Mechanism of Mcm10 Function during DNA Replication

by

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ABSTRACT

All life needs to replicate its genome completely and do so with limited errors. In eukaryotic cells, DNA replication is accomplished by a multi-stage process involving numerous protein assemblies. The core component of this process is the replicative DNA helicase, Mcm2-7. Mcm2-7 complexes are loaded around origins of DNA replication during G1 phase. As cells transition from G1 to S phase, the Mcm2-7 helicases are activated resulting in the recruitment of DNA polymerases and accessory proteins to begin DNA synthesis.

Activation of the Mcm2-7 replicative DNA helicase is the committed step in eukaryotic DNA replication initiation. Mcm2-7 helicase activation requires binding of the helicase-activating proteins, Cdc45 and GINS (forming the CMG complex). DNA unwinding cannot proceed, however, until an additional protein, Mcm10, associates with the CMG. Mcm10 continues to travel with the replication fork after stimulating initial DNA unwinding but a function for Mcm10 during DNA replication elongation had not been established.

Using a combination of molecular genetics and reconstituted biochemical assays, this thesis will outline the function of Mcm10 throughout DNA replication. I show that Mcm10 binds a conserved motif located between the OB-fold and A subdomain of Mcm2. Although buried in the interface between these domains in Mcm2-7 structures, mutations predicted to separate the domains and expose this motif restore growth to conditional-lethal MCM10 mutant cells. In addition to stimulating initial DNA unwinding, Mcm10 stabilizes Cdc45 and GINS association with Mcm2-7 and stimulates replication elongation in vivo and in vitro. Furthermore, a lethal allele of MCM10 that stimulates initial DNA unwinding but is defective in replication elongation and CMG binding is identified. These findings expand the roles of Mcm10 during DNA replication and suggest a new model for Mcm10 function as an activator of the CMG complex throughout DNA replication.

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Chapter I

Introduction
Genome duplication is critical to the survival of all living organisms. This process must occur in spite of numerous barriers including transcription, nucleosomes, and other DNA binding proteins. Complete genome duplication is required to ensure each daughter cell receives a copy of the parental DNA. In eukaryotic organisms, DNA replication requires numerous, conserved enzymes and intricate regulatory mechanisms to ensure this process occurs with remarkably limited errors and exactly once per cell cycle. The reason for this intense regulation of genomic integrity is apparent on the rare occasions when cells have adverse mutations in proteins involved in DNA replication, which leads to developmental abnormalities, cancer, or cell death (Blow and Gillespie 2008; Jackson et al. 2014).

My thesis work has focused on the committed step of eukaryotic DNA replication, activation of the replicative helicase. In this introduction, I will outline our current understanding of how eukaryotic cells first load inactive helicases at each origin of replication and then activate these loaded helicases to initiate DNA unwinding. I will then discuss the mechanisms controlling nascent DNA synthesis. Finally, I will provide an overview of the evolution of the biochemical studies of eukaryotic DNA replication, which I used extensively in my work.

All of my studies focus on the budding yeast, *Saccharomyces cerevisiae*, which represents a particularly useful organism to study eukaryotic DNA replication. Early budding yeast genetic screens identified and provided mutations in many DNA replication
proteins and proteins that regulate cell cycle events. (Hartwell 1976; Maine et al. 1984). Subsequent genetic-interaction studies in yeast revealed additional essential DNA-replication-initiation proteins (Kamimura et al. 1998; 2001; Takayama et al. 2003; Araki et al. 1995). In contrast to most other eukaryotic species, *S. cerevisiae* has defined origin of replication DNA sequences, and the location, timing, and efficiency of these origins has been well documented (Raghuraman et al. 2001; Yabuki et al. 2002). The identification of these sequences allowed the identification of proteins that bound and acted at these sites *in vivo* and *in vitro* (Labib et al. 2001; Blow and Laskey 1988; Bell and Stillman 1992; Cocker et al. 1996; Maiorano et al. 2000; Aparicio et al. 1997). Finally, the emergence of fully reconstituted biochemical assays to study DNA replication has provided critical tools to dissect these events (Remus et al. 2009; Evrin et al. 2009; Heller et al. 2011; Yeeles et al. 2015). Importantly, DNA replication proteins are conserved across eukaryotes. Thus, mechanisms discovered in budding yeast have guided studies in other eukaryotic organisms, including metazoans (Sansam et al. 2010; Kumagai et al. 2010; Sangrithi et al. 2005; Matsuno et al. 2006). Because my studies were performed using budding yeast cells and proteins, the following introduction will focus on our understanding of *S. cerevisiae* DNA replication.

**The Eukaryotic Cell Cycle**

Eukaryotic cell division is the result of a sequence of complex regulated events known collectively as the cell cycle. Transformation from a single cell to two daughter cells requires duplication of the cellular components necessary for survival and growth followed
by separation of these components into two daughter cells. Because propagation of the genome is central to cell division, two of the cell cycle stages are defined based on chromosomal events. Genome duplication occurs during S phase, and the segregation of the newly replicated chromosomes (mitosis) occurs during M phase. These temporally-separated phases ensure each daughter cell contains the same genomic contents as its parental cell.

The transitions between cell cycle phases are primarily dependent on the oscillation of Cyclin-Dependent Kinase (CDK) activity (Fig. 1). In the typical mitotic cell cycle, S and M phases are separated by gap phases that provide time to accumulate the necessary cellular machinery for the next step. These gap phases also act as a regulatory transition period ensuring the previous step has been completed. CDK levels are low during G1 phase and increase at the G1/S-phase transition. Although the specificity of CDK in each of the other phases is altered by binding different regulatory subunits known as cyclins (Kõivomägi et al. 2011), CDK activity remains high throughout S, G2, and M phase.

One of the functions of the oscillation in CDK activity is regulation of genome duplication and segregation. Preparation for DNA synthesis begins in G1 phase when CDK activity is low. Under these conditions, the inactive replicative Mcm2-7 helicase can be loaded on DNA. As cells enter S phase, increased CDK activity activates the Mcm2-7 helicase which drives subsequent initiation of DNA synthesis (Fig. 1). The resulting duplicated chromosomes are called sister chromatids and are held together by proteins called
Figure 1.

Figure 1. Eukaryotic cell cycle
Helicase loading is restricted to G1 phase when CDK levels are low. High CDK activity during S, G2 and M phase activate the helicase but prevent helicase loading. Once the genome has been duplicated, the resulting sister chromatids are equally divided into the two daughter cells in M phase.
cohesins. At the end of S phase, cells enter another gap phase, G2, to ensure they have completed genome duplication before dividing their DNA. During S and G2 phase, checkpoint proteins detect any aberrations in completion of DNA replication. When these atypical replication events occur, checkpoint proteins trigger a signaling pathway to prevent further cell cycle progression until the DNA replication defects are repaired (Nyberg et al. 2002). In M phase, sister chromatids are attached to microtubules radiating from opposite spindle poles. Cleavage of cohesin results in the sister chromatids segregating into a pair of daughter nuclei. The cell is then divided into two daughter cells (cytokinesis). As with DNA replication, there are checkpoint proteins that monitor the correct microtubule attachment and segregation of the sister chromatids (Musacchio 2015).

Oscillation in the potential for helicase loading and activation ensures that helicase loading and activation never occur at the same time. In budding yeast, this separation is achieved by oscillating CDK activity. However, the transition from high CDK to low CDK, or vice versa, presents a precarious situation as it is possible that intermediate CDK levels could lead to the undesirable outcome of simultaneous helicase loading and activation. To prevent both events from ever occurring simultaneously, the cell has implemented safeguards at the G1/S and M/G1 transitions when CDK activity is reversed. At the G1/S transition, this prevention is accomplished by controlling the specificity of CDK activity with cyclins. At the end of G1 phase, G1-phase cyclins (Cln1,2) are upregulated (forming G1-CDK) causing Cdc6 degradation and nuclear export of Mcm2-7 not bound to
chromatin (Drury et al. 2000; Labib et al. 1999). Importantly, G1-CDK cannot activate the helicase to begin DNA synthesis. Thus, neither helicase loading nor activation can occur during this short window of elevated G1-CDK activity but no S-CDK activity. Increased G1-CDK activity induces the synthesis of S-phase cyclins (Clb5,6) to form S-CDK. S-CDK then activates the Mcm2-7 helicase and also prevent helicase loading. Importantly, this inhibition of helicase loading is maintained through the end of mitosis by the S-phase and mitotic CDKs (M-CDKs). A similar mechanism is in place during the M/G1 transition to prevent simultaneous helicase loading and activation. In this case, the mechanism involves a second S-phase kinase, the Dbf4-dependent kinase (DDK). Like S-CDK, this kinase is required to activate the Mcm2-7 helicase. Dbf4, the regulatory subunit of DDK required for helicase activation, is degraded before inactivation of M-CDKs. This creates another short window where neither helicase activation or helicase loading (due to high M-CDK activity) can occur (Ferreira et al. 2000; Oshiro et al. 1999; Lu et al. 2014).

**Mcm2-7: The Central Motor of DNA Replication**

Like many cellular events, DNA replication requires the energy of ATP binding and hydrolysis to do work. Several DNA replication proteins are members of a family of proteins called ATPases Associated with various cellular Activities (AAA+). The ATP hydrolysis active sites of this class of enzymes are characterized by several motifs including Walker A and Walker B motifs that are critical for binding and hydrolyzing ATP, respectively (Duderstadt and Berger 2013). Proteins in this family typically hydrolyze ATP at active sites formed at the interface of two associated proteins. Thus, most of these
proteins function as either homo- or hetero-oligomers. The energy from ATP binding and hydrolysis is propagated to another region of the protein complex to induce conformational changes that perform molecular work (Hanson and Whiteheart 2005). Several functions of ATP hydrolysis during the process of helicase loading will be described below.

Mcm2-7 (Minichromosomal maintenance 2-7) is the core enzyme of the replicative helicase and forms a scaffold to which multiple replication factors bind. This complex is the only replication factor that is involved in every step of DNA replication. As mentioned above, Mcm2-7 must be loaded onto DNA before DNA replication is initiated in S phase. When initially loaded in G1 phase, Mcm2-7 is inactive, but its location defines potential origins. Thus, this process has been dubbed origin licensing (Blow and Laskey 1988). Once activated, Mcm2-7 becomes the central motor in the movement of DNA replication fork (see Mcm2-7 Activation).

Genetic and structural studies have shaped our current view of the architecture of Mcm2-7. The six genes of Mcm2-7 were first discovered in several genetic screens for genes involved in DNA replication and the cell cycle (Hennessy et al. 1991; Maine et al. 1984; Moir et al. 1982). They were later grouped together based on sequence similarity and biochemical characterization (Thömmes et al. 1997; Kubota et al. 1997). Mcm2-7 forms a heterohexameric complex and structural studies have provided insight into the conformation of this complex (Sun et al. 2013; 2015; Abid Ali et al. 2016; Zhai et al. 2017;
Figure 2. Mcm2-7 conformations

(A) Toroidal structure of Mcm2-7. The AAA+ ATPase active sites are located at the interfaces of the C-terminal domains of adjoining subunits. (image from Trakselis 2016; structure from Li et al. 2015).

(B) Cracked ring structure of Mcm2-7 with Cdt1 bound. (Zhai et al. 2017).

(C) Each Mcm2-7 N-terminal domain is comprised of an A subdomain, Oligo-saccharide/oligonucleotide-fold (OB-fold), and C subdomain. (Li et al. 2015).
Georgescu et al. 2017; Li et al. 2015; Yuan et al. 2016; Costa et al. 2011). Globally, the Mcm2-7 hexamer forms either an open ring or a toroidal shape with a central channel that encircles bound DNA (Fig. 2A and 2B). Biochemical interaction studies determined that the six subunits are arranged in a defined order (Bochman et al. 2008; Davey et al. 2003). When viewed from N-terminus, this order is: Mcm2-6-4-7-3-5. Open ring structures have a gap between Mcm2 and Mcm5, whereas these subunits interact in closed ring forms of Mcm2-7 (Costa et al. 2011; Zhai et al. 2017; Li et al. 2015).

Mcm2-7 is a AAA+ protein complex with six ATPase active sites located at the interfaces between the C-terminal domains of the adjacent subunits. When these sites are individually mutated, replication initiation is inhibited at different stages of the process, suggesting distinct functions for each ATPase site (Schwacha and Bell 2001; Kang et al. 2014; Coster et al. 2014). Each N-terminal domain is comprised of an oligosaccharide/oligonucleotide-fold (OB-fold), B subdomain, and A subdomain, as well as long unstructured tails at the N-terminus of Mcm2, 4, and 6 (Fig. 2C). Regulation of Mcm2-7 function through protein interactions and phosphorylation primarily occurs at the N-terminal domain. These regulatory events are propagated to the ATPase active sites in the C-terminal domain to control Mcm2-7 DNA unwinding function.

**Origin Licensing (aka Mcm2-7 Loading)**

The current model for initiation of DNA replication was first proposed in 1963. In this replicon model, an initiator protein recognizes and activates the replicator, a specific DNA
sequence that dictates the location of the initial DNA synthesis (Jacob et al. 1963). The specific DNA sites where initiation of DNA synthesis occurs are known as origins of replication. This basic model holds true for DNA replication initiation in most organisms but only explains the first step in the process. We now know that DNA replication initiation involves a much more complex coordinated series of protein assembly events.

The advent of molecular cloning led to the identification of replicators in several organisms. The first replicator was identified in *E. coli* by inserting fragments of its genome into exogenous plasmids and monitoring replication of the plasmids independent of chromosomal replication (Yasuda and Hirota 1977). In most bacteria, one replicator/origin is sufficient to duplicate their entire circular genome. In contrast, eukaryotic organisms initiate replication from many origins throughout the genome (Cairns 1963; Callan 1973). *S. cerevisiae* replicators were initially identified as autonomous replicating sequences (ARSs) and were discovered in a similar manner as the *E. coli* replicator (Stinchcomb et al. 1980; 1979; Chan and Tye 1980; Beach et al. 1980). ARS elements are typically 100-150 base pairs in length and include a highly-conserved ARS consensus sequence (ACS) (Marahrens and Stillman 1992; Broach et al. 1983; Celniker et al. 1984). In contrast to yeast, sequence-defined replicators have not been defined in most other eukaryotes. Instead, origins are likely defined by a more complex array of determinants including local DNA accessibility and chromatin states and are found to change location between different cell types (Leonard and Méchali 2013).
The initiator protein that first identifies and binds at eukaryotic origins is known as the Origin Recognition Complex (ORC). This six-subunit complex was first isolated biochemically based on its affinity for the most highly conserved element found in *S. cerevisiae* ARS elements, the ACS (Bell and Stillman 1992). In addition to recognizing origin DNA, ORC also facilitates loading of Mcm2-7 onto DNA. Structurally, ORC forms a partial ring that is found either in a spiral or flat-ring shape (Sun et al. 2013; Tocilj et al. 2017; Bleichert et al. 2015). This ring is composed of five of the six ORC subunits (Orc1-5), each of which has N-terminal winged helix domains and C-terminal regions that either are or are related to AAA+ ATPase domains. Of these subunits, only Orc1, Orc4, and Orc5 bind ATP, and only Orc1 can hydrolyze ATP (Klemm et al. 1997; Yuan et al. 2017). The ATP binding activity of Orc1 is critical for recognition of the ACS, and ATP hydrolysis by the same subunit facilitates Mcm2-7 helicase loading (Klemm and Bell 2001; Bowers et al. 2004; Bell and Stillman 1992). The smallest and least conserved subunit, Orc6, is not required for DNA binding, yet it is essential for Mcm2-7 loading (Li and Herskowitz 1993; Lee and Bell 1997; Chen et al. 2007).

Mcm2-7 loading and initial preparation for DNA replication occurs in G1 phase when ORC, along with Cdc6 (*C*ell *d*ivision *c*ycle 6) and Cdt1 (*C*DC10*-d*ependent *t*ranscript 1) load two Mcm2-7 rings around dsDNA at origins (Fig. 3). Initially, ORC then Cdc6 associates with replicator DNA. The resulting ORC/Cdc6 complex recruits a complex between Mcm2-7/Cdt1 to form a short-lived intermediate with all four proteins called the ORC/Cdc6/Cdt1/Mcm2-7 (OCCM) complex (Evrin et al. 2013; Sun et al. 2013). Once the
Figure 3. Mcm2-7 loading mechanism

ORC/Cdc6 bound to origin DNA recruit Mcm2-7/Cdt1 to load the first Mcm2-7 hexamer. After release of the first Cdc6 and Cdt1, a second Cdc6 associates with ORC and recruits the second Mcm2-7/Cdt1. The end result of this process is two Mcm2-7 hexamers interacting at their N-termini and loaded around dsDNA. (Ticau et al., 2015).
first Mcm2-7 is loaded, Cdc6 and Cdt1 are released, in that order. The ORC/Mcm2-7 complex that remains is capable of binding a second Cdc6 and Mcm2-7/Cdt1 complex to load a second Mcm2-7 (Ticau et al. 2015). At each origin, the two Mcm2-7 complexes are loaded with the N-termini facing each other, forming a head-to-head double hexamer (Evrin et al. 2009; Remus et al. 2009). At this stage, Mcm2-7 is inactive, but the two helicases are poised to leave the origin in opposite directions to create bidirectional replication forks.

