å resolution caught the yeast enzyme in the act of DNA synthesis, and revealed that the regulatory subunits Pol31 and Pol32 are positioned sideways with no access to the DNA [170]. Although all three major replicative polymerases are suggested to contain a 4Fe-4S cluster [171], only Pol δ has been structurally shown to coordinate the metal cluster with one

of its two cysteine-rich metal-binding modules in the C-terminal region of the catalytic Pol δ . The 4Fe-4S cluster was suggested to function like a keystone that nucleates the assembly of the 3-subunit Pol δ holoenzyme.

The proliferative cell nuclear antigen (PCNA) plays a key role in lagging strand synthesis. Three PCNA protomers form a ring-shaped homotrimeric complex that encircles dsDNA and provides a central platform to organize the Okazaki fragment synthesis and maturation activities [172]. PCNA stimulates the Pol δ activity up to 30-fold [173]. Recently, the structure of the human Pol δ holoenzyme—composed of the catalytic p125 and the regulatory p66 and p12—in complex with PCNA and DNA substrate was determined to 3.0 Å resolution by cryo-EM [174] (Fig. 10). Despite the presence of three PIP boxes in the human enzyme complex—one at the C-terminus of p66, one at the N-terminus of p12, and the last at the C-terminus of p125—the structure showed that only the p125 PIP motif, which is highly divergent from the conserved PIP sequence, actually interacts with PCNA. Consistent with the yeast Pol δ structure, the human regulatory subunits are projected laterally, away from the DNA.

The PCNA toolbelt model posits that the three major enzymes involved in lagging-strand DNA synthesis, Pol δ , the flap endonuclease Fen1, and the DNA nick ligase, are able to bind the PCNA trimer simultaneously; and that they take turns accessing the DNA substrate [175]. In agreement with the model, the recent cryo-EM study showed that at least the human FEN1 and Pol δ can bind the PCNA ring at the same time. FEN1 is relatively small, binds to a second PCNA protomer via its C-terminal PIP box, and occupies a wedge-shaped gap between Pol δ and PCNA ring [174]. Also in support of the toolbelt model is an earlier study of the archaeal system in which the hyperthermophilic *Sulfolobus* Fen1, DNA ligase, and DNA polymerase PolB1 simultaneously bind the PCNA ring to form the so-called Okazakisome [176]. However, the 30-Å resolution of the negative-stain EM map was insufficient to ascertain whether all three enzymes were present at a stoichiometric ratio in the *in vitro*-assembled complex. Furthermore, the ligase is of moderate size and may need to gain full access to the DNA substrate for its function [177]. It is also unclear whether Pol δ , without binding to DNA, can remain stably associated with PCNA when the ligase takes over the DNA. It was found that a mutant PCNA trimer with only one functional binding site supports Okazaki fragment maturation, suggesting that sequential switching, rather than simultaneous binding, of the three partners may be the underlying mechanism for the PCNA-mediated Okazaki fragment maturation [178].

f) Pol η and Pol δ collaborate to bypass DNA lesions and restart DNA replication.—When a DNA lesion (such as a UV-induced cyclobutane pyrimidine dimer) is encountered by Pol δ on the lagging strand, the E2–E3 ubiquitin ligase complex Rad6–Rad18 comes in to mono-ubiquitinate PCNA, leading to the dissociation of Pol δ from the Ub–PCNA and the binding of Pol η for lesion bypass [179, 180]. However, when Pol ϵ encounters the lesion on the leading strand, the catalytic domain disengages the DNA while Pol ϵ remains associated with the helicase, leading to the uncoupling of DNA unwinding and DNA synthesis [181]. A recent study clarified how lesion bypass is accomplished on the leading strand, showing that the lesion vacated by Pol ϵ on the leading strand is quickly bound by Pol δ . Subsequently, PCNA mono-ubiquitination leads to the dissociation of Pol δ

and the binding of Pol η [182]. Therefore, the lesion bypass mechanisms for the leading and lagging strands are rather similar. The main difference is that leading-strand bypass involves an additional polymerase switching step: after lesion bypass and PCNA deubiquitination, Pol δ will rebind the leading strand to synthesize DNA in order to catch up with the CMG helicase. And when it reaches the replication fork, Pol δ dissociates and Pol ϵ reengages the DNA, leading to recoupling of the DNA unwinding and canonical DNA synthesis on the leading strand. Therefore, lesion bypass on both the leading and lagging strands requires the functions of both Pol η and Pol δ .

