Helicase Loading at Chromosomal Origins of Replication

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Abstract:

Loading of the replicative DNA helicase at origins of replication is of central importance in DNA replication. The first of the replication fork proteins to assemble at chromosomal origins of replication, the loaded helicase is required for the recruitment of the rest of the replication machinery. In this chapter, we review the current knowledge of helicase loading at *E. coli* and eukaryotic origins of replication. In each case, this process requires both an origin recognition protein as well as one or more additional proteins. Comparison of these events shows intriguing similarities that suggest a similar underlying mechanism as well as critical differences that likely reflect the distinct processes that regulate helicase loading in bacterial and eukaryotic cells.

Introduction

Replicative DNA helicase loading is a critical and highly regulated event in chromosomal replication. The DNA helicase is the first of the replication fork proteins recruited to and loaded onto origins of replication and the loaded helicase is required for the recruitment of the rest of the replication machinery (Kaguni 2011; Remus and Diffley 2009). Indeed, the replicative DNA helicase links the replication machinery to the parental DNA (O'Donnell 2006). In *E. coli* cells, the DnaB replicative helicase binds to the RNA primase and the sliding clamp loader that in turn binds the DNA polymerases. Although the polymerases are also linked to the template DNA by sliding clamps, when these interactions are broken the polymerases association with the sliding clamp loader and the helicase keeps them at the site of replication. The interactions that tether the DNA polymerases to the eukaryotic replication fork are less clear but are very likely to involve direct and indirect interactions with the Mcm2-7 replicative helicase (Calzada et al. 2005).

Helicase loading is carefully regulated to control the location and frequency of replication initiation. In eukaryotic cells, helicase loading is tightly restricted to the G1 phase of the cell cycle. This constraint is a key part of the mechanisms that ensure that no origin can initiate more than once per cell cycle (Chapter 23). In addition, the sites of eukaryotic replicative helicase loading define the potential sites of replication initiation in the cell (although not all loaded helicases are used during a given S phase, Chapter 7). Although the central regulated event in bacterial chromosome duplication is the recruitment of the ATP-bound initiator protein DnaA (Chapter 22), the loading of the replicative helicase represents a key committed step during initiation.

In this chapter we will discuss the mechanism of helicase loading in bacteria and eukaryotic cells. Much of the discussion will focus on studies in the bacterium *E. coli*, the yeast *S. cerevisiae* and the frog *X. laevis*, where the events of helicase loading are best understood. Comparison of these mechanisms shows important similarities and differences between the

domains of life. In both bacteria and eukaryotic cells, multiple AAA+ proteins use ATP binding and hydrolysis to direct helicase loading and both helicases are initially loaded in an inactive form. On the other hand, the eukaryotic helicase is loaded around double-stranded DNA (dsDNA) and as a double hexamer, whereas the bacterial helicase is loaded around ssDNA as a single hexamer. These distinctions are very likely due to the very different regulation of DNA replication in bacteria and eukaryotic cells.

Helicase loading in *E. coli*

The *E. coli* replication origin (*oriC*) has two essential functions. One is to serve as a site where the replication fork machinery assembles. DNA sequence motifs in this chromosomal locus and their roles are described in detail elsewhere in this volume (Chapter 2). The second is to act as a site where the process of DNA replication is controlled. As described in Chapter 22, several separate mechanisms modulate the frequency of initiation of DNA replication. At this stage, a critical event is the loading of the replicative helicase named DnaB (Chapter 17), which must be complexed with its partner, DnaC. A summary of the functions of DnaA, DnaB and DnaC serves as the foundation for a description of the helicase loading process.

DnaA is the replication initiator in bacteria.