The Mcm2-7 ring must be broken before loading onto DNA and then subsequently closed to stably encircle DNA. This DNA entry gate is located at the interface between Mcm2 and Mcm5 (Samel et al. 2014). Before being loaded, Mcm2-7 exists in an open conformation that is competent for DNA entry into its central channel (Costa et al. 2011; Zhai et al. 2017). This open conformation is independent of Cdt1 binding, but, once Mcm2-7 is loaded, Mcm3/5 ATP hydrolysis (and possibly other Mcm2-7 ATPases) promotes closure of the Mcm2-7 ring and release of Cdt1 (Ticau et al. 2017).

Cdc6 is another AAA+ protein that is part of the same family as Orc1-5. Cdc6 binds the Orc1 and Orc2 subunits to complete the ORC protein ring (Tocilj et al. 2017; Speck et al. 2005) (Fig. 4). Importantly, this ORC/Cdc6 ring is found to encircle the bound dsDNA, which involves DNA interactions with both ORC and Cdc6 (Sun et al. 2013; Yuan et al. 2017). Consistent with Cdc6 completing a protein ring that encircles DNA, interaction of Cdc6 with ORC increases the specificity of ORC DNA binding (Duzdevich et al. 2015;
Figure 4

Figure 4. Orc1-5/Cdc6 ring
Cdc6 binds Orc1 and Orc2 to complete a ring encircling dsDNA. (Yuan et al. 2017).
Speck and Stillman 2007). Although Cdc6 ATP hydrolysis is not required for helicase loading, it is required for cell viability. The reason for this paradox is that Cdc6 ATPase activity is required for its release from Mcm2-7 after loading, and retention of Cdc6 inhibits downstream steps of replication initiation (Chang et al. 2015). Cdc6 ATPase activity is also involved in the removal incompletely-loaded Mcm2-7 from the DNA (Kang et al. 2014; Coster et al. 2014).

Although Cdt1 does not have ATPase activity, it is still essential for helicase loading. Before Mcm2-7 is loaded, Cdt1 is stably associated with Mcm2-7 to form a heptomeric complex (Tanaka and Diffley 2002). Cdt1 also interacts with the Orc6 subunit of ORC, which helps recruit Mcm2-7 to ORC (Chen and Bell 2011; Chen et al. 2007; Yuan et al. 2017). In addition, recent single-molecule studies showed that closure of the Mcm2-7 ring around dsDNA is temporally correlated with release of Cdt1 (Ticau et al. 2017). These data suggest that either Cdt1 release leads to Mcm2-7 ring closure or ring closure causes Cdt1 release.

To prevent duplication of the genome more than once during a cell cycle, Mcm2-7 loading is inhibited after cells enter S phase. Even limited re-replication of the genome leads to extensive DNA damage and cell death (Green and Li 2005). The Mcm2-7 loading components, ORC, Cdc6, and Cdt1, are not required for DNA replication after they have served their purpose in G1 phase (Hua and Newport 1998; Rowles et al. 1999) and thus, are inhibited by CDK as cells enter S phase. In budding yeast, CDK inhibits helicase
Figure 5. CDK regulation of rereplication
CDK inhibits additional loading of Mcm2-7 during S phase by phosphorylating ORC, Cdc6, and Mcm2-7. Phosphorylation of ORC inhibits its function during loading. Phosphorylation of Mcm2-7 results in its exportation from the nucleus. Phosphorylation of Cdc6 results in its degradation. (Nguyen et al., 2001).
loading by the following overlapping phosphorylation-dependent mechanisms: 1) ORC phosphorylation inhibits its function during Mcm2-7 loading (Chen and Bell 2011; Wilmes et al. 2004), 2) Mcm2-7 phosphorylation leads to the export of Mcm2-7 and associated Cdt1 from the nucleus (Nguyen et al. 2000; Tanaka and Diffley 2002), and 3) Cdc6 expression is decreased and phosphorylation of Cdc6 leads to its ubiquitin-dependent degradation (Drury et al. 2000) (Fig. 5). Importantly, these events do not affect pre-existing loaded Mcm2-7. When all of these CDK-dependent events are inhibited, cells can simultaneously load and activate the Mcm2-7 helicase leading to untimely rereplication of their genome resulting in cell death (Nguyen et al. 2001). In contrast to budding yeast, metazoan organisms prevent loading of Mcm2-7 outside of G1 phase by degradation of Cdt1 and expression of the Cdt1 inhibitor, geminin (Zhong et al. 2003; Li et al. 2003; Wohlschlegel et al. 2000; Arias and Walter 2005; Nishitani et al. 2001).

**Origin Activation throughout S Phase**

Upon entry into S phase, not all Mcm2-7 double hexamers at origins initiate simultaneously. Instead, initiation of replication occurs throughout S phase, with each origin exhibiting a characteristic average time of activation within S phase. A fundamental difference between early- and late-initiating origins is their ability to compete for limiting replication factors. Overexpression of these limiting replication-initiation proteins causes early initiation of typically late-initiating origins (Tanaka et al. 2011a; Mantiero et al. 2011). Although the exact determinants remain unclear, there is evidence that both local chromatin states (Ferguson and Fangman 1992; Raghuraman et al. 2001) and proximal
binding of the Forkhead DNA binding protein (Knott et al. 2012; Lõoke et al. 2013) contribute to origin timing. The temporal distribution of origin initiation across S phase is important to complete replication between two convergent replication forks that have both stalled. In this situation, a previously unactivated but licensed origin between the two forks (which would likely have been passively replicated otherwise) can initiate replication to finish duplication of the region between the stalled forks (Fig. 6).

Although there are over 700 potential origins in a budding yeast cell, only a fraction of these origins will initiate DNA replication in any given cell cycle (Santocanale et al. 1999; Vujcic et al. 1999; Feng et al. 2006; Wyrick et al. 2001). The likelihood that a particular origin initiates during a cell cycle is referred to as the efficiency of that origin. In many instances, origin efficiency is correlated with origin timing. Origins that initiate earlier in S phase tend to be more efficient than those that initiate later in S phase. In part, this difference in efficiency is the result of late origins being more likely to be passively replicated by replication forks originating from neighboring early-initiating origins. In addition, as described above, it is clear that early origins compete more effectively for limiting replication-initiation proteins, making initiation from these origins more likely. Nevertheless, there is clearly a stochastic element to replication timing and efficiency. This is most clearly illustrated by single-molecule analysis of the replication of an individual yeast chromosome using DNA combing, a method that reveals whether or not specific origins have initiated (Czajkowsky et al. 2008). These studies found a wide array of patterns of initiation including chromosomes with typically late and inefficient origins.
Figure 6. Replication origin timing
Late origins are activated when converging replication forks stall. (left) Unencumbered converging replication forks from early-firing origins can passively replicate potential origins that have not fired. (right) When converging replication forks stall, the dormant middle origins can be activated to complete duplication of the region between stalled forks.
initiating in the absence of initiation from origins that typically initiate early and are more efficient. Importantly, the average patterns of origin initiation from the individual chromosomes agreed with the origin initiation efficiencies observed in population-based studies (Raghuraman et al. 2001; Yabuki et al. 2002).

**Mcm2-7 Activation**

Several characteristics of Mcm2-7 strongly suggest that it is the central motor of the replication fork helicase. First, Mcm2-7 travels with replication forks during S phase (Aparicio et al. 1997). Second, Mcm2-7 is required throughout DNA replication elongation (Labib et al. 2000; Pacek and Walter 2004). Third, direct biochemical studies show that Mcm2-7 is an AAA+ protein that can use ATP hydrolysis to catalyze DNA unwinding (Bochman et al. 2008).

Although Mcm2-7 is a very weak helicase on its own, Cdc45 and GINS association with Mcm2-7 dramatically stimulates this activity (Ilves et al. 2010). Cdc45 and GINS binding bridges the dynamic Mcm2/5 gate by interacting with the N-terminal domains of Mcm2, Mcm3, and Mcm5 (Zhou et al. 2017; Costa et al. 2011; Yuan et al. 2016; Abid Ali et al. 2016) (Fig. 7). Together, these three proteins form the Cdc45/Mcm2-7/GINS (CMG) helicase (Moyer et al. 2006). The recruitment of these two proteins to the loaded Mcm2-7 helicase at each origin is a key event in helicase activation.
Figure 7. Cdc45/Mcm2-7/GINS active helicase
Cdc45 binds the N-terminal domain of Mcm2, whereas GINS primarily binds the N-terminal domain of Mcm5. These interactions bridge the Mcm2/5 gate. (Costa et al. 2011).
GINS (from the Japanese Go-Inchi-Ni-San meaning 5-1-2-3) is a stable complex of four proteins, Sld5 (Synthetic lethal with dpb11-1 5), Psf1 (Partner of Sld five 1), Psf2, and Psf3 (Takayama et al. 2003; Kubota et al. 2003). This heterotetramer forms a ring with a small central pore (Kubota et al. 2003; Chang et al. 2007). GINS activates Mcm2-7 at origins and remains associated with Mcm2-7 at replication forks (Kanemaki and Labib 2006; Calzada et al. 2005; Pacek et al. 2006). If GINS is inactivated during S phase, further replication fork progression is blocked (Kanemaki et al. 2003). In addition to stimulating the helicase activity of Mcm2-7, GINS indirectly recruits Pol α/primase to the replication fork through an interaction with the Pol α-binding protein, Ctf4 (see DNA synthesis).

Similar to GINS, Cdc45 stimulates Mcm2-7 helicase activity and remains associated with Mcm2-7 throughout replication fork function (Pacek and Walter 2004; Gambus et al. 2006; Kanemaki and Labib 2006; Zou and Stillman 2000; Tercero et al. 2000). Cdc45 contains a DNA interaction domain that is homologous to the bacterial ssDNA exonuclease, RecJ. It has been proposed that this domain enables Cdc45 to interact with single-stranded DNA (ssDNA) generated by the CMG helicase (Petojevic et al. 2015). Given its role in DNA synthesis, it is noteworthy that critical residues necessary for RecJ exonuclease activity are absent in Cdc45 (Pellegrini 2016).

Two kinases and several additional helicase-activating proteins are required to recruit Cdc45 and GINS to form the CMG. In the first stage of helicase activation, DDK promotes
Figure 8

(A) Phosphorylation of Mcm2-7 by DDK stimulates the recruitment of Sld3, Sld7 and Cdc45. (B) CDK phosphorylation of Sld2 results in the formation of the pre-loading complex, consisting of Sld2, Dpb11, GINS and Pol ε. This complex is recruited to Mcm2-7 by interactions between CDK-phosphorylated Sld3 and Dpb11. (C) Mcm10 then associates with the CMG to generate ssDNA that is stabilized by RPA. Dpb11, Sld3, Sld7 and Sld2 are all released during the process of helicase activation.

Figure 8. Activation of the CMG helicase
the association of Cdc45 with loaded Mcm2-7 (Fig. 8A). DDK associates with Mcm2-7 via an interaction with Mcm2 and Mcm4 (Ramer et al. 2013; Sheu and Stillman 2006) and phosphorylates the extended, unstructured N-terminal serine/threonine-rich domains (NSD) of Mcm2, Mcm4, and Mcm6 (Randell et al. 2010; Lei et al. 1997; Weinreich and Stillman 1999; Masai et al. 2006). Deletion of the Mcm4 NSD bypasses the essential activity of DDK, albeit weakly (Sheu and Stillman 2010). This finding suggests that phosphorylation of the NSDs relieves an inhibitory conformation of Mcm2-7. In agreement with DDK phosphorylation stimulating a Mcm2-7 conformational change, another DDK bypass mutant, mcm5-bob1 (Hardy et al. 1997), was inserted into an archaeal homohexameric MCM complex, and this mutation resulted in the rearrangement of the MCM N-terminal domains (Fletcher et al. 2003). Once Mcm2-7 is phosphorylated, Sld3, Sld7, and Cdc45 are recruited to Mcm2-7 through an association of Sld3 with the DDK-phosphorylated N-terminal tails of Mcm4 and Mcm6 (Deegan et al. 2016; Heller et al. 2011). It is unclear how cells with a deletion of the Mcm4 NSD can bind Sld3 and bypass DDK. However, Mcm2-7 is phosphorylated by other kinases (CDK and Mec1) (Randell et al. 2010; Deegan et al. 2016) and these phosphorylation events may be sufficient for Sld3 to function. Sld7 is not essential for cell viability or Cdc45 recruitment to Mcm2-7 but is important for robust replication initiation (Tanaka et al. 2011b). Exactly how Cdc45 recruitment is stimulated by Sld3 binding to phosphorylated Mcm2-7 remains to be determined. These associations occur in the absence of CDK activity and can be observed at a subset of early origins during G1 phase (Tanaka et al. 2011b; Löoke et al. 2013; Aparicio et al. 1997; Heller et al. 2011; Deegan et al. 2016). Although DDK activity
is much lower in G1 compared to S phase, these Cdc45 associations in G1 phase remain DDK-dependent (Tanaka et al. 2011a).

GINS is recruited to Sld3/Sld7/Cdc45-associated Mcm2-7 in a CDK-dependent manner (Fig. 8B). The accumulation of two cyclins, Clb5 and Clb6, triggers entry into S phase by associating with the catalytic subunit of CDK (Cdc28, which is also known as Cdk1) to form S-CDK (Schwob and Nasmyth 1993). S-CDK phosphorylates Sld3 and Sld2, which are the only S-CDK targets that are required for DNA replication initiation (Tanaka et al. 2007; Zegerman and Diffley 2007). Phosphorylated Sld2 binds Dpb11 (DNA polymerase B possible subunit 11). This interaction is mediated by two C-terminal phosphopeptide-binding BRCA1 C-terminal (BRCT) domains in Dpb11 that recognize a specific phosphopeptide in Sld2 (Tak et al. 2006). Although the mechanism is less clear, CDK activity also facilitates the formation of a semi-stable pre-loading complex (pre-LC), consisting of Sld2, Dpb11, GINS and DNA polymerase ε (Pol ε) (Muramatsu et al. 2010). One of the essential scaffolding components in the pre-LC is an interaction between the Dbp2 subunit of Pol ε and GINS (Sengupta et al. 2013). Dbp2 is dispensable for Pol ε in vitro polymerase activity (Isoz et al. 2012) but is essential for cell viability. These findings suggesting that the main function of Dpb2 is aiding in CMG formation and tethering Pol ε to the CMG during replication elongation. Two additional N-terminal BRCT motifs in Dpb11 recognize phosphorylated Sld3 recruiting the pre-LC (including GINS) to Mcm2-7 (Zegerman and Diffley 2007; Tanaka et al. 2007).
Despite the formation of the CMG complex at this stage, DNA unwinding is not initiated until a final Mcm2-7 activation protein, Mcm10, acts (Kanke et al. 2012; Watase et al. 2012; Wohlschlegel et al. 2002; van Deursen et al. 2012) (Fig. 8C). Once activated, the ssDNA that is generated by the CMG is stabilized by the ssDNA binding protein, Replication Protein A (RPA) and serves as the template for the RNA primer and initial DNA synthesis. Of the helicase-activating proteins, only Cdc45, GINS, and Pol ε remain associated with the replication fork. Sld2, Sld3, Sld7, and Dpb11 do not travel with the fork and are released at an unknown stage of helicase activation/replication initiation (Kanemaki and Labib 2006; Gambus et al. 2009; Yu et al. 2014).

Major changes in both the DNA and the Mcm2-7 complex must occur to transition into a replication fork. After helicase loading, two tightly associated Mcm2-7 complexes encircle dsDNA. In contrast, a single Mcm2-7 is present at each replication fork (Moyer et al. 2006; Gambus et al. 2006; Yardimci et al. 2010). Additionally, the CMG helicase travels along ssDNA in the 3’ to 5’ direction on the leading strand template and unwinds dsDNA by occluding the lagging strand template from the center of Mcm2-7 (Fu et al. 2011). To accomplish this transition, the two Mcm2-7 complexes in the double hexamers must separate from one another, the origin DNA needs to be melted, and the Mcm2-7 ring must be reopened to allow opposite strands of the DNA to be extruded from each Mcm2-7 complex (Yardimci et al. 2010; Fu et al. 2011). The order of these events is currently unknown. Although structural studies have captured Mcm2-7 during initial loading and in the CMG complex (Yuan et al. 2016; Li et al. 2015; Abid Ali et al. 2016; Sun et al. 2013;
Georgescu et al. 2017), the conformational changes necessary for DNA unwinding and translocation along DNA are unknown. A proposed Mcm10-dependent conformational change in Mcm2-7 will be described in Chapter II.