g) Is replisome a locomotive or a sewing machine?—Arthur Kornberg posed this question in his “10 commandments” review on the bacterial replication [183]. Twenty years later, we still don’t have a clear answer to his question either in bacteria or in eukaryotes. There is evidence supporting both concepts. Single-molecule and DNA combing studies show that the sister replisomes move apart when both ends of the DNA are fixed [63, 184]. However, *in vivo* light microscopy at increasingly better resolution of bacteria, yeast, and human cells seem to suggest that the replication foci are large stationery, and it is the parental DNA that is pulled into the protein factory and the nascent daughter DNA pushed out [185–188]. A recent cryo-EM study may tip the balance toward a stationary replisome, i.e., the sewing machine model [161]. It was found that the Ctf4 trimer is able to organize without any steric conflict two or even three very large CMG helicases (Fig. 11). The interface between each Ctf4 subunit and its associated CMG is identical to that observed in the complex of Ctf4 with a single CMG, which is very extensive. Given the high affinity, it is possible that one Ctf4 trimer *in vivo* may coordinate two sisters CMG helicases that are assembled from the same origin. The possibility suggests that the sister replisomes established from the same replication origin are physically linked by the Ctf4 trimer.

Two CMGs on a Ctf4 trimer leaves one Ctf4 protomer for other Ctf4-interacting factors such as Pol α -primase. The Ctf4 trimer can bind to only one Pol α -primase [161]. But Ctf4 has several additional functional partners, including Chl1, a helicase that may unwind the inadvertently paired DNA region near the replication fork, and Dna2, a nuclease-helicase that is involved in lagging-strand DNA synthesis as well as damaged and stalled fork processing [163, 164]. In the linked sister replisome model (Fig. 11), the two CMG helicases dock on the Ctf4 trimer with their respective MCM N-tier rings nearly facing each other, placing the two lagging strands toward the center and two leading strands out the sides. The sole Pol α -primase is centrally and flexibly tethered to the remaining Ctf4 protomer, apparently capable of priming for both sister replisomes. Despite the physical linkage, the sister replisomes may be dynamic, and one CMG helicase or the Pol α -primase may dissociate to allow access of Chl1 or Dna2 to the Ctf4 trimer when the fork stalls or a damaged DNA site is encountered.

3. Molecular Mechanism of Chromatin Maintenance

a) Parental epigenetic marks are reestablished after DNA replication.—

Eukaryotic genomes are compacted by the nucleosomes into chromatin structures to prevent DNA tangling and to facilitate cell division [189]. The nucleosome is a histone octamer containing an H3–H4 tetramer and two H2A–H2B dimers wrapped round by approximately

147 bp of DNA [190]. Epigenetic modifications on both DNA and histones determine gene expression patterns, regulate replication timing, and specify cellular identities [191, 192]. Histones can be acetylated, methylated, phosphorylated, ubiquitinated, and ADP-ribosylated [193]. The epigenetic information is maintained during DNA replication. The DNA methylation pattern on the parental strand is copied into the nascent strand by DNA methyltransferase 1 (DNMT1). The parental nucleosomes ahead of the DNA replication fork are disassembled and stripped off the DNA to facilitate duplex unwinding. Parental histones, together with an equal number of newly synthesized histones, are repackaged into nucleosomes that organize the daughter DNA behind the fork in a process termed DNA replication-coupled nucleosome assembly [194]. The modifications in parental H3–H4 histones are kept intact during DNA replication. Newly synthesized H3–H4 histones are acetylated on H4K5, H4K12, or H3K56 to increase their affinity for the histone chaperones and promote nucleosome assembly. Surprisingly, a recent study demonstrated that nucleosomes do not disperse in the wake of the replisome, and they are redeposited to exactly the same positions they had in the parent DNA before replication [195]. Recent studies using next-generation sequencing and biochemical and structural approaches have revealed that several components of the replisome, such as the replicative helicase, Pol ϵ , and Pol α -primase, participate in and often direct the deposition of the parental histones. Therefore, replication-coupled nucleosome assembly is a highly regulated process.