E. coli DnaA performs a central role in the initiation of DNA replication at *oriC*, recognizing specific DNA sequences named the DnaA box, I-site and τ-site within *oriC* (Chapter 2). Studies reveal that this protein, which is highly conserved among bacteria, can be divided into four domains (http://www.molgen.mpg.de/~messer/; reviewed in (Leonard and Grimwade 2011; Mott and Berger 2007; Ozaki and Katayama 2009)). Starting from the N-terminus, domain 1 (amino acids 1-90 of *E. coli* DnaA) interacts with a variety of proteins, including DnaB (Sutton et al. 1998), and DnaA itself in the assembly of the DnaA oligomer at *oriC* (Simmons et al. 2003;

Abe et al. 2007; Felczak and Kaguni 2004). These interactions are described in more detail below.

Other proteins that interact with domain 1 are HU (Chodavarapu et al. 2008a), DiaA (Keyamura et al. 2009; 2007), Dps (Chodavarapu et al. 2008b), ribosomal protein L2 (Chodavarapu et al. 2011), and RNA polymerase (Flåtten et al. 2009). The interaction with RNA polymerase correlates with the role of DnaA as a transcriptional activator in stabilizing the binding of RNA polymerase at the *gidA* and *λPR* promoters (Flåtten et al. 2009; Szalewska-Pałasz et al. 1998), and possibly at the *glpD* and *fliC* promoters (Messer and Weigel 1997; Mizushima et al. 1994). With HU or DiaA, their respective binding stabilizes DnaA oligomerized at *oriC* to stimulate initiation in vitro (Chodavarapu et al. 2008a; Keyamura et al. 2009; 2007). By comparison, L2 inhibits initiation by impairing the formation of a DnaA oligomer at *oriC* (Chodavarapu et al. 2008b; Chodavarapu et al. 2011). Like L2, Dps bound to domain 1 interferes with strand opening of *oriC* in vitro, which correlates with less frequent initiation in vivo when the Dps level is elevated (Chodavarapu et al. 2008b; Chodavarapu et al. 2011). These observations suggest that domain 1 acts as a sensor, responding to proteins that modulate the initiation process by affecting the assembly of DnaA at *oriC*.

Among DnaAs of bacteria (http://www.molgen.mpg.de/~messer/), domain 2 (residues 90-130 of *E. coli* DnaA) is not conserved in amino acid sequence or length. For *E. coli* DnaA, the removal of residues 96-120 in domain 2 leads to reduced activity in DNA replication, but deletion of other consecutive sequences from this region has no apparent effect on activity (Molt et al. 2009; Nozaki and Ogawa 2008). These observations suggest that domain 2 acts as a flexible link between domain 1 and 3.

DnaA complexed to ATP is the active form for DNA replication. As such, Domain 3 (residues 130-347 of *E. coli* DnaA) is responsible for ATP binding and hydrolysis (Duderstadt and Berger 2008). Like other members of the AAA+ family of ATPases, this region of DnaA carries the Walker A (P loop) and B boxes that are involved in ATP binding. The sensor I, II (box

VII) and box VIII motifs are thought to coordinate ATP hydrolysis with a change in conformation (reviewed in Erzberger and Berger 2006)). The adenine nucleotide bound to a shortened variant of *Aquifex aeolicus* DnaA lacking domain 1 and 2 has a striking effect on its X-ray crystal structure (Erzberger et al. 2006; Erzberger et al. 2002). With ADP, six monomers of this DnaA assemble as a closed ring. With ATP instead, an open ring with a right-handed pitch forms by virtue of a distended conformation of domain 3.

Acidic phospholipids in a fluid bilayer bind to a segment spanning the junction between domain 3 and 4 (reviewed in (Boeneman and Crooke 2005). If DnaA is complexed to an adenine nucleotide, for example ADP that remains bound after ATP hydrolysis, this interaction promotes nucleotide release (Sekimizu and Kornberg 1988). As the concentration of ATP is higher than ADP in vivo, this effect may favor the ATP-bound state of DnaA, and is the basis for speculation that this interaction may regulate the activity of DnaA. Although fluorescence microscopy indicates that DnaA associates with the inner membrane in vivo (Boeneman et al. 2009) as well as with the *oriC* region (Nozaki et al. 2009), it is not known if interactions with the inner membrane promotes nucleotide exchange in living cells.