Several hypotheses have been proposed for the mechanism of initial DNA unwinding by the CMG (Bochman and Schwacha 2009; Takahashi et al. 2005). One model is that a strand is actively ejected from the center of the Mcm2-7 by its own action or that of another protein (Fig. 9A). This model posits that the CMG sterically occludes one strand at the onset of DNA unwinding (Kaplan et al. 2003). In a second model, known as the rotary pump model, the CMG helicases rotate the dsDNA in opposite directions causing a rotational stress and ssDNA formation (Laskey and Madine 2003) (Fig. 9B). A third model, based on electron microscopy studies of the T-antigen helicase function (Wessel et al. 1992), suggests that the double hexamers pump dsDNA towards the interface between the two helicases resulting in ssDNA generation (Fig. 9C). Finally, the ploughshare model (Takahashi et al. 2005) proposes that the CMG moves on dsDNA and a protein wedge behind the CMG splits the two DNA strands apart after the DNA passes through the CMG (Fig. 9D). Regardless of the mechanism of initial DNA unwinding, the lagging strand template must be extruded from the center of Mcm2-7 to unwind dsDNA by steric exclusion during replication elongation (Fu et al. 2011).

Although the configuration of loaded Mcm2-7 double hexamers is known (Evrin et al. 2009; Remus et al. 2009), the directionality of CMG movement has not been resolved.
Figure 9. Models for initial DNA unwinding

(A) Steric exclusion. DNA is unwound by extruding one strand from the central channel and is blocked from re-entering the channel during translocation. (B) Rotary pump. The CMG rotates dsDNA in opposite directions to create torque and generate ssDNA. (C) dsDNA pump. DNA is pumped into the interface of the double hexamer and ejects ssDNA. (D) Ploughshare. ssDNA is generated by protein that inserts itself between dsDNA to pry the two strands apart. (Bochman and Schwacha, 2009).
One hypothesis for the polarity of the CMG movement is that interactions at the N-termini of Mcm2-7 double hexamers are broken and individual CMG complexes travel in opposite directions with the C-terminus leading DNA unwinding. This model is supported by structural studies of *Drosophila melanogaster* CMG helicases in complex with a DNA substrate that has a biotin/streptavidin at one end to provide a frame of reference (Costa et al. 2014). In contrast, a structure of the *S. cerevisiae* CMG with a similar DNA fork substrate showed that the N-terminal face of the CMG is leading unwinding (Georgescu et al. 2017). In this model, activation of the Mcm2-7 double hexamer would result in the two CMG complexes passing each other on opposite strands. It is unlikely that these two species unwind DNA with different CMG polarity due to the high sequence conservation of the proteins involved. Further biochemical and structural studies will be needed to resolve these conflicting reports.

**Mcm10**

Mcm10 was identified in the same screen as three of the six Mcm2-7 subunits (Maine et al. 1984) leading to its similar name. Despite this, Mcm10 bears no sequence or functional homology to Mcm2-7 subunits. The protein consists of an N-terminal coiled-coiled domain, an internal domain (ID) harboring a OB-fold and a putative PCNA-interacting peptide (Das-Bradoo et al. 2006), and a poorly-conserved C-terminal domain (Robertson et al. 2008). Because Mcm10 does not have any known enzymatic functions, it most likely acts by tethering together or stimulating the activity of other proteins.
Although the importance of Mcm10 for helicase activation is clear, how Mcm10 contributes to CMG activation is not. Recruitment of Cdc45 or GINS to origins is independent of Mcm10, but Mcm10 is required for CMG helicase activation. Cells lacking Mcm10 activity show defects in RPA recruitment to unwound origin DNA (Kanke et al. 2012; Wohlschlegel et al. 2002; van Deursen et al. 2012; Watase et al. 2012; Yeeles et al. 2015) and separation of the Mcm2-7 double hexamer (Quan et al. 2015). The C-terminal region of Mcm10 preferentially binds to Mcm2, Mcm4, and Mcm6 subunits of Mcm2-7, and deletion of the Mcm10 C-terminus delays Mcm2-7 double-hexamer separation (Douglas and Diffley 2016; Quan et al. 2015). Mcm10 also travels with replisome progression complex during S-phase (Gambus et al. 2006; Ricke and Bielinsky 2004), although the biological relevance of this interaction is unclear.

Mcm10 interacts with ssDNA and several replication proteins but how these interactions contribute to Mcm10 function remains to be determined. Biochemical and structural studies support Mcm10 binding ssDNA (Warren et al. 2009; Robertson et al. 2008; Di Perna et al. 2013), but in vivo experiments supporting this function have not been performed. In addition, Mcm10 interacts with Pol α/primase, PCNA, and Mec3 (a DNA damage checkpoint protein) under certain conditions. The Mcm10/Pol α interaction implicates Mcm10 in tethering Pol α to the replisome. Elimination of the Mec3- and PCNA-interacting regions of Mcm10 caused mild growth defects (Das-Bradoo et al. 2006; Alver et al. 2014). It is presently unclear how any of these interactions are connected to the
function of Mcm10 during DNA replication, but could provide a mechanism for regulating these proteins at the replication fork.

**DNA Synthesis**

Once ssDNA has been generated by the activated CMG, two additional polymerases, along with accessory proteins, are recruited to unwound origin DNA to initiate DNA synthesis (Gambus et al. 2009; Heller et al. 2011; Walter and Newport 2000). DNA synthesis is initiated by a RNA-DNA primer synthesized by the DNA Pol α/primase holoenzyme. Because the DNA strands are antiparallel and DNA synthesis is semi-conservative (duplication of the genome results in dsDNA containing one parental and one nascent strand) (Meselson and Stahl 1958), only one of the strands can be synthesized continuously with fork movement. The strand that is synthesized continuously from a single RNA priming event is known as the leading strand and is synthesized by Pol ε, whereas the lagging strand is synthesized in a discontinuous fashion and is synthesized by Pol δ (Fig. 10). The lagging strand requires multiple RNA priming events resulting in 150-250 base pair units, known as Okazaki fragments (Smith and Whitehouse 2012; Georgescu et al. 2015; Ishimi et al. 1988; Okazaki et al. 1968). In budding yeast, replication forks synthesize DNA at a rate of ~1.7 kilobase pairs per minute (Sekedat et al. 2010; Hodgson et al. 2007) allowing cells to finish genome duplication in ~20 minutes under optimal conditions.
Figure 10. Eukaryotic DNA replication fork
DNA synthesis begins after intial CMG unwinding. Pol $\alpha$ synthesizes a short RNA/DNA primer, before Pol $\varepsilon$ begins synthesis on the leading strand and Pol $\delta$ on the lagging strand. RPA stabilizes the exposed ssDNA. PCNA, loaded by RFC, stimulates the processivity of the polymerases. (Burgers, 2009).
Pol α/primase plays a critical role during eukaryotic DNA replication initiation. The primase component of the Pol α/primase holoenzyme synthesizes the essential RNA primer, which is rapidly extended by the Pol α DNA polymerase component (Kuchta et al. 1990). The resulting RNA-DNA is only 20-25 nucleotides in length due to the limited processivity of each enzyme (Santocanale et al. 1993). Unlike the other two replicative DNA polymerases, Pol α lacks a proofreading exonuclease. However, current models suggest that the RNA-DNA primer synthesized by Pol α is removed and replaced by Pol δ synthesized DNA (see below). An accessory protein, Ctf4 (Chromosome transmission fidelity 4), localizes Pol α/primase to the CMG complex by forming a homotrimer that simultaneously binds GINS and Pol α/primase. These interactions are mediated by conserved Ctf4-interacting peptides in the Sld5 subunit of GINS and the Pol1 catalytic subunit of Pol α/primase (Gambus et al. 2009; Simon et al. 2014). Interestingly, Ctf4 is not essential (Miles and Formosa 1992) suggesting that there are other mechanisms that can recruit Pol α/primase to the replication fork.

Stand-specific mutation-rate analysis and chromatin immunoprecipitation studies strongly support the hypothesis that Pol ε synthesizes the leading strand, whereas Pol δ synthesizes the lagging strand (Nick McElhinny et al. 2008; Yu et al. 2014; Pursell et al. 2007). Both Pol δ and Pol ε contain 3’ to 5’ exonucleases that increase the fidelity of DNA replication by ~100-fold (Pavlov et al. 2001). As described above, Pol ε is required for CMG formation, and this places Pol ε in an ideal position to synthesize the leading strand as soon as ssDNA is generated by the CMG complex (Sengupta et al. 2013). In contrast,
Pol δ does not associate with the CMG (Gambus et al. 2009). Instead, Pol δ is thought to act independently from the replication fork. The processivity of Pol δ is enhanced by associating with a processivity clamp, PCNA (Proliferating Cell Nuclear Antigen), which is loaded onto DNA by the clamp loader, RFC (Replication Factor C). Although PCNA is capable of stimulating both Pol ε and Pol δ in vitro, Pol δ is stimulated to a greater degree and has a higher affinity for PCNA (Chilkova et al. 2007).

In addition to synthesizing the lagging strand, Pol δ also participates in repair of Okazaki fragments to create a continuous lagging strand DNA product. This process requires removal of the RNA-DNA primers, replacement with DNA, and ligation of Okazaki fragments (Fig. 11). When Pol δ encounters an RNA-DNA primer of the adjacent Okazaki fragment, it continues DNA synthesis by displacing the primer. As the RNA-DNA primer is displaced, it is cleaved by the Flap endonuclease, Fen1. Reiterative cycles of RNA/DNA displacement and Fen1 cleavage continues until a DNA-DNA ligation can be made by DNA ligase I (Turchi and Bambara 1993; Johnston and Nasmyth 1978; Garg et al. 2004; Stith et al. 2008). When the displaced flap becomes ~30 base pairs, RPA binds and inhibits Fen1 cleavage (Bae et al. 2001). In this case, Dna2 cleaves most of the long flap leaving a short flap that is subsequently processed by Fen1 (Bae and Seo 2000). It is currently unclear how much DNA beyond the RNA primer is replaced with Pol δ synthesized DNA. Although, the amount of DNA replaced has been proposed to be influenced by the presence of nucleosomes, with Pol δ reaching no farther than the midpoint of the nucleosomal DNA (Smith and Whitehouse 2012). An additional pathway
Figure 11. Removal of Pol $\alpha$ synthesized RNA-DNA primer
Pol $\delta$ is capable of displacing the RNA-DNA primer synthesized by Pol $\alpha$/primase. Once a small flap of ssRNA or ssDNA has been generated, Fen1 cleaves the flap. This cycle is repeated until the RNA is removed and DNA ends can be ligated by DNA ligase I. When the displaced RNA/DNA becomes longer, Dna2 first cleaves the resulting long flap before Fen1 cleaves the Dna2-cleaved flap. The RNA-DNA primer can also be removed through the action of RNase H and Exol. (Liu et al. 2017).
for removal of the RNA-DNA primers has also been observed involving RNase H and a DNA exonuclease, Exo1 (Liu et al. 2017) (Fig. 11). The distribution of each of these pathways for Okazaki fragment maturation remains unresolved.

Addition accessory proteins are localized to the replication fork to aid in various functions associated with faithful DNA replication (On et al. 2014; Gambus et al. 2009; 2006; Yu et al. 2014). Topoisomerases I and II (Top1 and Top2) are important for relieving the topological strain induced by unwound DNA by the CMG helicase (Kim and Wang 1989). Top1 is not essential, whereas Top2 is required for decatenation of chromosomes after DNA replication (Holm et al. 1985; Thrash et al. 1985). The histone chaperone, FACT, is important for navigating nucleosomes during DNA replication (Kurat et al. 2016; Wittmeyer and Formosa 1997). Mrc1 associates with the replisome and contributes to normal replisome speed (Yeeles et al. 2016; Hodgson et al. 2007). A complex of Mrc1, Tof1, and Csm3 is important for activation of the replication checkpoint during DNA damage (Katou et al. 2003; Dalgaard and Klar 2000; Alcasabas et al. 2001).

Biochemical Studies of DNA Replication

Although genetic studies were critical for determining the essential proteins involved in DNA replication and the general consequences of their loss (Maine et al. 1984; Araki et al. 1995; Kamimura et al. 1998; 2001; Hennessy et al. 1991; Takayama et al. 2003), developing biochemical assays to study this process has been vital in determining the mechanistic details controlling DNA replication. Because cells tightly regulate the
oscillation between the potential for loading and activating Mcm2-7 to ensure DNA replication occurs only once per cell cycle, creating \textit{in vitro} assays to study this process has required mimicking these cell cycle transitions. Over time, the role of CDK and the necessary proteins became clearer and led to the development of fully reconstituted assays, which are more amenable to studying the mechanism of DNA replication initiation.

As our knowledge of DNA replication has evolved, so too have the biochemical studies of this process. One of the first biochemical DNA replication studies utilized unfertilized \textit{Xenopus laevis} frog eggs as a rich source of DNA replication proteins. Purified DNA was replicated by injecting it into the frog eggs, along with radioactive deoxythymidine to detect replication of the injected DNA (Gurdon et al. 1969). A cell-free system was later developed by making extracts from Simian Virus 40 (SV40) infected monkey cells. Several of the DNA polymerases and accessory replication factors were first shown to contribute to replication of SV40-origin-containing plasmids using this system (Stillman 1989)). On the other hand, the multifunctional SV40 large T-antigen bypasses the function of many replication proteins, most notably the replicative helicase and the initiator, and SV40 DNA replication is not cell cycle regulated. Thus, while this assay resulted in the identification of a number of elongation proteins, it could not be used to study initiation or the function of the replicative helicase.
The development of a replication assay that biochemically separated origin licensing from activation using a nucleus-free assay opened a new era in in vitro replication studies (Walter et al. 1998). All previous cell-free DNA replication assays relied on the presence nuclear membranes to replicate DNA (Newport 1987; Blow and Laskey 1986; Lohka and Masui 1983). This nucleus-free system replicated DNA by sequential incubations of Xenopus egg extracts that mimicked the G1 and S phase states. Incubation of plasmid DNA with a G1-phase-like cytosolic egg extract was necessary for loading Mcm2-7. Addition of an S-phase-like nucleoplasmic extract activated the helicase to initiate DNA replication (Walter et al. 1998). These studies eliminated a then-prominent model that nuclear structures were required for eukaryotic DNA replication. Using this system, the protein requirements for DNA unwinding and the order of kinase function during helicase activation were determined (Walter and Newport 2000; Walter 2000; Wohlschlegel et al. 2002).

More recently, a similar extract-based assay for DNA replication was developed using budding yeast and was dependent on defined origins of replication (Heller et al. 2011). Incubations with G1-phase and subsequently, S-phase extract from yeast resulted in origin-dependent DNA replication. In this assay, the DNA was coupled to magnetic beads to isolate DNA-associated proteins and determine the order of events during activation of the helicase and association of DNA polymerases (Heller et al. 2011).
Although useful in aiding in our understanding of the order of events during helicase
loading activation, the study of mutant proteins and manipulation of protein extracts (e.g.
protein depletion) can be difficult. Thus, efforts have been made to reconstitute replication
initiation and elongation with purified proteins. A completely reconstituted assay to
monitoring loading of Mcm2-7 using purified ORC, Cdc6, and Mcm2-7/Cdt1 has aided in
our understanding of the details of Mcm2-7 loading, such as the role of ATP hydrolysis
(Remus et al. 2009; Evrin et al. 2009; Coster et al. 2014; Kang et al. 2014). In addition,
this reconstituted assay has led to single-molecule studies of Mcm2-7 loading, which have
provided insight into the stoichiometry of proteins, the dynamics of protein associations,
and the mechanism of Mcm2-7 ring opening and closing around DNA (Ticau et al. 2017;
2015; Duzdevich et al. 2015).

Recently, Mcm2-7 activation and replication elongation were reconstituted with a minimal
set of purified proteins (Yeeles et al. 2015). Purified helicase-activating proteins, DNA
polymerases, and replication-fork proteins were added to loaded Mcm2-7 helicases to
initiate DNA replication. Importantly, this assay has many hallmarks of in vivo DNA
replication, including dependence on prior Mcm2-7 loading, both S-phase kinases (DDK
and CDK), and all essential helicase-activating proteins. Using this assay, Mrc1, Tof1,
and Csm3 were shown to be necessary for a replisome progression speed that is
comparable to rates measured in vivo (Yeeles et al. 2016). In addition, chromatinized
DNA templates were utilized to determine the effect of nucleosomes on lagging strand
synthesis products and how chromatin-associated proteins, such as FACT, affect DNA
replication (Kurat et al. 2016; Devbhandari et al. 2017). In chapter II, a modified version of this assay will be used to study the essential function of Mcm10 during DNA replication.
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Chapter II

Mcm10 Regulates DNA Replication Elongation by Stimulating the CMG Replicative Helicase

An earlier version of this chapter was published as referenced below.