b) DNMT1 is recruited to the replication fork to maintain DNA methylation patterns in the daughter DNA.—DNA methylation is the addition of the 5' methyl group to a cytosine base of DNA. DNA methylation is necessary after every DNA replication cycle and is typically repressive of gene transcription [196]. After DNA replication, only the old but not the new strand is methylated. The first mechanism for copying DNA methylation is through the specialized function of DNMT1 in recognizing hemimethylated DNA. Therefore, DNMT1 is primarily responsible for maintaining the methylation pattern following DNA replication. DNMT1 is a large protein composed of a N-terminal DMAP domain, a PIP motif for PCNA interaction, a replication foci targeting domain (RFTS), a DNA-binding CXXC domain, a pair of bromo-adjacent homology (BAH1 and BAH2) domains, and a C-terminal catalytic methyltransferase domain (MTase) [197, 198]. The RFTS domain limits DNMT1 to function during DNA replication and prevents the enzyme from modifying the genome beyond replication [199]. Although DNMT3A and DNMT3B are *de novo* DNA methylation enzymes, yet their localization in genome may also contribute to the maintenance of DNA methylation [200].

The second mechanism of maintaining DNA methylation pattern is through the recruitment of DNMT1 to the replisome and the newly replicated chromatin. DNMT1 is actively recruited to newly synthesized DNA and becomes an integral component of the replication progression complex [201, 202]. DNMT1 can be directly recruited to the DNA replication sites by interaction with PCNA [203, 204]. DNMT1 can also be recruited to the replication sites via an UHRF1-mediated ubiquitination mechanism, either on PAF15 (PCNA-associated factor 15) at early replication sites or on the H3 tail at the late replication sites [205, 206]. UHRF1 is a five-domain protein with an N-terminal ubiquitin-like domain (UBL), a set and ring-associated domain (SRA), a tandem tudor domain, and a C-terminal

RING finger that has the E3 ligase activity. UHRF1 is an epigenetic integrator that functions in both DNA methylation and histone code maintenance, and it has emerged as a key oncogene that may be targeted for anti-cancer drug development and used as a biomarker for cancer diagnosis [207]. In late S phase, the C-terminal RING finger ligase domain of UHRF1 adds two ubiquitin molecules to the H3 tail [208]. DNMT1 has an N-terminal ubiquitin-interacting motif (UIM). DNMT1 can be recruited to the nucleosome by interactions of its UIM either with the dual mono-ubiquitylation of histone H3 or with the UHRF1 UBL domain [209, 210]. The UHRF1 UBL domain interacts with E2 ligase and coordinates with other UHRF1 domains to recognize the hemimethylated DNA and histone H3 substrates [211, 212]. A detailed picture of DNMT1 recruitment to and function on chromatin DNA has been lacking and requires further structural and biochemical efforts.

c) Replisome facilitates the nucleosome assembly.—In an *in vitro* replication system, nucleosomes can randomly disperse, and DNA mechanics and DNA looping seem to facilitate passive nucleosome transfer to nascent DNA [213]. However, it is generally accepted that replisome and other histone chaperones coordinate to shuttle parental histones and deposit nascent histones to newly replicated DNA [194, 214]. Two categories of histone chaperone activities are involved in replication-coupled nucleosome assembly: the canonical histone chaperones such as CAF-1 and FACT and the replisome components like Mcm2 and Dpb3–Dpb4 that possess additional histone chaperone activities [215]. CAF1 is a heterotrimer and chaperones H3–H4 [216]. CAF-1 is recruited to the replication fork via a specific interaction with PCNA [217]; this interaction mediates DNA replication-coupled nucleosome assembly and also facilitates heterochromatin establishment [218]. The CAF-1 subunit Cac1 is responsible for high-affinity binding to the H3–H4 dimer [219]. Two CAF-1 chaperones, each binding an H3–H4 dimer, are recruited to DNA via their respective winged helix domains to assemble the H3–H4 tetramer [220].