*indicates equal contribution. I purified the replication proteins and developed/performed the reconstituted replication assays. M.L. generated Mcm10 and Mcm2 mutants and performed dilution plating and FACS analysis.
ABSTRACT

Activation of the Mcm2-7 replicative DNA helicase is the committed step in eukaryotic DNA replication initiation. Although Mcm2-7 activation requires binding of the helicase-activating proteins, Cdc45 and GINS (forming the CMG complex), an additional protein, Mcm10, drives initial origin DNA unwinding by an unknown mechanism. We show that Mcm10 binds a conserved motif located between the OB-fold and A subdomain of Mcm2. Although buried in the interface between these domains in Mcm2-7 structures, mutations predicted to separate the domains and expose this motif restore growth to conditional-lethal *MCM10* mutant cells. We found that in addition to stimulating initial DNA unwinding, Mcm10 stabilizes Cdc45 and GINS association with Mcm2-7 and stimulates replication elongation *in vivo* and *in vitro*. Furthermore, we identify a lethal allele of *MCM10* that stimulates initial DNA unwinding but is defective in replication elongation and CMG binding. Our findings expand the roles of Mcm10 during DNA replication and suggest a new model for Mcm10 function as an activator of the CMG complex throughout DNA replication.
INTRODUCTION

Eukaryotic DNA replication initiation requires the sequential assembly of protein complexes at origins of replication. In G1 phase, the Mcm2-7 replicative helicase is loaded onto double-stranded DNA (dsDNA) as a head-to-head double hexamer in an inactive state (Evrin et al. 2009; Ticau et al. 2017; 2015; Remus et al. 2009). As cells progress into S phase, two kinases, S-phase cyclin-dependent kinase (S-CDK) and the Dbf4-dependent Cdc7 kinase (DDK), promote the association of two helicase activators, Cdc45 and GINS, with Mcm2-7. DDK phosphorylation of Mcm2-7 stimulates the association of Cdc45, Sld3, and Sld7 (Deegan et al. 2016; Heller et al. 2011) followed by the S-CDK-dependent recruitment of a complex between Sld2, Dpb11, DNA polymerase ε (Pol ε), and GINS (Muramatsu et al. 2010; Yeeles et al. 2015; Tanaka et al. 2007; Zegerman and Diffley 2007). Cdc45 and GINS association with Mcm2-7 forms the replicative DNA helicase, the Cdc45/Mcm2-7/GINS (CMG) complex (Moyer et al. 2006; Ilves et al. 2010), but initial DNA unwinding by this assembly and commitment to replication initiation require Mcm10 (van Deursen et al. 2012; Kanke et al. 2012; Watase et al. 2012). The resulting single-stranded DNA (ssDNA) facilitates recruitment of the remaining DNA synthesis machinery (Heller et al. 2011).

The process of helicase activation requires the loaded Mcm2-7 double hexamer and its associated DNA to undergo major conformational changes. Initially, Mcm2-7 double hexamers encircle dsDNA (Remus et al. 2009; Evrin et al. 2009). In contrast, activated CMG complexes at replication forks contain a single Mcm2-7 complex and encircle
ssDNA (Fu et al. 2011; Yardimci et al. 2012; Sun et al. 2015; Georgescu et al. 2017). Structural studies have captured Mcm2-7 at multiple stages during helicase loading and in the CMG complex (Yuan et al. 2016; Li et al. 2015; Abid Ali et al. 2016; Sun et al. 2013; Georgescu et al. 2017). These structures have provided important insights into Mcm2-7 loading and the interactions of Mcm2-7 with Cdc45 and GINS. Nevertheless, the Mcm2-7 conformational changes necessary for DNA unwinding are controversial (Yuan et al. 2016; Abid Ali et al. 2016; Georgescu et al. 2017), and the events that drive the transition from the initially-loaded, inactive Mcm2-7 double hexamer to the activated CMG complex are poorly understood.

Although the general consequences of Mcm10 loss are understood, how it activates the CMG complex to initiate DNA unwinding is unclear. Recruitment of Cdc45 or GINS to Mcm2-7 is independent of Mcm10 (van Deursen et al. 2012; Watase et al. 2012; Kanke et al. 2012; Yeeles et al. 2015). In contrast, Mcm10 is required for initial DNA unwinding at origins of replication (van Deursen et al. 2012; Watase et al. 2012; Kanke et al. 2012; Yeeles et al. 2015) and has been implicated in the separation of the Mcm2-7 double hexamer (Quan et al. 2015). It is possible that the double hexamer of the Mcm2-7 complex inhibits DNA unwinding, and Mcm10 activates unwinding by causing double-hexamer separation (Quan et al. 2015). Alternatively, Mcm10 could facilitate extrusion of ssDNA from the Mcm2-7 central channel, enabling the transition from Mcm2-7 encircling dsDNA to ssDNA (Costa et al. 2014). Finally, Mcm10 binding could directly activate CMG DNA unwinding, indirectly leading to the separation of the Mcm2-7
Several lines of evidence suggest that Mcm10 acts by interacting with Mcm2-7. Although unrelated to the Mcm2-7 proteins, Mcm10 binds to the Mcm2, Mcm4, and Mcm6 subunits of Mcm2-7 (Quan et al. 2015; Douglas and Diffley 2016). In addition, genetic studies suggest an important interaction between Mcm10 and Mcm2 (Lee et al. 2010; Homesley et al. 2000; Apger et al. 2010). Finally, Mcm10 associates with the replisome under certain conditions (Gambus et al. 2006; Ricke and Bielinsky 2004), although the biological significance of this interaction is unclear. Despite these observations, a specific Mcm10-binding site has not been identified on any Mcm2-7 subunit.

In this study, we use a combination of molecular genetics and reconstituted DNA replication assays to investigate Mcm10 function. Using Mcm2-Mcm10 interaction data, we identify a conserved Mcm10-binding motif in Mcm2. Although obscured in current Mcm2-7 structures, mutants designed to expose the Mcm10-binding motif bypassed conditional-lethal MCM10 mutations. Consistent with a direct effect of Mcm10 binding on Mcm2-7, Mcm10 stabilized Cdc45 and GINS association with Mcm2-7. Additionally, we observe that Mcm10 stimulated replication elongation both in vivo and in vitro and characterize an Mcm10 separation-of-function mutant that is specifically defective in this elongation function. Our findings expand the roles of Mcm10 and illuminate its mechanism of function.
RESULTS

Mcm10 binds a conserved region in the Mcm2 N-terminal domain

During Mcm10 purification, we observed three co-purifying proteins. Two of the proteins co-migrated with Mcm6 and Mcm4 during SDS-PAGE (Fig. 1A). Consistent with recent findings (Douglas and Diffley, 2016; Quan et al., 2015), mass spectrometry confirmed these proteins were Mcm4 and Mcm6 and identified Mcm2 as the third protein (Fig. 1B).

To understand the target of Mcm10 in more detail, we sought to identify the binding site for Mcm10 on Mcm2, Mcm4, or Mcm6. Consistent with prior studies that identified MCM2 mutants as suppressors of mcm10-1 (Lee et al. 2010), we found that Mcm10 showed robust interactions with Mcm2 and much weaker interactions with Mcm4 and Mcm6 (Fig. 2A). No binding to Mcm5 or Mcm7 was detected. Thus, we focused on localizing the strong Mcm10 binding site on Mcm2. All Mcm2-7 subunits include three folded domains, the A subdomain, the oligonucleotide/oligosaccharide-fold (OB-fold) and the C-terminal AAA+ ATPase domain (Li et al. 2015). Testing Mcm10 binding to truncated forms of Mcm2 (Fig. 2B and 2C) showed that the A subdomain, but not the OB-fold or AAA+ domains, bound Mcm10. Importantly, Mcm10 binding to Mcm2 required residues 290-299 of the A subdomain, and mutating these residues in a larger Mcm2 fragment eliminated Mcm10 binding (Fig. 2C, lanes 19 and 20). Importantly, the region of Mcm2 bound by Mcm10 (referred to here as the Mcm10-binding motif) is highly conserved across eukaryotic species (Fig. 2Di), but is absent in the other Mcm2-7 subunits (Fig. 2Dii), strongly suggesting that Mcm10 binding to Mcm2 is conserved.
Figure 1

(A) Purified Mcm2-7 (lane 1) or indicated eluates during Mcm10-FLAG purification were separated by SDS-PAGE and stained with Coomassie. Treatment of the anti-FLAG eluate with λ-phosphatase resolved three proteins in an equimolar ratio. The middle protein migrated more slowly after λ-phosphatase treatment, a characteristic of Mcm2 dephosphorylation. (B) Mass spectrometry analysis of the top ten Mcm10-FLAG-co-purifying proteins with the highest enrichment.
Figure 2

A

Mcm2-7

Mcm10-FLAG

B

Mcm2

N-terminal tail

A-subdomain

OB-fold

MBP

C

Input

IP Flag

IP Mcm10-FLAG

Input

MBP

MBP

MBP

MBP

mmb

D

i

MCM2

S. cer

S. pom

D. mel

X. lae

M. mus

H. sap

ii

S. cer

MCM3

MCM4

MCM5

MCM6

MCM7

E

MCM2 allele

-URA

5-FOA

290-299 sequence

MCM2

mcm2-mbm

mcm2- Δ290-299

HSEIHVRISD

SAKISVEIAK
Figure 2. Mcm10 binds to a highly-conserved region of Mcm2

(A) Mcm10 preferentially binds Mcm2. Purified Mcm10-FLAG was incubated with individual purified Mcm subunits followed by anti-FLAG immunoprecipitation (IP) and separated by SDS-PAGE and stained with Krypton (lanes 6-10). Control IPs lacking Mcm10-FLAG (lane 1-5), the equivalent amounts of Mcm2-7 subunits added to the IPs (lanes 11-15) and purified Mcm2-7 (lane 16) were separated on the same gel. (B) Diagram of Mcm2 domain structure and the truncations used in this study. For each truncated protein, the included amino acids and epitope tag used for purification are indicated. (C) Mcm10 binding requires the linker region between the A subdomain and the OB-fold of Mcm2. Purified Mcm2 truncations were tested for co-IP with FLAG-Mcm10-V5 followed by separation by SDS-PAGE and Coomassie staining (lanes 11-20). The equivalent amounts of the Mcm2 truncation proteins added to the co-IP experiments were similarly analyzed (lanes 1-10). (D) The Mcm10-binding motif on Mcm2 is conserved across eukaryotes but not in other Mcm2-7 subunits. (i) Alignment of the Mcm10-binding motif of Mcm2 for *Saccharomyces cerevisiae* (*S. cer*), *Schizosaccharomyces pombe* (*S. pom*), *Drosophila melanogaster* (*D. mel*), *Xenopus laevis* (*X. lae*), *Mus musculus* (*M. mus*), *Homo sapiens* (*H. sap*). (ii) Alignment of the Mcm10-binding motif of *S. cer* Mcm2-7 subunits. Limited homology between the Mcm10-binding motif in Mcm2 and Mcm4 is indicated. (E) The Mcm10-binding motif of Mcm2 is essential. In all strains, the endogenous *MCM2* gene is deleted and a copy of WT *MCM2* is present on a *URA3*-containing plasmid. *MCM2* mutants that eliminated (*mcm2-Δ290-299*) or mutated (*mcm2-mbm*) the Mcm10-binding motif were integrated into the *LEU2* locus. Growth on -URA media retains WT *MCM2* and indicates that the mutants are not dominant. Growth on 5-FOA selects against cells containing WT *MCM2* plasmid revealing the functionality of *mcm2-Δ290-299* or *mcm2-mbm* alleles. Five-fold serial dilutions of cells were grown on the indicated media for 3 days at 30°C.
We next tested the importance of this Mcm10-binding motif for Mcm2 function. When present as the only copy of the MCM2 gene, yeast strains lacking (mcm2-Δ290-299) or with substitution mutations (mcm2-mbm) in the Mcm10-binding motif in Mcm2 showed strong growth defects or cell death, respectively (Fig. 2E, 5-FOA panel). These mutations are not dominant as normal cell growth is detected when wild-type (WT) MCM2 is also present (Fig. 2E, -URA).

Disrupting interactions in the Mcm2 N-terminal domain bypasses Mcm10 depletion

The structures of the initially-loaded Mcm2-7 complex (Li et al. 2015) and the CMG complex (Yuan et al. 2016) showed that the Mcm10-binding motif in Mcm2 is buried between the A subdomain and the OB-fold of Mcm2, restricting the accessibility of these residues (Fig. 3A). This finding suggests that either Mcm10 captures or induces the displacement of the A subdomain to access the Mcm10-binding motif. To investigate the importance of the interaction between the Mcm2 OB-fold and A subdomain, we generated mutants at the interface between these domains (Fig. 3B). Each of these alleles was viable when present as the only copy of MCM2 (Fig. 3C).

To test the hypothesis that Mcm10 displaces the Mcm2 A subdomain, we asked if the mutations at the interface of the Mcm2 OB-fold and A subdomain complemented the lethal depletion of Mcm10 from the nucleus. We used the anchor-away method (Haruki et al., 2008) to deplete Mcm10 linked to a rapamycin-binding protein (Mcm10-FRB) from
Figure 3

(A) The Mcm10-binding motif of Mcm2 is buried in the absence of Mcm10. (Left) The cryo-EM structure of the CMG complex (PDB: 3JC5, Yuan et al., 2016). (Right) Space-filling representation of Cdc45, Mcm2 A subdomain and OB-fold. Residues mutated in the Mcm2-mbm mutant are shown in red. (B) Ribbon diagram of the Mcm2 A subdomain (cyan) and OB-fold domain (blue). The residues predicted to be involved in the A subdomain/OB-fold interaction and mutated in (C) and (D) are labeled. (C) Viability of MCM2 mutants predicted to disrupt the A subdomain/OB-fold interaction. The indicated MCM2 mutants were tested for complementation of a MCM2 deletion (5-FOA). Growth on CSM media retains WT MCM2. Five-fold serial dilutions of cells were grown on the indicated media for 3 days at 30°C. (D) mcm2-bom1 and mcm10-bom2 bypass the lethal depletion of Mcm10-FRB. Genetic complementation of the Mcm10 anchor-away phenotype by indicated alleles of MCM2 or MCM10. Cells were spotted and grown as in (B).
the nucleus (Fig. 3D, top). Importantly, yeast strains containing mcm10-FRB showed rapamycin-dependent cell death that was rescued by expressing WT MCM10 (Fig. 3D, rows 1 and 2). For most of the MCM2 mutants, depletion of Mcm10 from the nucleus remained lethal. Remarkably, two of the mutants (mcm2-bom1 [bypass of Mcm10] and mcm2-bom2) restored viability to cells depleted of Mcm10 (Fig. 3D). Supporting the hypothesis that disrupting the OB-fold/A subdomain interaction complements Mcm10 depletion, the residues mutated in Mcm2-bom1 and Mcm2-bom2 are located opposite from one another on the OB-fold and A subdomain, respectively.

To further explore the ability of the mcm2-bom1 and mcm2-bom2 alleles to bypass Mcm10 function, we tested two other MCM10 conditional-lethal alleles, mcm10-1 (Homesley et al. 2000; Merchant et al. 1997) and mcm10-1td (van Deursen et al. 2012) and a complete MCM10 deletion (∆mcm10). Under conditions that are lethal for mcm10-1 and mcm10-1td, we found that mcm2-bom1 or mcm2-bom2 restored cell viability (Fig. 4A and 4B). Despite restoring growth to the conditional-lethal alleles, ∆mcm10 could not be bypassed by either mcm2-bom1 or mcm2-bom2 (Fig. 4C). The inability to bypass ∆mcm10 suggests that mcm2-bom1 and mcm2-bom2 require Mcm10, but at much lower levels than WT MCM2 (see Discussion). Consistent with this hypothesis, further depletion of Mcm10-1td protein (by induction of the Ubr1 protein) led to reduced growth rates in the presence of mcm2-bom1 and mcm2-bom2 (compare Fig. 4Bi and 4Bii). Nevertheless, the ability to rescue multiple conditional-lethal alleles of MCM10 by mutating the Mcm2 OB-fold and A subdomain interface strongly supports the conclusion
**Figure 4. Viability of MCM2 mutants with MCM10 conditional-lethal alleles**

Five-fold serial dilutions of yeast cells were spotted on the indicated plates and grown for 3 days. (A) Complementation of *mcm10-1* lethality. The viability of *mcm10-1* strains with WT MCM2, mcm2-bom1, or mcm2-bom2 was at 25°C and 37°C. (B) Complementation of *mcm10-1td* lethality. The viability of *mcm10-1td* strains with WT MCM2, mcm2-bom1, or mcm2-bom2 was tested at 25°C and 37°C. (i) YP-glucose. E3 ubiquitin ligase, *UBR1*, expression is OFF. (ii) YP-galactose. *UBR1* expression is ON. (C) Complementation of Δ*mcm10* lethality. The viability of Δ*mcm10* strains with WT MCM2, mcm2-bom1, or mcm2-bom2 was tested. The WT copy of *MCM10* is present on a *URA3*-containing plasmid and is selected against on 5-FOA-containing media.
that binding to this region of Mcm2 is critical for Mcm10 function.

**Mcm10 stabilizes the CMG complex**

We used a modified reconstituted DNA replication assay (Yeeles et al. 2015) to further investigate the mechanism and importance of the Mcm10-Mcm2 interaction during DNA replication. To mimic the *in vivo* order of replication events, we sequentially incubated subsets of purified replication proteins (Fig. 5A) with a replication-origin-containing, circular DNA template coupled to magnetic beads. This assay has many hallmarks of eukaryotic DNA replication, including dependence on the S-CDK and DDK kinases and all of the helicase-activating proteins (Fig. 5B). The polymerases and accessory DNA replication proteins used in the assay were also shown to be functional (Fig. 5C and 5D).

We initially assessed the requirements for Mcm10 binding during CMG formation, as it is controversial whether only Mcm2-7 loading (van Deursen et al. 2012; Wohlschlegel et al. 2002; Karnani and Dutta 2011) or full CMG formation (Kanke et al. 2012; Watase et al. 2012; Heller et al. 2011; Douglas and Diffley 2016) is required for recruitment of Mcm10 to origin DNA. We assembled CMG complexes using a simplified assay involving three steps: Mcm2-7 loading, DDK phosphorylation and CMG formation (Fig. 6A). Pol α, Pol δ and all nucleotides except ATP were omitted from the final step, allowing CMG formation and activation (Fig. 6B) but preventing DNA synthesis. We measured Mcm10 association with the DNA template after each step of the assay. Mcm10 did not associate with DNA alone or with loaded Mcm2-7 in the absence of
Figure 5

(A) Purified DNA replication proteins. The indicated purified protein preparation was separated by SDS-PAGE and stained with Coomassie. (B) DNA replication assay is dependent on essential replication proteins. Pol δ, PCNA, Ctf4, and RFC were omitted from these reactions. Assays were performed in the absence of the indicated proteins. (C) RFC/PCNA stimulates Pol δ DNA synthesis. DNA synthesis by Pol δ and PCNA with or without RFC was initiated from an oligo annealed to circular M13 ssDNA. The products were separated on a 0.7% native gel. (D) Replication fork-associated proteins effect DNA replication products. Assays were performed in the absence of the indicated proteins.
Figure 6

Figure 6. Mcm10 addition stabilizes the CMG complex

(A) Mcm10 preferentially associates with CMG complexes. Reaction scheme for CMG formation assay (left). The indicated purified proteins were sequentially incubated with ARS1-containing 3.7 kb plasmids coupled to magnetic beads. The previous reaction mix was removed prior to addition of the next without washing the beads. DNA beads or the indicated DNA-associated complexes formed at the end of each incubation were incubated with the indicated amount of Mcm10 for 1 hour (right). Bead-associated proteins were washed with low-salt buffer and detected by immunoblot.

(B) Mcm10-dependent formation of salt-stable CMG complexes. CMG formation was performed as in (A) except after the final incubation the reactions were washed with low-salt (LSW; 0.3 M KGlut) or high-salt (HSW; 0.5 M NaCl) containing buffers. Assays were performed in the presence and absence of Mcm10 or DDK as indicated (right). Omission of DDK was used as a control for non-specific DNA binding of Cdc45, GINS, RPA, and Pol ε (Mcm2-7 loading is DDK-independent).

(C) Salt-stable CMG complexes
are competent for DNA replication. The reaction scheme is illustrated (left). After CMG formation, as in (A), the DNA beads were washed with the indicated buffer followed by addition of the indicated proteins and [α-32P]-dCTP. Where indicated, Mcm10 and DDK were omitted during both CMG formation and DNA replication. Replication products were separated on a 1% alkaline agarose gel and imaged using a phosphorimager.
DDK-treatment (Fig. 6A). DDK phosphorylation of loaded Mcm2-7 resulted in detectable Mcm10 binding but only at high Mcm10 concentrations. Importantly, Mcm10 showed ~10-fold higher affinity for the CMG complex relative to DDK-phosphorylated Mcm2-7 (Fig. 6A). Thus, both DDK-phosphorylation of Mcm2-7 and CMG complex formation contribute to Mcm10 recruitment.

We next evaluated the role of Mcm10 in CMG formation and activation. Consistent with previous findings, Mcm10 was required for the recruitment of the ssDNA binding protein, RPA, a marker for DNA unwinding (Fig. 6B; van Deursen et al. 2012; Watase et al. 2012; Yeeles et al. 2015). Also in agreement with previous data (Kanke et al. 2012; van Deursen et al. 2012; Heller et al. 2011; Yeeles et al. 2015), we found similar levels of DNA-associated Cdc45 and GINS regardless of the presence of Mcm10 after washing with a low-salt buffer (Fig. 6B, lanes 2 and 3). Washing the same reactions with a stringent high-salt buffer (containing 0.5 M NaCl) revealed that only CMG complexes treated with Mcm10 were retained on the DNA (Fig. 6B, lanes 5 and 6), while Pol ε and RPA were released. Interestingly, this increased stability of Cdc45 and GINS association did not require continued Mcm10 binding, as the high-salt wash also released Mcm10 from the template. Together, these data show that Mcm10 associates with and alters the CMG in a manner that stabilizes Cdc45 and GINS association with Mcm2-7.

To determine if the high-salt washed CMG complexes were true intermediates in the
replication-initiation process and competent for DNA replication, we added replication-elongation proteins (Pol ε, Pol α, Pol δ, Top2, Ctf4, RPA, RFC, PCNA, and Mcm10 [as indicated]) and all nucleotides to initiate DNA synthesis (Fig. 6C). Because free Cdc45 and GINS were removed during the high-salt wash, new CMG formation was prevented during this last incubation. No DNA synthesis was observed when Mcm10 or DDK was omitted from these reactions (Fig. 6C). When Mcm10 and DDK were included, DNA synthesis initiated from the high-salt-resistant CMG complexes (Fig. 6C, lane 6), indicating that they are functional replication intermediates. The reduced DNA replication initiating from the high-salt washed relative to low-salt washed CMG complexes (Fig. 6C, lanes 3 and 6) was likely caused by the higher amounts of Cdc45 and GINS retained after the low-salt wash (Fig. 6B) that were subsequently activated by Mcm10 present during the final DNA replication step.

**Mcm2 mutants that bypass Mcm10 function increase replication-product lengths**

To further explore the significance of the Mcm10-Mcm2 interactions *in vitro*, we purified Mcm2-7/Cdt1 complexes containing the *mcm2-mbm*, *mcm2-bom1*, or *mcm2-bom2* mutations. We compared the WT and mutant complexes in the CMG formation assay followed by a low-salt wash. We detected significantly weaker binding of Mcm10 to CMG complexes formed with Mcm2-7*2-mbm* and Mcm2-7*2-bom2* (Fig. 7A), consistent with these mutants altering the Mcm10-binding motif. In contrast, Mcm10 association with Mcm2-7*2-bom1*, which does not alter the Mcm10-binding motif, was near WT levels. In addition to Mcm10-binding defects, Mcm2-7*2-mbm* and Mcm2-7*2-bom2* mutant complexes
Figure 7

A

B

C

D

E
Figure 7. Mcm10 interactions with mutant Mcm2-7 complexes

(A) Mutants in the Mcm2 A subdomain and OB-fold domain are defective for Mcm10 binding and CMG formation. CMG formation assays were performed with Mcm2-7WT or Mcm2-7 including Mcm2-mbm (Mcm2-72-mbm), Mcm2-bom1 (Mcm2-72-bom1), or Mcm2-bom2 (Mcm2-72-bom2). All reactions were washed with low-salt buffer. (B) Mcm2-72-mbm is defective for DNA replication. The reaction scheme was the same as Fig. 6A, except the indicated replication proteins were included in the final step to allow DNA replication initiation. Replication products were monitored as in Fig. 6C. (C) Mcm2-72-bom1 and Mcm2-72-bom2 bypass Mcm10 function in vitro. Mcm2-7WT, Mcm2-72-bom1, or Mcm2-72-bom2 were tested for their ability to participate in DNA replication in vitro. Assays were performed with and without DDK and Mcm10, as indicated. DNA replication assays were performed and replication products analyzed as described in (B). (D) Titration of Mcm10 in the DNA replication reaction. Replication reactions were performed as described in (B) with the indicated amount of Mcm10. Note that 1.5 fmoles of Mcm10 is not sufficient to stimulate DNA replication in this reaction. (E) Mcm2-7WT and mutant Mcm2-7 complexes have an undetectable amount of Mcm10. One picomole of the indicated Mcm2-7 complexes (the same amount used in the replication reactions) and the indicated amounts of Mcm10 were were run on SDS-PAGE gel and analyzed by immunoblot. Anti-FLAG and Anti-Mcm10 antibodies were used to detect Mcm3 and Mcm10, respectively. Based on our findings, if there is any Mcm10 in these preparations, it would be significantly less than 1.5 fmoles, an amount that is not sufficient to stimulate DNA replication (see D).
exhibited weak CMG formation defects even in the absence of Mcm10. These findings suggest that the Mcm10-binding region contributes to initial CMG formation.

We also assessed the replication capacity of the mutant Mcm2-7 complexes. For these assays, we added the proteins required for CMG formation and initiation of DNA synthesis to a single final incubation (Fig. 7B and 7C). Consistent with the Mcm10-binding and CMG-formation defects observed for Mcm2-7^{mbm}, DNA synthesis was reduced in reactions containing this mutant complex (Fig. 7B). Although replication with WT Mcm2-7 was fully dependent on Mcm10, the Mcm10 bypass mutants (Mcm2-7^{bom1} and Mcm2-7^{bom2}) replicated plasmid DNA in the absence of Mcm10 (Fig. 7C). It was possible that the ability to replicate DNA without Mcm10 was due to co-purification of Mcm10 with Mcm2-7^{bom1} or Mcm2-7^{bom2}. In contrast to this possibility, the amount of Mcm10 associated with these complexes was undetectable and lower than the amount required for in vitro DNA replication (Fig. 7D and 7E) Intriguingly, when Mcm10 was added to reactions containing Mcm2-7^{bom1} or Mcm2-7^{bom2}, the resulting replication products were longer than those observed with WT Mcm2-7 (Fig. 7C). This effect on the length of replication products raised the possibility that Mcm10 functions during replication elongation.

**Mcm10 stimulates DNA replication elongation**

To address the hypothesis that Mcm10 is involved in replication elongation, we titrated the amount of Mcm10 added to the 3-step, reconstituted DNA replication assay (see
Fig. 7B) and examined the resulting replication products (Fig. 8A). Consistent with Mcm10 stimulating replication elongation, decreasing amounts of Mcm10 resulted in shorter replication products. Interestingly, the concentrations of Mcm10 that reduce replication-product lengths remain saturating for DNA unwinding and CMG stabilization during initiation (Fig. 8B). This difference in the effective Mcm10 concentration suggests that either the affinity of Mcm10 binding necessary to activate initiation and elongation differs or Mcm10 functions differently during the two events.

The effect of Mcm10 titration on replication-product length was not observed for other helicase-activating proteins. Titrations of Cdc45 or Dpb11 reduced the amount but not the length of the DNA replication products (Fig. 8C and 8D), consistent with an effect on initiation but not elongation. In contrast, titration of the known processivity factor, PCNA (Prelich et al. 1987), showed altered replication-product lengths (Fig. 9A). Because previous studies have suggested that Mcm10 interacts with PCNA (Das-Bradoo et al. 2006), we asked if the presence of PCNA was required to observe the Mcm10-dependent effects on replication-product length. Although replication products were shorter in the absence of PCNA, reducing Mcm10 levels in this condition further decreased replication product length (Fig. 9B). Thus, Mcm10 impacts replication elongation independent of PCNA.

Because CMG stabilization and replication elongation occurred in the same step in the previous assays, we modified our assay to isolate the effect of Mcm10 on elongation
Figure 8

(A) Decreased Mcm10 leads to shorter replication products. Replications reactions were performed as described in Fig. 7B with the indicated amounts of Mcm10. Replication product intensities are plotted with the colors corresponding to the box above the given lane. Red lines indicate the midpoint of the top 10% of replication product intensity for a given lane.

(B) Titration of Dpb11 does not alter replication-product lengths. DNA replication reactions were performed with the indicated amounts of Dpb11 and analyzed as described in Fig. 7B. Replication-product distribution for lanes 4-6 were analyzed as described in (A).

(C) Titration of Cdc45 does not alter replication-product lengths. DNA replication reactions were performed with the indicated amounts of Cdc45 and analyzed as described in Fig. 7B. Replication-product distribution for lanes 4-6 were analyzed as described in (A).
Figure 9

(A) Titration of PCNA alters replication-product lengths. DNA replication reactions were performed with the indicated amounts of PCNA and analyzed as described in Fig. 7B. Replication product distributions for lanes 1, 3, and 6 were analyzed as described in Fig. 8A. 

(B) Titration of Mcm10 alters replication-product lengths in the absence of PCNA. DNA replication reactions were performed as described in Fig. 7B with the indicated amounts of Mcm10. Replication product distributions for lanes 3-5 were analyzed as described in Fig. 8A.

Figure 9. Mcm10 affects replication elongation independent of PCNA

(A) Titration of PCNA alters replication-product lengths. DNA replication reactions were performed with the indicated amounts of PCNA and analyzed as described in Fig. 7B. Replication product distributions for lanes 1, 3, and 6 were analyzed as described in Fig. 8A. 

(B) Titration of Mcm10 alters replication-product lengths in the absence of PCNA. DNA replication reactions were performed as described in Fig. 7B with the indicated amounts of Mcm10. Replication product distributions for lanes 3-5 were analyzed as described in Fig. 8A.
Figure 10. Isolation of the stimulation of replication elongation by Mcm10

High-salt-resistant CMG complexes were formed and DNA replication was initiated with the same proteins as in Fig. 6C except except Mcm10 was either omitted or the indicated amount was added during the replication step. Replication product intensities were analyzed as described in Fig. 8A.
(see Fig. 6C). After CMG assembly, Mcm10 was removed with a high-salt wash. Subsequently, DNA synthesis was activated by addition of DNA polymerases and accessory factors with or without Mcm10. In agreement with an elongation role, addition of Mcm10 to the separate DNA synthesis step resulted in longer DNA replication products (Fig. 10).

**An Mcm10 mutant that is unable to function during elongation**

To further understand Mcm10 function, we sought to identify functionally important regions of Mcm10. To this end, we generated *MCM10* truncations (Fig. 11A) and analyzed their ability to complement the lethal *mcm10-FRB* anchor-away phenotype (see Fig. 3D). Deletion of the N-terminal domain of Mcm10 resulted in no growth defects. In contrast, several C-terminal domain truncations revealed a region of Mcm10 (residues 399-434) that was critical for viability (Fig. 11B). Alanine scanning of this region identified a mutant (*mcm10-A3*) that was unable to support cell growth (Fig. 11B).