FACT is a heterodimer of SPT16 and SSRP1. FACT is recruited to the replisome via interactions with the replicative helicase and Pol α -primase [221], and it functions in both nucleosome disassembly in front of the fork and nucleosome reassembly on the nascent daughter DNA behind the fork, as well as in DNA repair and gene transcription [222, 223]. An smFRET study demonstrated that FACT uncoils over 70% of the DNA in a nucleosome [224]. The recent cryo-EM structure showed that human FACT rides like a unicyclist on a partially assembled nucleosome with 79-bp DNA, with the SPT16 dimerization domain straddling DNA at the nucleosome dyad; the middle domains of SPT16 and SSRP1 clamp on both sides of the nucleosome [225] (Fig. 12). The structural and biochemical study underscored the importance of the interaction plasticity of FACT with nucleosome intermediates during nucleosome disassembly and reassembly in DNA replication and transcription.

The replisome core contains multiple histone-binding activities to facilitate nucleosome assembly and maintain epigenetic state. Mcm2 is a subunit of the CMG helicase, yet it competes with DNA to bind the H3–H4 tetramer via its highly conserved N-terminal histone-binding domain (HBD) [226–228] (Fig. 13). Mcm2 acts as a histone chaperone for both H3–H4 dimers and tetramers in their free form or on chromatin. Two recent studies showed that Mcm2 regulates histone segregation: mutations in the Mcm2 HBD disrupt the

interaction with H3–H4, resulting in a leading-strand bias for the parental histone mark H4K20me2 and a lagging-strand bias for the new histone mark H4K5ac [229, 230]. Therefore, Mcm2 facilitates H3–H4 deposition on the parental lagging strand DNA. The histone chaperone activity may be unique to the Mcm2 N-terminal peptide; the N-terminal peptides of Mcm5 and Mcm7 participate in Mcm2–7 double-hexamers assembly, but they are not known to bind any histone protein.

An early proteomic study showed that Pol ϵ can pull down all four core histones [231]. The Pol ϵ component Dpb3–Dpb4 was found to maintain the silent state of heterochromatin [120, 232]. The deletion of Dpb3 or Dpb4 dramatically increased the deposition of parental histones to the lagging strand, indicating that Dpb3–Dpb4 guides the parental histones to the leading strands [233]. This is consistent with the *in vitro* pull-down of H3–H4 with Dpb3–Dpb4 (Fig. 13). Another study in mammalian cells found that POLE3–POLE4, the Dpb3–4 equivalent, binds to both the H3–H4 dimer or the tetramer [234]. Similarly, deletion of POLE3–POLE4 impairs both the deposition of newly synthesized histones and the recycling of parental histones, leading to global defects in chromatin assembly. Thus, Dpb3–Dpb4 has a conserved histone chaperone activity towards H3–H4 and functions to bridge the replisome and the nucleosome. Because the recent structure of the Pol ϵ holoenzyme does not show how Dpb3–Dpb4 might interact with H3–H4 [122], and Dpb3–Dpb4 association with catalytic Pol2 is dynamic in solution, it is currently unclear if the histone chaperone activity of Dpb3–Dpb4 is within or outside the Pol ϵ holoenzyme.