Given its lethal phenotype, we investigated Mcm10-A3 function *in vitro*. Like WT Mcm10, Mcm10-A3 co-purified with Mcm2/4/6 and bound to purified Mcm2 and Mcm6 with similar affinity (Fig. 12A and 12B). However, in the context of the CMG complex, Mcm10-A3 showed a ~10-fold reduction in binding affinity (Fig. 12Ci). Despite this binding defect, Mcm10-A3 was comparable to WT Mcm10 in establishing high-salt resistant CMG complexes (Fig. 12Cii and 12D) and stimulating initial DNA unwinding.
**Figure 11**

**A**

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**B**

Figure 11. Identification of biologically important regions of Mcm10

(A) Schematic of the Mcm10 protein and the mutants analyzed. Mcm10 domain organization is shown above a set of truncation mutants tested for Mcm10 complementation. All proteins included a FLAG tag and ORC2 nuclear-localization sequence (NLS) at the C-terminus. For residues 399-434, six alanine-scanning mutations were constructed in the context of full-length Mcm10. (B) Mcm10 mutant complementation. (Top) Genetic complementation scheme. Addition of rapamycin results in depletion of Mcm10-FRB from the nucleus. (Bottom) Genetic complementation of mcm10-FRB anchor-away phenotype with the indicated MCM10 alleles inserted to LEU2 locus. Five-fold serial dilutions of cells were spotted on the indicated media and incubated at 30°C for 3 days.
Figure 12

A

Mcm6, Mcm2, Mcm4
Mcm10-A3

B

Input, Mcm10 FLAG IP, Mcm10-A3 FLAG IP

C

i

DDK phosphorylation
CMG formation

ii

LSW
HSW

D

WT Mcm10, Mcm10-A3
Mcm10 input (pmol)

E

DDK phosphorylation
CMG formation and DNA replication

F

WT Mcm10, Mcm10-A3
Mcm10 used during:
CMG formation
DNA replication and elongation

G

Figure 12
Figure 12. Mcm10-A3 is defective for stimulating replication elongation

**A** Mcm2/4/6 copurifies with Mcm10-A3. The indicated eluates from the Mcm10-A3-Flag purification were separated by SDS-PAGE and stained with Coomassie. **B** Mcm10-A3 binds to Mcm2 and Mcm6. Purified Mcm10-Flag or Mcm10-A3-Flag was incubated with Mcm2, Mcm6, or Mcm7 followed by anti-Flag IP. IPs were separated by SDS-PAGE and stained with Coomassie. **C** Mcm10-A3 is competent for CMG activation. The indicated amount of WT Mcm10 (lane 3) or Mcm10-A3 (lanes 4-7) was used for (i) CMG formation followed by a low-salt wash or (ii) high-salt wash. The reaction scheme for (i) and (ii) was the same as Fig. 3A. Immunoblots of DNA-associated proteins are shown. **D** WT Mcm10 and Mcm10-A3 have comparable abilities to stabilize the CMG. The reaction scheme was the same as Fig. 6A. The indicated amount of Mcm10 or Mcm10-A3 was used for CMG formation followed by a high-salt wash. Immunoblots of DNA-associated proteins are shown. **E** Mcm10-A3 is unable to stimulate replication elongation. The reaction scheme was the same as Fig. 7B with the indicated amount of WT Mcm10 (lane 3) or Mcm10-A3 (lanes 4-7). Labeled DNA replication products were analyzed as described in Fig. 6C. **F** Mcm10 but not Mcm10-A3 facilitates DNA replication after CMG formation. High-salt-resistant CMG complexes were formed with the indicated Mcm10 protein. DNA replication was initiated as in Fig. 6C except WT Mcm10 or Mcm10-A3 was included (or omitted) as indicated. DNA replication products were analyzed as described in Fig. 6C.
(as measured by RPA recruitment, Fig. 12Ci). In contrast, when incorporated into the complete replication assay, Mcm10-A3 resulted in reduced and shorter replication products compared to WT Mcm10 (Fig. 12E).

To further address whether salt-stable CMG complexes formed with Mcm10-A3 were functional for replication initiation and elongation, we performed replication assays with separate CMG formation and DNA replication steps (see Fig. 6C). CMG complexes were assembled with either WT Mcm10 or Mcm10-A3 followed by a high-salt wash to remove Mcm10 and unstable CMG complexes. In both cases, subsequent addition of WT Mcm10 during the DNA replication elongation step resulted in substantial replication (Fig. 12F). In contrast, addition of Mcm10-A3 during the elongation stage showed background levels of replication independent of whether WT Mcm10 or Mcm10-A3 was present during initial CMG formation. These findings establish that Mcm10-A3 is a separation-of-function mutant that is competent to stabilize the CMG complex and activate initial DNA unwinding but is defective in the stimulation of replication elongation.

**Mcm10 stimulates replication elongation in vivo**

Although our *in vitro* studies showed that Mcm10 stimulates replication elongation, it was important to determine if Mcm10 contributes to replication elongation *in vivo*. To this end, hydroxyurea was used to arrest *mcm10-1td* cells in early S-phase, and Mcm10-1td was degraded by shifting cells to 37°C. At this arrest point, any roles of
Mcm10 in CMG formation and initial replisome formation at early-replicating origins have been completed. In addition, CDC7 was replaced with cdc7-1 to prevent the activation of new origins after release from HU treatment (Bousset and Diffley 1998; Donaldson et al. 1998). Thus, under non-permissive conditions, only replication elongation by replisomes formed before the HU arrest will determine the rate of completing genome duplication as measured by analysis of DNA content by flow cytometry.

After release from the early-S-phase arrest, comparison of cdc7-1 and cdc7-1 mcm10-1td cells revealed that Mcm10-1td degradation resulted in a significant delay in completing S phase (Fig. 13). Importantly, the elongation defects observed after Mcm10-1td degradation were rescued in cells that expressed MCM10 from another locus. Consistent with a defect in elongation stimulation, expression of mcm10-A3 failed to rescue the elongation defect of cdc7-1 mcm10-1td cells (Fig. 13). These findings indicate that Mcm10 contributes to replication elongation in vivo and that the stimulation of replication elongation by Mcm10 observed in vitro is not an artifact due to the formation of an incomplete or defective replication forks.
Figure 13. Mcm10 facilitates replication elongation in vivo  
Flow cytometry analysis of the DNA content of cdc7-1 cells with indicated alleles of MCM10. Cells, grown in YP-glucose, were first arrested in G1 phase with α-factor and then in early S phase with hydroxyurea (HU) at 25°C. Next, mcm10-1td was degraded by shifting cells to 37°C in HU. Subsequently, cells were released from HU arrest at 37°C into nocodazole-containing media at 37°C. To test the complementation of the mcm10-1td allele, an additional copy of MCM10 or mcm10-A3 was inserted at the LEU2 locus.
DISCUSSION

Our findings provide multiple insights into the function of Mcm10 during DNA replication. We identified a Mcm10-binding motif at the interface between the OB-fold and A subdomain of Mcm2 and found that mutants predicted to expose this region restore growth to conditional-lethal alleles of *MCM10*. We demonstrate that Mcm10 alters the CMG complex in a manner that stabilizes Cdc45 and GINS association with Mcm2-7. Importantly, our data indicate that, in addition to its previously known role during initial helicase activation, Mcm10 stimulates replication elongation. Together, these data support a model in which Mcm10 activates the CMG complex throughout DNA replication.

**Mcm10 remolds the CMG complex**

We identified a highly-conserved motif in Mcm2 as a binding site for Mcm10. Previous genetic, biochemical and two-hybrid interaction studies support the importance of Mcm10-Mcm2 interactions (Lee et al. 2010; Quan et al. 2015; Douglas and Diffley 2016; Homesley et al. 2000; Apger et al. 2010) but had not mapped an Mcm10-binding site. The identified Mcm10-binding motif is buried between the Mcm2 A subdomain and OB-fold in all current Mcm2-7 structures (Li et al. 2015; Yuan et al. 2016). It is possible that mutants in this motif prevent Mcm10 binding by disrupting a composite Mcm10 binding site that is formed at the interface of the OB-fold and A subdomain. However, several observations argue against this hypothesis: (1) deletion of the Mcm10-binding motif inhibits Mcm10 binding in the absence of the OB-fold (Fig. 2C, lanes 15 and 19); (2)
Mcm10 does not bind the OB-fold alone (Fig. 1C, lane 16); and (3) a protein fragment including the OB-fold and the A subdomain does not bind Mcm10 better than the A subdomain alone (Fig. 1C, lanes 12 and 15).

Instead of binding to a site formed by both the OB-fold and A subdomain, we propose that Mcm10 induces or captures a conformational change in Mcm2 that exposes the Mcm10-binding motif resulting in CMG activation. Consistent with this hypothesis mutations on both sides of the Mcm2 OB-fold/A subdomain interface designed to expose the Mcm10-binding motif restore viability to cells with conditional-lethal $MCM10$ alleles (Fig. 3 and 4). In addition, Mcm2-7 complexes containing these mutations allow replication initiation in the absence of Mcm10 $\textit{in vitro}$ (Fig. 7C). Although the Mcm10-binding motif is buried in current $S. \textit{cerevisiae}$ Mcm2-7 structures (Yuan et al. 2016; Li et al. 2015), the A subdomain is rotated and the Mcm10-binding motif exposed in the only full-length structure of an active archaeal MCM complex (Miller et al. 2014; Fig. 14). We note that this archaeal MCM complex is a hybrid protein with the N-terminal domain (including both the A subdomain and the OB-fold) from $S. \textit{sulfolobus}$ and the C-terminal AAA+ domain from $P. \textit{furiosus}$. Nevertheless, this hybrid MCM is an active helicase and there are no unusual interactions between the N- and C-terminal domains that would drive movement of the A subdomain.

Further evidence in favor of Mcm10 altering CMG conformation stems from our observation that Mcm10 stabilizes and activates the CMG complex (Fig. 6, 8, and 13).
Figure 14. Structural comparison of the A subdomain/OB-fold interface
(A) Conformational comparison of the Mcm2 (yellow) and MCM (blue) N-terminal domains from *S. cerevisiae* (*S. cer*) and *S. solfataricus* (*S. sol*), respectively. The *S. cer* Mcm2 N-terminal domain from the G1-purified inactive double-hexameric Mcm2-7 structure (PDB: 3JA8, Li et al., 2015) was aligned with the *S. sol* N-terminal domain of the MCM crystal structure (PDB:2VL6, Liu et al, 2008). (B) The A subdomain is rotated in a chimeric structure of archaeal MCM. The N-terminal domain of *S. cer* Mcm2 was aligned with the *S. sol* N-terminal domain that was expressed as a fusion protein with the *P. fur* MCM C-terminal ATPase domain (PDB:4R7Y, Miller et al, 2014). The A subdomain of the archaeal MCM is rotated, exposing the site of the Mcm10-binding motif in Mcm2 (red).
Consistent with the Mcm10-dependent CMG stabilization being due to a conformational change, we find that stabilization does not require the continued presence of Mcm10 (Fig. 6B). It is unclear what molecular event causes CMG stabilization and when it occurs relative to helicase activation. Mcm10-dependent movement of the Mcm2 A subdomain could reveal additional interaction regions on Mcm2-7 for Cdc45 and GINS, resulting in enhanced stability and helicase activation. Alternatively, Mcm10-dependent stabilization of the CMG complex could occur as a consequence of helicase activation or extrusion of ssDNA from the Mcm2-7 central channel (Fu et al. 2011). For example, the ssDNA generated by one or both of these events could interact with Cdc45 or GINS (Costa et al. 2014) resulting in stabilized CMG complexes. Supporting this possibility, Cdc45 is related to the bacterial RecJ ssDNA nuclease and has been shown to bind ssDNA (Bruck and Kaplan 2013; Petojevic et al. 2015). Finally, given the potential role of OB-fold domains in ssDNA interactions (Froelich et al. 2014; Ashton et al. 2013), it is also possible that release from the A subdomain allows the Mcm2 OB-fold domain to form more productive interactions with translocating ssDNA. These possibilities are not mutually exclusive.

Our studies combined with previous data suggest that the Mcm2 A-domain/OB-fold interface is a nexus for interactions that regulate Mcm2-7 activity. In addition to inhibiting Mcm10 binding, mutations at this interface also lead to reduced Cdc45 and GINS recruitment (Fig. 3D). These defects are consistent with interactions between Cdc45 and Mcm2 A subdomain observed in the CMG structure (Fig. 2A; Yuan et al. 2014).
Interestingly, of the three OB-fold/A subdomain interface mutants we tested in vitro, the stronger Mcm10 bypass allele (\textit{mcm2-bom1}) has only minor CMG formation defects (Fig. 7A). Thus, bypassing Mcm10 function may involve a balance between opening the OB-fold/A subdomain interface while not disrupting interactions necessary for Cdc45 and GINS binding.

Several explanations are possible for \textit{mcm2-bom1} and \textit{mcm2-bom2} not being able to bypass a complete \textit{MCM10} deletion (Fig. 4C). It is possible that a small amount of residual Mcm10 function is required to allow cells to grow in the presence of the bypass alleles. Furthermore, the inability to bypass \textit{\Delta mcm10} could be due to incomplete disruption of the A subdomain/OB-fold interaction in \textit{mcm2-bom1} or \textit{mcm2-bom2}. Given that Mcm10 catalyzes the committed step of replication initiation, another possibility is that Mcm10 bypass may lead to a deleterious loss of coordination between replication initiation events. Alternatively, Mcm10 could have an additional essential function beyond helicase activation.

**Mcm10 stimulates replication elongation**

We provide both \textit{in vivo} (Fig. 13) and \textit{in vitro} (Fig. 10) evidence that Mcm10 stimulates replication elongation. Consistent with a role for Mcm10 in elongation, previous studies have found that Mcm10 travels with the replisome (Gambus et al. 2006; Pacek et al. 2006; Ricke and Bielinsky 2004). Furthermore, a temperature-sensitive allele of \textit{MCM10} (\textit{mcm10-1}) causes replication fork pausing at the restricted temperature (Homesley et
al. 2000; Merchant et al. 1997). Supporting the importance of this function, we note that the elongation-defective \textit{mcm10}-A3 allele is unable to complement the lethal depletion of Mcm10-FRB (Fig. 11B).

Although a precise mechanism for Mcm10 stimulation of elongation remains to be determined, our studies provide insights into this control. The finding that Mcm10 stabilizes the CMG complex (Fig. 6B) raises the possibility that Mcm10 binding stimulates elongation by enhancing the processivity of the CMG complex. In addition, Mcm2-7^{2-bom1} and Mcm2-7^{2-bom2} both lead to longer replication products suggesting that conformational changes in the OB-fold/A subdomain interface contribute to elongation. It is possible that Mcm10 binding drives changes in the OB-fold/A subdomain interface and that this has a direct impact on the stability or speed of the CMG. Alternatively, changes induced by Mcm10 binding could alter interactions of Cdc45 and GINS with Mm2-7. Further detailed biochemical studies will be required to test these possibilities.

**Does Mcm10 activate initiation and elongation by the same mechanism?**

Whether Mcm10 functions during replication initiation and elongation by the same or different mechanisms remains to be determined. The simplest model is that Mcm10 stimulates both events by the same mechanism. Consistent with this idea, our \textit{in vitro} analyses of the Mcm10-bypass mutants suggest that both the initiation and elongation functions of Mcm10 are impacted by these mutants. The ability to detect replication products in these assays indicates that these mutants facilitate initiation in the absence
of Mcm10 (Fig. 7C). Two observations suggest that the elongation function of Mcm10 is also altered by these mutations. First, in the absence of Mcm10, the length of \textit{in vitro} replication products correlates with the strength of the Mcm10 bypass allele. Second, when WT Mcm10 is present, Mcm2-7$^{2-\text{bom}1}$ and Mcm2-7$^{2-\text{bom}2}$ produce longer replication products (Fig. 7C).

On the other hand, we identified an \textit{MCM10} allele (\textit{mcm10-A3}) that shows differential effects on replication initiation and elongation. This protein is defective for stimulation of replication elongation (Fig. 12E) and binding to the CMG (Fig. 12Ci), but exhibits similar capabilities as WT Mcm10 to form salt-stable CMG complexes and stimulate initial DNA unwinding (Figs. 12C and 12D). These findings suggest that stable binding to the CMG correlates with the ability to stimulate replication elongation and that a different interaction is involved in stabilizing the CMG and stimulating initial DNA unwinding. Further experiments will be necessary to determine whether and how the Mcm10 mechanism of function differs between initiation and elongation.
MATERIALS AND METHODS

Yeast strains and plasmids

All *S. cerevisiae* strains were congenic with W303 (*ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-1 can1-100*) and the genotypes are summarized in Table 1. Protein expression plasmids are summarized in Table 2.

Protein Purification

Mcm2-7/Cdt1, ORC, Cdc6, Ctf4 and Top2 were purified as previously described (Yeeles et al. 2015; Kang et al. 2014). Purifications of the remaining proteins are described below.

Buffers

The following buffers were used for protein purification: buffer H (50 mM HEPES-KOH [pH 7.6], 1 mM EDTA, 1 mM EGTA, 5 mM MgOAc, 10% glycerol), buffer I (buffer H, 0.02% NP-40, 0.3 M potassium glutamate [KGlut], 10 mM imidazole), buffer M (buffer H, 0.02% NP-40, 0.3 M KCl), buffer D (buffer H, 0.3 M KOAc, 0.02% NP-40), buffer E (buffer H, 0.4 M NaOAc, 0.01% NP-40), buffer R (50 mM HEPES-KOH [pH 7.6], 10% glycerol, 7 mM MgOAc, 0.01% NP-40, 1 mM ATP) and buffer C (25 mM Tris-Cl [pH 7.2], 10% glycerol, 1 mM DTT).

Yeast cell growth and lysis

All yeast strains were grown in selective media before being inoculated into 8L of YEP +
2% glycerol at 30°C. Cell were grown to an OD<sub>600</sub> ~1 before induction with galactose (2% final conc.). After 4-6 hours, the cells were harvested and washed with 200 ml of chilled water + 0.2 mM PMSF. The cells were then resuspended in approximately 1/2 packed cell volume of the indicated lysis buffer containing a protease inhibitor tablet and frozen dropwise into liquid nitrogen. The frozen cells were lysed using a SPEX SamplePrep Freezer/Mill. Lysed cell powder was transferred to ultracentrifugation tubes and thawed on ice. The lysate was cleared by centrifugation in a Beckman ultracentrifuge at ≥140k x g for ≥1 hour. All steps were done at 4°C.

**FLAG-affinity purification**

Cleared lysates were incubated with the indicated amount of packed anti-FLAG M2 affinity gel (Sigma) for 2 hours at 4°C. After a column wash, the bound proteins were eluted with the indicated buffer including 0.2 mg/ml 3xFLAG peptide (MDYKDHDG DYKDHDIDYKDDDDK, Koch Institute Swanson Biotechnology Center). The first eluate was collected by flowing over 1 column volume (CV) of elution buffer. The next four eluates were collected after a 30-minute incubation with the elution buffer.

**S-CDK**

Clb5-FLAG and Cdc28-6xHis were over-expressed from ySK119. Clb5 was expressed with a deletion of residues 1-94 to remove a destruction box (Cross et al. 1999). Cells were resuspended in buffer H, 1 M sorbitol, 0.02% NP-40, 2 mM ATP, 0.5 M KCl. After cell lysis, the cleared lysate was diluted to 0.3 M KCl with buffer H. The lysate was then
incubated with 1 ml anti-FLAG M2 affinity gel equilibrated with buffer M. The resin was
washed with 20 CV of buffer M followed by 10 CV of buffer I + 3xFLAG peptide. S-CDK
was eluted in buffer I. S-CDK-containing fractions were flowed over cOmplete His-tag
resin (Roche) twice, washed with 20 CV of buffer I, and eluted with buffer I + 250 mM
imidazole. Peak fractions were pooled and applied to a Superdex 200 column (GE
healthcare) equilibrated with buffer H, 0.01% NP-40, 1 mM ATP, 0.3 M KGlut.

**Sld3/Sld7**

Sld3-3xFLAG and Sld7-VSV-G were over-expressed from ySK123. Sld3 was expressed
with a deletion of residues 1-104 to remove a putative destruction box. Cells were
resuspended in buffer H, 1 M sorbitol, 0.02% NP-40, 2 mM ATP, 0.8 M KCl. After cell
lysis, the cleared lysate was diluted to 0.3 M KCl with buffer H. The diluted lysate was
incubated with 1.5 ml anti-FLAG M2 affinity gel equilibrated with buffer M. The resin was
washed with 30 CV of buffer M and eluted in buffer M + 3xFLAG peptide. Sld3/Sld7
containing fractions were diluted to 0.2 M KCl with buffer H immediately before being
applied to a 1 ml HiTrap SP HP column (GE healthcare). The column was washed with
buffer H, 0.02% NP-40, 330 mM KCl and eluted with buffer H, 0.02% NP-40, 640 mM
KCl.

**Sld2**

3xFLAG-3C-Sld2 was over-expressed from ySK127. Cells were resuspended in buffer
H, 1 M sorbitol, 0.02% NP-40, 2 mM ATP, 0.8 M KCl. After cell lysis, the cleared lysate
was dialyzed overnight (16 hours) in buffer M with 3 mM ATP and 1 mM PMSF. The lysate was cleared a second time by spinning for 15 min at 11k rpm. Sld2 was purified using 1 ml anti-FLAG resin as described above for Sld3/Sld7 except 1 mM ATP was added to buffer M. Sld2 containing fractions were diluted to 0.2 M KCl with buffer H immediately before being applied to a 1 ml HiTrap SP HP column. Sld2 was eluted with a 15 CV gradient of 0.2 M – 1 M KCl in buffer H, 0.02% NP-40, 1 mM ATP.

**Dpb11**

Dpb11-FLAG was over-expressed from yRH154. Dpb11 was purified in a similar manner as Sld2 except for the following modifications. Fractions containing Dpb11 from the anti-FLAG column were diluted to 0.1 M KCl with buffer H immediately before being applied to a 1 ml HiTrap SP HP column. Dpb11 was eluted with an 18 CV gradient of 0.1 M – 1 M KCl in buffer H, 0.02% NP-40, 1 mM ATP. The peak fractions were dialyzed against buffer D.

**Cdc45**

Cdc45-3xFLAG was over-expressed from yMM016. Purification of Cdc45 was based on a previously published protocol (Yeeles et al., 2015) with the following modifications. Cells were resuspended in buffer H, 1 M sorbitol, 3 mM ATP, 500 mM KGlut. After lysis, the lysate was incubated with 1.5 ml anti-FLAG M2 affinity gel equilibrated with buffer H, 500 mM KGlut, 2 mM ATP for. The resin was washed with 20 CV of buffer H, 500 mM KGlut, 2 mM ATP followed by 10 CV of 20 mM potassium phosphate buffer (pH 7.4),
150 mM KOAc, 10% glycerol. Cdc45 was eluted in the previous buffer + 3xFLAG peptide. After the hydroxyapatite column, Cdc45 was dialyzed against buffer H, 0.3 M KGlut.

**GINS**

Sld5, Psf1, Psf3, and Psf2-3C-6xHis-FLAG were over-expressed from ySK136. Cells were resuspended in buffer H, 1 M sorbitol, 0.02% NP-40, 2 mM ATP, 0.5 M KCl. After lysis, the cleared lysate was diluted to 0.3 M KCl with buffer H. The lysate was then incubated with 1.5 ml anti-FLAG M2 affinity gel equilibrated with buffer M. The resin was washed with 20 CV of buffer M followed by 10 CV of buffer H, 0.02% NP-40, 0.1 M KCl. GINS was eluted in the previous buffer + 3xFLAG peptide. The FLAG tag on Psf2 was removed with an overnight incubation (16 hours) with HRV 3C protease. GINS was flowed over complete His-tag resin to remove uncut GINS and HRV 3C protease before applying the flow through to a 1 ml HiTrap Q HP column (GE healthcare). GINS was eluted with a 20 CV gradient of 0.1 M – 1 M KCl in buffer H, 0.02% NP-40. The peak fractions were dialyzed against buffer D.

**Polymerase ε**

Pol2-3C-5xFLAG, Dpb3, Dpb4-3C-6xHis, and Dpb2-3C-FLAG were over-expressed from yMH28. Cells were resuspended in buffer E. After cell lysis, the cleared lysate was incubated with 1.5 ml anti-FLAG M2 affinity gel equilibrated with buffer E. The resin was washed with 20 CV of buffer E and eluted in buffer E + 3xFLAG peptide. The FLAG tags
were removed with a 2-hour incubation with HRV 3C protease. Polymerase ε was concentrated using a 10K MWCO spin column (Sartorius) before being applied to a Superdex 200 column equilibrated with buffer E.

**Polymerase α/primase**

Pri1, Pri2, Pol1, and Pol12-3C-FLAG were over-expressed from yAS3. Polymerase α/primase was purified in a similar manner as polymerase ε except buffer H, 0.3 M KGlut, 0.01% NP-40 was used for cell resuspension and all chromatography steps and an additional cOmplete protease inhibitor tablet was added during the anti-FLAG incubation.

**DDK**

Dbf4-FLAG and Cdc7 were over-expressed from yRH146. Cells were resuspended in buffer H, 0.3 M KGlut, 0.01% NP-40. After lysis, the cleared lysate was incubated with 1.5 ml anti-FLAG M2 affinity gel equilibrated with buffer H, 0.3 M KGlut, 0.01% NP-40. The resin was washed with 30 CV and eluted in the previous buffer + 3xFLAG peptide.

**RPA**

The purification was based on a previously published protocol (Gibb et al. 2014) with the following modifications. Rosetta 2 E. coli cells were transformed with p11d-tscRPA-30MxeHis6 and 2 L of culture were grown at 37°C in 2xYT+amp+cm. After the Ni-NTA and chitin column, RPA was applied to a Superdex 200 column equilibrated with 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 20% glycerol.
**Mcm10 and Mcm10-A3**

WT Mcm10-FLAG and Mcm10-A3-FLAG were over-expressed from MLY049 and MLY136, respectively. FLAG-3C-Mcm10-V5 was over-expressed from MLY048. Cells were resuspended in buffer H, 1 M sorbitol, 0.05% NP-40, 0.5 M KCl. After lysis, the cleared lysate was diluted to 0.25 M KCl with buffer H, 0.05% NP-40. The lysate was then incubated with 1.5 ml anti-FLAG M2 affinity gel equilibrated with buffer H, 0.05% NP-40, 0.25 M KCl. The resin was washed with 30 CV of buffer H, 0.05% NP-40, 0.25 M KCl and eluted in the same buffer. The eluted protein was diluted to 0.15 M KCl with buffer H, 0.05% NP-40 immediately before being applied to a 1 ml HiTrap SP HP column. Mcm10 was eluted with a 15 CV gradient of 0.15 M – 1.5 M KCl in buffer H, 0.05% NP-40.

**Polymerase δ**

Purification of Polymerase δ was based on a previously published protocol (Langston and O'Donnell 2008) with the following modifications. 8 L of both yeast and *E. coli* cultures were used. Pol3-FLAG was over-expressed from yAS26. Rosetta 2 *E. coli* cells were co-transformed with pMM051 and pMM053 and grown at 37°C in 2xYT media with 100 μg/ml ampicillin, 50 μg/ml kanamycin, and 34 μg/ml chloramphenicol. (2xYT+amp+kan+cm). At an OD$_{600}$ ~0.6, cells were moved to 25°C and induced with 1 mM IPTG. After a 4-hour induction, cells were harvested and resuspended in 15 mL (per 1L culture) of buffer H, 0.01% NP-40, 0.3 M KCl. The resuspended *E. coli* cells
were treated with 0.1 mg/ml lysozyme (from chicken egg white, Sigma) for 30 minutes followed by 6 cycles of sonication (30% amplitude, 10 seconds ON, 10 seconds OFF) with a Branson digital sonifier (Emerson Industrial Automation). Yeast cells were resuspened in buffer H, 0.01% NP-40, 0.3 M KCl. After lysis and clarification, yeast and *E. coli* lysates were combined and Pol δ was purified in a similar manner as Dpb11.

**RFC**

Purification of RFC with a deletion of RFC1 from residues 1-274 was based on a previously published protocol (Gomes et al. 2000) with the following modifications. Rosetta 2 *E. coli* cells were transformed with pBL481 and 4 L of culture were grown at 37°C in 2xYT+amp+cm. At an OD_{600} ~0.7, cells were moved to 30°C and induced with 0.5 mM IPTG. After a 3-hour induction, cells were harvested in 20 mL (per 1L culture) of buffer R, 0.2 M NaCl plus a cOmplete protease inhibitor tablet. The resuspended cells were treated with lysozyme and sonicated as described for Pol δ. The cleared lysate was applied to 2 ml Ni-NTA resin equilibrated with buffer R, 0.2 M NaCl. The resin was washed with 50 CV of buffer R, 0.2 M NaCl and eluted with the same buffer with 300 mM imidazole. The eluted protein was diluted to 0.15 M NaCl with buffer R before being applied to a 1 mL HiTrap SP HP column. RFC was eluted with a 24 CV gradient from 0.15 M – 0.75 M NaCl in buffer R. Peak fractions were pooled and applied to a Superdex 200 column equilibrated with buffer R, 0.15 M NaCl.
PCNA

Rosetta 2 *E. coli* cells were transformed with pMM054 and 1 L of culture was grown at 37°C in 2xYT+amp+cm. At an OD$_{600}$ ~0.6, cells were induced with 1 mM IPTG. After 3 hours, cells were harvested in 20 mL of 50 mM HEPES-KOH (pH 7.6), 0.1 M KCl, 10% glycerol, 0.01% NP-40, 10 mM imidazole plus a protease inhibitor tablet. The resuspended cells were treated with lysozyme and sonicated as described for Pol δ. The cleared lysate was applied to 3 ml Ni-NTA resin equilibrated with the previous buffer. The resin was washed and then eluted with 300 mM imidazole in the same buffer. PCNA containing fractions were applied to a 1 mL HiTrap SP HP column and eluted with a 20 CV gradient from 0.1 M – 1 M KCl in buffer H.

**Mcm2, Mcm4, Mcm5, Mcm6, Mcm7 and Mcm2 truncations**

Mcm2 (pNI001), Mcm4 (pNI002), Mcm5 (pNI003), Mcm6 (pNI004), Mcm7 (pNI005), Mcm2 1-483 (pML028), Mcm2 1-195 (pML027) were C-terminally 6xHis tagged and expressed in Rosetta 2 *E. coli* cells. Resuspension of the bacterial pellet and purification was done in buffer H (without EDTA and EGTA), 0.25 M KCl, 10 mM Imidazole. Mcm2 474-868 (pML035), Mcm2 196-299 (pML030), Mcm2 300-473 (pML034), Mcm2 196-240 (pML032), Mcm2 241-299 (pML033), Mcm2 196-289 (pML031) and Mcm2 196-473-mbm (pML050) were N-terminally MBP tagged and expressed in Rosetta 2 *E. coli* cells. Resuspension of the bacterial pellet and purification was done in buffer H, 250 mM KCl. For both 6xHis and MBP purifications cells were resuspended in 50 ml of buffer and treated with lysozyme and sonicated as described for Pol δ. The cell lysate
was cleared by a 30-minute centrifugation (20,000 g). 2 ml of Ni-NTA resin was used for 6xHis purifications and 2 ml of amylose resin was used for MBP purifications. Before elusion, resin was washed with 30 ml of buffer and proteins were eluded with 350 mM imidazole from the Ni-NTA resin and with 10 mM maltose from the amylose resin.

**Reconstituted DNA replication and CMG formation**

The DNA plasmid template, pUC19-ARS1, was randomly biotinylated and coupled to streptavidin-coated magnetic beads as previously described (Heller et al. 2011). Each incubation step was performed in a thermomixer (Eppendorf) shaking at 1150 rpm at 25°C. Supernatants of each step were removed by applying the reaction to a DynaMag-2 Magnet (ThermoFisher Scientific) to isolate the DNA coupled to magnetic streptavidin beads from the supernatant. Mcm2-7 loading was performed by incubating 0.25 pmol ORC, 0.5 pmol Cdc45, and 1 pmol Mcm2-7/Cdt1 with 0.125 pmol pUC19-ARS1 in 25 mM HEPES (pH 7.6), 10 mM MgOAc, 0.1 mM ZnOAc, 1 mM DTT, 300 mM KGlut, 20 mM phosphocreatine (PC), 6 mM ATP, 0.1 mM EDTA, 0.02% NP-40, 10% glycerol and 0.2 μg creatine kinase (CK). The Mcm2-7 loading step was done in a volume of 10 μl and was incubated for 30 minutes. After removal of the supernatant, DDK phosphorylation was performed as described previously (Kang et al. 2014) in a 10 μl reaction volume for 25 minutes. After removal of the DDK reaction supernatant, the following amounts of protein were added to the DDK-phosphorylated Mcm2-7: 1 pmol Cdc28/Cib5, 0.3 pmol DDK, 1 pmol Sld3/Sld7, 5 pmol Cdc45, 2 pmol Sld2, 0.6 pmol Dpb11, 5 pmol GINS, 0.15 pmol Mcm10 (or as indicated), 1.85 pmol Pol ε, and 2 pmol
RPA. The buffer used for CMG formation contained 25 mM HEPES, 12 mM MgOAc, 0.1 mM ZnOAc, 1 mM DTT, 20 mM PC, 6 mM ATP, 10% glycerol, 0.04 mg/ml BSA and 0.3 μg CK. The CMG formation step was done in a volume of 30 μl and was incubated for 1 hour. Reactions were washed with the indicated buffer, and proteins were released from the DNA by incubating with 5 U DNase (Worthington) in 15 μl buffer H, 150 mM KGlut, 0.01% NP-40 for 30 minutes at 25°C before immunoblotting.

To initiate DNA replication, the following amounts of proteins were added along with the proteins from the CMG formation step: 2.5 pmol Pol α, 0.5 pmol Top2, 3 pmol Ctf4, 1 pmol RFC, 6 pmol PCNA, 2 pmol Pol δ. 0.2 mM rNTP, 0.04 mM dNTP, and 10 μCi [α-P32] dCTP were included in the buffer to initiate and monitor DNA replication. The DNA replication step was done in a volume of 30 μl and was incubated for 1 hour. Reactions were washed with buffer H, 500 mM NaCl, 0.05% NP-40 before being resuspended in alkaline gel-loading buffer (50 mM NaOH, 4 mM EDTA, 4.5% Ficoll400, 0.01% bromocresol green). DNA replication products were separated in a 1% alkaline agarose gel, dried and imaged using a phosphor screen. When CMG formation and DNA replication were performed in separate steps, the supernatant from the CMG formation step was removed after 1 hour before adding the DNA replication proteins. DNA replication was initiated by omitting all the proteins used for CMG formation except Pol ε, RPA, and Mcm10 (as indicated). The CMG formation step and DNA replication step were both done in a volume of 30 μl and were incubated for 1 hour each.
The following antibodies were used for immunoblotting: α-Cdc45 (HM7135), α-GINS (HM7128), α-Mcm10 (HM6465), α-Pol ε (HM7602), α-Mcm2 (yN-19, Santa Cruz), α-Mcm5 (yN-19, Santa Cruz), and α-Rfa1 (gift from Steven Brill).