As described above, Pol α -primase is tethered to the CMG helicase by Ctf4 [161, 164]. The Ctf4 tethering of Pol α -primase to the replisome is not essential for replication in yeast, but it is essential to maintain epigenetic silencing of telomeres and the silent mating type genes. The latter function relies on a novel histone-binding motif at the N-terminal region of catalytic Pol1 subunit that binds H2A–H2B [235] (Fig. 13). Mutations that disrupt the Pol α –H2A–H2B interaction or the Pol α –Ctf4 interaction lead to silencing loss in telomeres [236]. Disruption of the Pol α –Ctf4 interaction can also lead to preferred deposition of parental H3–H4 histones on the leading strand, indicating that Pol α -primase guides the parental histones to the lagging strand. Notably, abolishing the H2A–H2B-binding site of Pol α will disrupt the Pol α –Mcm2 interaction, while not affecting the Pol α –Ctf4 interaction. Together, Mcm2–Ctf4–Pol α may also work as a whole platform to recycle parental histone to the lagging strand. However, the inheritance pattern of H2A–H2B is not currently known. The behavior of H2A–H2B during DNA replication and its interaction with the replisome are still open questions.

CONCLUSION AND PERSPECTIVES

The past several years have witnessed amazing progress in our understanding of eukaryotic DNA replication and replication-coupled chromosome maintenance. Major events during DNA replication have been reconstituted *in vitro*, key molecular steps have been analyzed by single-molecule techniques, and the structures of core molecular machines involved in DNA replication have been determined by cryo-EM. Major progress has also been made in other replication-related areas not mentioned in this review, including replication termination [36],

coordination and conflict resolution between DNA replication and transcription [237], and replication stress [238].

However, our understanding is far from complete. We don't have a complete structure of the Pol α -primase, so it is unclear how the primase and polymerase of the holoenzyme work in tandem to synthesize RNA-DNA hybrid primer with defined length. The structure of the leading-strand replisome core is being built up by structural determination of multiple replication factors bound to the helicase individually or in combination, including Pol ϵ , Ctf4, Mrc1–Tof1–Csm3, and Pol α -primase, but we still don't have the complete picture of leading-strand replisome. The structures of key components of the lagging-strand synthesis machinery have been determined, but the structure of the entire Okazakisome remains unknown. We have very limited knowledge of how the various components of the leading-strand replisome and lagging-strand Okazakisome work together or of how these two machines are coordinated to ensure the copying of the leading and lagging strands at the same rate. We know very little how the replication machines work with nucleosomes and the DNA- and histone-modification enzymes to maintain epigenetic inheritance. A question central to the replication field is whether sister replisomes emerging from the same replication origins are coupled and stationary or whether they move away from each other during uninterrupted DNA replication. Another major question is the role and significance of LLPS in DNA replication and chromatin maintenance. These questions provide exciting opportunities for future studies.

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Abbreviations

AAA+	ATPases associated with various cellular activities
ACS	ARS consensus sequence
ARS	autonomously replicating sequence
BP	basic patch
CDK	cyclin-dependent kinase
CMG	Cdc45–Mcm2–7–GINS
Cryo-EM	cryoelectron microscopy
Ctf4	chromosome transmission factor 4
DDK	Dbf4-dependent kinase
FACT-1	facilitates chromatin transcription 1
GINS	go-ichi-ni-san

HD	helical domain
ISM	initiator-specific motif
LLPS	liquid-liquid phase separation
Mcm	minichromosome maintenance
OB	oligosaccharide/oligonucleotide
ORC	origin recognition complex
OCCM	ORC–Cdc6–Cdt1–Mcm2–7
Pol α	DNA polymerase α
Pol δ	DNA polymerase δ
Pol ϵ	DNA polymerase ϵ
RFC	replication factor C
smFRET	single-molecule Förster resonance energy transfer
WHD	winged helical domain

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Figure 1. Major steps in ORC–Cdc6 loading of the Cdt1-bound Mcm2–7 hexamers onto origin DNA.

ORC alone on origin DNA is inactive. Cdc6 binding leads to the assembly of the active loading platform. ORC-Cdc6 loads the Cdt1-bound Mcm2–7 on DNA to form the OCCM complex. Loading of the second Cdt1-bound Mcm2–7 leads to the assembly of Mcm2–7 double hexamer on double-stranded origin DNA.