**Anchor-away**

The base strain (MLy054) for protein anchoring was obtained by crossing Y40434 (Euroscarf; Haruki et al., 2008) and OAy470 to obtain a bar1::hisG MATa version of the Y40434 strain. Next MCM10 or CDC7 were C-terminally FRB tagged using plasmids pFA6a-FRB-KanMX6 or pFA6a-FRB-His3 (Euroscarf), respectively. All alleles for mcm10-FRB complementation were expressed from the MCM10 promoter and were inserted into LEU2 locus as a single-copy integration. To drive protein anchoring, solid media was supplemented with DMSO (1% final conc.) and rapamycin (5 μg/ml final conc.).

**Mass Spectrometry**

An eluate from anti-FLAG M2 affinity gel (Sigma) of an Mcm10-FLAG purification was separated on an SDS-PAGE gel, and the band corresponding to Mcm10-FLAG was excised. The remainder of the gel lane was subjected to mass spectrometry using standard methods.

**Immunoprecipitation (IP)**

Purified Mcm10-FLAG was bound to anti-FLAG M2 affinity gel in buffer H, 250 mM KCl,
0.05% NP-40 for 1 hour at 4°C. Purified Mcm2, Mcm4, Mcm5, Mcm6, Mcm7, or variants of Mcm2 were added to bound Mcm10, incubated at 25°C for 30 minutes and washed 3 times in buffer H, 250 mM KCl, 0.05% NP-40 (unless stated otherwise in the figure legend). Precipitated proteins were eluded in buffer H, 250 mM KCl, 0.05% NP-40 with 0.15 mg/ml 3xFLAG peptide.

**Flow cytometry**

Cells were arrested in G1 phase with 20 μg/ml α-factor on the hour and arrested in early S phase with 150 mM HU. Cells were released from HU arrest into media containing 1.5 μg/ml nocodazole. For each time point, 0.5 ml of cells (OD₆₀₀ ~0.6) were fixed in 10 ml of 70% ethanol for at least 15 minutes. The cells were then washed once with 1ml of 50 mM sodium citrate. RNA was degraded with 10 μg/ml RNase A in 500 μl of 50 mM sodium citrate for 16 hours at 37°C, followed by 30 minutes 20 μg/ml Proteinase K treatment at 42°C. DNA was stained with 10×SYTOX Green in 100 μl of 50 mM sodium citrate for 30 minutes and analyzed with CytoFLEX Flow Cytometer (Beckman Coulter).
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Chapter III

Expanded Discussion and Future Directions
This thesis has focused on the function of the essential Mcm10 protein throughout DNA replication. Previous studies have shown that Mcm10 is required for initial DNA unwinding at origins of replication and accomplishes this function through interactions with the Mcm2, Mcm4, and Mcm6 subunits of Mcm2-7 helicase (van Deursen et al. 2012; Wohlschlegel et al. 2002; Kanke et al. 2012; Watase et al. 2012; Quan et al. 2015; Douglas and Diffley 2016). In Chapter II, an Mcm10-interaction region on Mcm2-7 was narrowed down to a 10-amino-acid motif in the N-terminal domain of Mcm2. Mutations near this motif not only reduced Mcm10 binding but also bypassed conditional-lethal alleles of MCM10, likely due to a conformational change in the N-terminal domain of Mcm2. Additionally, Mcm10 was shown to stimulate the CMG not only during initial DNA unwinding but throughout DNA replication. This work has expanded our knowledge of how and when Mcm10 functions during the entire process of DNA replication.

Although this work has furthered our understanding of Mcm10 functions, many questions remain regarding the molecular mechanism of Mcm10, as well as the contribution of each of the helicase-activation proteins. In chapter II, we showed that Mcm10 stimulates replication elongation. However, whether Mcm10 is affecting the speed or the processivity of the CMG is currently unclear. In addition, Mcm10 associates with various other replication-associated proteins (Alver et al. 2014; Das-Bradoo et al. 2006; Ricke and Bielinsky 2004), but the consequences and importance of these interactions is unclear. Finally, Mcm10 is required for initial DNA unwinding at origins, but determining when unwinding occurs relative to double-hexamer separation will provide insight into a
mechanism for initial CMG DNA unwinding. In this chapter, I will outline the motivation for these questions and provide possible approaches to answering them.

**Mcm10 affecting CMG speed vs. processivity**

One of the unresolved aspects of Mcm10 function is how it affects replication elongation. Mcm10 could act as a processivity factor for the CMG, or it could stimulate the speed of the CMG. Another possibility is that the CMG has an intrinsic period of time it stays on the DNA. Thus, by increasing the speed of the CMG, Mcm10 would also cause the CMG to be more processive.

Two approaches could be used to determine the mechanism by which Mcm10 stimulates replication elongation. One method is to monitor completion of genome duplication *in vivo* after depletion of Mcm10 during S phase. In chapter II (Fig. 13), we showed that S phase progression was delayed without Mcm10, but it was unclear if the replication forks were slowed or collapsed. If the absence of Mcm10 slows replication forks, cells will still finish DNA replication (albeit slower than with Mcm10) and continue into G2, M, and G1 phase. However, if Mcm10 affects the processivity of the CMG, I would expect a similar outcome as depletion of other CMG components during S phase; an inability to complete genome duplication which would be reflected in the DNA replication checkpoint remaining active (Tercero et al. 2000; Labib et al. 2001).
Speed versus processivity can also be tested *in vitro*. A pulse-chase of radioactive nucleotides has been used in the reconstituted DNA replication assay to measure replication fork speed (Yeeles et al. 2016). If Mcm10 is affecting replication fork speed, then omission of Mcm10 during elongation will result in a slower increase in replication product size over time. In contrast, if Mcm10 is affecting CMG processivity, the maximum length of replication products at the completion of the reaction will be smaller without Mcm10.

A role for Mcm10 in replication elongation provides additional means to regulate fork movement. Two observations support such a role for Mcm10. First, Mcm10 remains associated with replication forks when forks are stalled by a barrier but is released when DNA polymerases are uncoupled from the helicase (Pacek et al. 2006). The release of Mcm10 upon replication stress would allow ssDNA formation that is necessary for DNA-damage signaling (Navadgi-Patil and Burgers 2011) but would limit the extent of DNA unwinding. Second, phosphoproteomic studies reveal that Mcm10 is phosphorylated by the DNA damage induced kinase, Chk1 (Bodenmiller et al. 2010). This raises the possibility that Chk1 phosphorylation could regulate association of Mcm10 with the replication fork and therefore, fork progression. It is also possible that this phosphorylation could regulate the replication initiation functions of Mcm10.

The consequence of Chk1 phosphorylation of Mcm10 could be determined by mutating the Mcm10 residues targeted by Chk1 during replication stress. The residues could be
mutated to alanine or aspartic acid (as a phosphomimetic) and tested in the following two assays: 1) complementation of an $MCM10$ deletion and 2) function in the reconstituted replication assay. If the mutant $MCM10$ alleles are viable as the sole copy of $MCM10$, the mutant strains can then be tested for sensitivity to DNA damaging agents or replication stress (e.g. hydroxyurea). If Mcm10 phosphorylation is involved in regulating replication fork progression during replication stress, mutating the residues targeted for phosphorylation to alanine will prevent the cells from being able to properly respond to the stress. The consequences of Chk1 phosphorylation could also be tested \textit{in vitro} with purified Chk1. Addition of the Chk1-phosphorylated Mcm10 to the reconstituted DNA replication assay could reveal if phosphorylating Mcm10 affects its ability to bind Mcm2-7 and function during initial DNA unwinding or elongation. Together, these assays would provide insight into whether Chk1 phosphorylation is regulating Mcm10 function.

\textbf{Other Mcm10 functions during DNA replication}

Mcm10 interacts with several replication-associated proteins suggesting other functions for Mcm10 in addition to CMG stimulation. An interaction between Mcm10 and Pol $\alpha$ implicates Mcm10 in tethering Pol $\alpha$ to the replication fork (Warren et al. 2009; Ricke and Bielinsky 2004; Fien et al. 2004). However, this function was based on a key observation that Mcm10 depletion caused Pol $\alpha$ to be degraded, a finding that was later shown to be an artifact of the intrinsic instability of Pol $\alpha$ (Ricke and Bielinsky 2006; 2004; van Deursen et al. 2012). A possible ubiquitinated form of Mcm10 binds the processivity factor, PCNA. Interestingly, when a putative PCNA-interaction motif in Mcm10 was mutated, the cells
exhibited growth defects (Das-Bradoo et al. 2006). However, I found that the Mcm10-dependent stimulation of replication elongation was independent of PCNA (Chapter II, Fig. 9B). Another Mcm10 interaction partner is Mec3, a component of the DNA damage response. Binding to Mec3 is mediated through the N-terminal domain of Mcm10 (Alver et al. 2014). However, cells with a deletion of MEC3 and the N-terminal domain of Mcm10 showed an additive growth defect during replication stress, suggesting Mcm10 and Mec3 function in separate pathways.

Although it is clear that Mcm10 interacts with these various proteins, the other, putative Mcm10 functions need to be studied in parallel with its essential function of stimulating the CMG helicase. The directed mutations in Mcm10 that eliminate binding to PCNA (Das-Bradoo et al. 2006) and Mec3 (Alver et al. 2014) could be tested in our in vitro replication assay. Because Mcm10 function is independent of PCNA (Chapter II, Fig. 9B) and Mec3 in our reconstituted replication assay, the mutant Mcm10 proteins can be purified and tested for any defects in CMG formation or stimulating DNA replication. These assays would indicate if the observed growth defects with the Mcm10 mutants can be separated from defects in origin DNA unwinding or replication elongation.

In addition to binding Mcm2, Mcm10 interacts with other Mcm2-7 subunits (Quan et al. 2015; Douglas and Diffley 2016). In Chapter II, we focused on the Mcm10-binding site on Mcm2 because it displayed the tightest interaction with Mcm10. However, the interactions with other Mcm2-7 subunits need to be investigated further to determine if these
interactions also contribute to Mcm10 function. In support of a role for these interactions, when the mcm2-mbm mutant was incorporated into the Mcm2-7 complex, Mcm10 binding was clearly diminished, but not eliminated (Chapter II, Fig. 7A). This residual binding suggests that Mcm10 is binding additional regions of Mcm2, another Mcm2-7 subunit, or another replication-initiation protein (e.g. Cdc45). One method to determine other Mcm10-interacting proteins is to chemically cross-link the CMG complex with Mcm10, fragment the proteins, and use mass spectrometry to determine inter- and intra-cross-linked peptides (Shi et al. 2014). Using this technique, interactions between the components of the CMG and Polε were determined with high resolution and agreed with cryo-EM structures (Sun et al. 2015). Mutations in the regions of Mcm10 that are crosslinked to other proteins could then be engineered and tested for in vivo complementation and in vitro DNA replication defects.

**DNA unwinding relative to double-hexamer separation**

Although Mcm10 is required for DNA unwinding by the CMG, when unwinding happens relative to separation of the two CMG helicases is of interest. This separation is not caused by DDK phosphorylation of Mmc2-7 (On et al. 2014), but likely happens at some stage after Cdc45 and GINS association. There are several possible mechanisms for double-hexamer separation. It is possible that Mcm10 causes separation of the two CMG complexes, which leads to DNA unwinding (Quan et al. 2015). Mcm10 could also interact with the DNA after initial DNA unwinding to extrude ssDNA from the central channel of the CMG, and this transition from Mmc2-7 encircling dsDNA to ssDNA could facilitate
CMG separation. Finally, CMG formation may cause double-hexamer separation, and Mcm10 then stimulates DNA unwinding of individual CMG complexes after separation has occurred. EM studies of the CMG complex have already revealed where Cdc45 and GINS interact with Mcm2-7 and possible CMG conformational changes during DNA unwinding (Georgescu et al. 2017; Yuan et al. 2016; Sun et al. 2015; Abid Ali et al. 2016). Further structural characterization of the CMG in the absence of Mcm10 and with different DNA templates may help determine when DNA unwinding is occurring relative to double-hexamer separation. Isolating CMG complexes from cells depleted of Mcm10 and determining its structure would provide insight into the order of these events.

Another method to determine the timing of double-hexamer separation relative to DNA unwinding is to utilize a single-molecule approach. Recently, our lab has collaborated with Jeff Gelles’ lab at Brandeis University to study Mcm2-7 loading using colocalization single-molecule spectroscopy (CoSMoS) (Ticau et al. 2017; 2015). This technique uses total-internal-reflection-fluorescence (TIRF) microscopy to measure real-time dynamics of macromolecular interactions (Friedman et al. 2006). Our lab has exploited this single-molecule approach by fluorescently labeling DNA and helicase-loading proteins and monitoring protein dynamics at hundreds of DNA molecules attached to a microscope slide. This assay could be expanded to include a helicase-activation step with fluorescently-labeled helicase-activation proteins.
To monitor DNA unwinding and double-hexamer separation simultaneously, FRET between Mcm2-7 double hexamers and association of RPA, which is indicative of DNA unwinding, can be monitored. When two different fluorescently-labeled Mcm2-7 complexes are loaded onto DNA as a double hexamer, the emission energy from one fluorophore is able to excite the other resulting in FRET (Ticau et al. 2015). By monitoring FRET loss (while also monitoring the presence of both Mcm2-7 to rule out FRET loss due to photobleaching) and fluorescently-labeled RPA association, the order of DNA unwinding and double-hexamer separation can be determined.
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Appendix A

Mcm2-7 ATP Hydrolysis during Helicase Activation
Because Mcm2-7 has six different ATPase active sites, I sought to determine the requirement of Mcm2-7 ATP hydrolysis during CMG formation and helicase activation. Individual mutations at each of these ATPase active sites result in defects during distinct events of DNA replication initiation, including helicase activation (Kang et al. 2014; Coster et al. 2014). The slow-hydrolyzing ATP analog, ATP\(_\gamma\)S, can be used to inhibit ATP hydrolysis by the Mcm2-7 helicase. When added to an \textit{in vitro} Mcm2-7 loading reaction, ATP\(_\gamma\)S prevents Mcm2-7 from stably loading around dsDNA and captures an intermediate containing ORC, Cdc6, Cdt1, and Mcm2-7 (Evrin et al. 2013).

To determine how ATP hydrolysis affects CMG formation, ATP\(_\gamma\)S was added in excess during the final step of the reconstituted CMG assay. The assay was performed by first loading Mcm2-7 and subsequently phosphorylating Mcm2-7 with DDK in ATP-containing buffers. To determine if any step during CMG formation was prevented, excess ATP\(_\gamma\)S was included with the helicase-activation proteins and CMG formation was monitored by immunoblot. CDK was able to use ATP\(_\gamma\)S as a phosphate donor to phosphorylate Sld2 and Sld3, in agreement with previous studies of CDK (Allen et al. 2007). CMG formation was unaffected by addition of excess ATP\(_\gamma\)S. However, RPA binding to unwound dsDNA was not detected with ATP\(_\gamma\)S addition, indicative of inhibition of Mcm2-7 ATP hydrolysis (Fig. 1, compare lanes 2 and 4). As seen previously (Chapter II, Fig. 6B), addition of Mcm10 results in CMG complexes that are resistant to washes with high-salt buffer (0.5 M NaCl). In contrast to ATP-containing reactions, excess ATP\(_\gamma\)S addition results in CMG complexes that are resistant to a high-salt wash regardless of the presence of Mcm10.
Figure 1

**Figure 1. Mcm2-7 ATP hydrolysis during helicase activation**

The reaction scheme was the same as Chapter II Fig. 6A, except ATPγS was added in >1000-fold excess of ATP during the CMG formation step in lanes 3-4 and 7-8. Reactions were washed with a low-salt buffer (lanes 1-4) or high-salt buffer (lanes 5-8). Immunoblots of DNA-associated proteins are shown.
(Fig. 1, compare lanes 5-6 and 7-8). Interestingly, Mcm10 is also retained at low levels in the presence of ATPγS after a high-salt wash.

This result suggests that Mcm2-7 ATP hydrolysis is not required for CMG formation, but is required for initial DNA unwinding. The high-salt stability of ATPγS-bound CMG regardless of the presence of Mcm10 could be the result of favoring a specific, more stable conformation of the CMG, whereas ATP hydrolysis would allow sampling of different conformations. This hypothesis is supported by cryo-EM studies in which multiple conformations of the CMG were observed (Abid Ali et al. 2016; Yuan et al. 2016). Alternatively, ATPγS could be trapping the CMG complex in an off-pathway intermediate causing it to be atypically stable. Thus, it is unclear whether the ATPγS-bound CMG is stabilized by the same mechanism as the Mcm10-dependent stabilization of the CMG when ATP is present.
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