Figure 2. Cryo-EM structure of *S. cerevisiae* ORC in complex with ARS305 origin DNA.

This is an edge-on surface view of the ring-like ORC structure (PDB ID 5ZR1). The right panel shows the DNA interacting elements of ORC and the 51° bend of the origin DNA (B1 element) outside the central channel of ORC.

Figure 3. Loading onto the origin DNA of the first Mcm2–7 by ORC-Cdc6.

a) A surface view of the structure of the *S. cerevisiae* OCCM complex (PDB 5V8F). The origin DNA is horizontal in this view but is invisible because it is inside the central channels of ORC-Cdc6 and Mcm2–7. b) Three-step loading process of the first Mcm2–7 hexamer. See text for detail.

Figure 4. Molecular mechanism of loading the second Cdt1-bound Mcm2-7 hexamer onto origin DNA.

After the formation of OCCM, ORC and Cdc6 dissociate from the C-tier AAA+ side of the loaded Mcm2-7. ORC binds to B2 element of the origin DNA and the N-tier side of the first Mcm2-7, forming the MO complex. Cdc6 next binds to MO to recruit the second Cdt1-bound Mcm2-7, leading to the eventual assembly of the Mcm2-7 double hexamer.

Figure 5. Structure of the *S. cerevisiae* Mcm2-7 double hexamer.

Left panel sketches the N-tier to N-tier or head-to-head binding of the double hexamer.

Right panel is a semi-transparent surface view of the double hexamer structure (PDB ID 5BK4), with the internal dsDNA shown in gold cartoon.

Figure 6. Two helicases bypass each other in order to leave the origin.

Because the eukaryotic CMG helicase translocates on the leading strand DNA N-tier first, the two helicases can leave the origin only when the lagging strand is extruded from the central channel of each helicase to make it possible for the helicases to pass each other. Cdc45 and GINS are omitted and only Mcm2-7 hexamers are shown for simplicity.

Figure 7. Duplex DNA unwinding by CMG helicase.

a) Domain architecture of an Mcm protein. b) Side view of the CMG-forked DNA structure (PDB ID 6U0M) showing only Mcm4 and Mcm5 for clarity. c) The proposed unwinding mechanism.

Figure 8. Structure of Pol e and a model of a leading-strand replisome.

a) Pol2 domain map. b) Structure of the Pol e holoenzyme in cartoon (PDB ID 6WJY). The OB domain is highlighted in red shade. c) Model of a leading-strand replisome. The OB domain is highlighted in red to mark its central location in the potential path of the leading strand DNA from the helicase to the polymerase.

Figure 9. The binding positions of MTC, Mcm10, and Ctf18-RFC in leading-strand replisome. The three replisome interacting factors are labeled in red. The Mcm10 location is undetermined and is for illustration only.

Figure 10. Structure of human Pol δ holoenzyme bound to FEN1 and DNA substrate. The protein structure (PDB ID 6TNZ) is in cartoon and colored individually. The template/primer is shown in orange surface. The bound Zn²⁺ and 4Fe-4S clusters are in spheres.

Figure 11. A Ctf4 trimer organizes the sister leading-strand replisomes and Pol α -primase into the core of the replication factory.

This sketch is based on the cryo-EM structure of Ctf4 trimer bound to two CMG helicases (PDB ID6PTN).

Figure 12. FACT rides on top of the partially assembled nucleosome.

The cryo-EM structure is shown in edge (left) and side (right) views (PDB ID 6UPL). FACT and DNA are shown in surface, and histones are in cartoon.

Fig. 13. Replisomes actively participate in nucleosome reassembly.

The sketch highlights three components of a replisome that are shown to possess histone chaperone activity: the Mcm2 N-terminal histone binding domain, the Pol ϵ subunit Dpb3–Dpb4 heterodimer, and the N-terminal domain of Pol α . FACT, not shown here, participates in both nucleosome disassembly in front of the fork and the nucleosome reassembly following the replication fork.