Domain 4 (residues 347-467) acts in binding to the DnaA box. NMR and X-ray crystallographic analysis of this domain reveal a helix-turn-helix amino acid motif and a basic loop (Erzberger et al. 2002; Fujikawa et al. 2003). Combined with the biochemical characterization of mutant DnaAs bearing specific amino acid substitutions that impair the recognition of the DnaA box, we have an atomic understanding of how DnaA binds to the DnaA box (Sutton and Kaguni 1997; Blaesing et al. 2000).

DnaB is the replicative helicase, and interacts with DnaC to form the DnaB-DnaC complex.

In *E. coli*, DnaB is the replicative helicase whose native structure is a toroid of six identical subunits oriented in the same direction (Arai et al. 1981; Bailey et al. 2007; Donate et al. 2000; Reha-Krantz and Hurwitz 1978). Whereas Mg^{2+} ion stabilizes this native structure,

removal of the metal ion by dialysis or chelation causes the DnaB hexamer to dissociate into trimers and monomers (Bujalowski et al. 1994). X-ray crystallography of the homologous proteins from *Geobacillus kaustophilus* and *Geobacillus stearothermophilus* provide a detailed view of this helicase and the relative arrangement of its smaller N-terminal and larger C-terminal domain (Bailey et al. 2007; Lo et al. 2009).

Studies of *E. coli* DnaB and others in the DnaB helicase family indicate that the enzyme unwinds DNA by translocating in the 5'-to-3'direction on the single-stranded DNA to which it is bound (LeBowitz and McMacken 1986; Lee et al. 1989; Matson et al. 1983; Richardson and Nossal 1989; Venkatesan et al. 1982). Fluorescent energy transfer experiments suggest that 20 ± 3 nucleotides of single-stranded DNA pass through the central cavity of DnaB during translocation (Jezewska et al. 1996; Kaplan 2000). DnaB is uniquely oriented on a forked DNA molecule with a 5' ssDNA tail (Jezewska et al. 1998). On the basis of this orientation, the smaller N-terminal domain is upstream to the C-terminal domain, which is at the apex of the replication fork.

At the stage of initiation at *oriC*, the form of DnaB that is required is as a complex with its partner, DnaC. Early studies suggested that ATP bound to DnaC is necessary for DnaC to form an isolable complex with DnaB, and to protect DnaC from inactivation by N-ethylmaleimide (Kobori and Kornberg, 1982; Wickner and Hurwitz, 1975). More recent studies indicate that ATP is not needed for DnaC to interact with DnaB (Davey et al. 2002; Galletto et al. 2003; Mott et al. 2008). On the basis of the independent methods of cryoelectron microscopy of the DnaB-DnaC complex in comparison with DnaB, and fluorescence energy transfer experiments, DnaC is bound to the larger (C-terminal) end of the DnaB toroid (Bárcena et al. 2001; Galletto et al. 2003). Relative to the direction of helicase movement after DnaC dissociates, DnaC in the DnaB-DnaC complex is positioned proximal to the replication fork junction (Figure 1).

Because amino acid substitutions within residues 10-44 near the N-terminus of DnaC impair the interaction between DnaC and DnaB, this region of DnaC apparently binds directly to

DnaB to form the DnaB-DnaC complex (Ludlam et al. 2001). Initial studies showed that six DnaC monomers bind per DnaB hexamer (Kobori and Kornberg 1982; Lanka and Schuster 1983; Wickner and Hurwitz 1975). Subsequent sedimentation velocity analysis to determine the affinity of DnaC for DnaB in combination with estimates of the in vivo levels of DnaC and DnaB suggest that the cellular DnaB-DnaC complex is heterogeneous in composition with a fraction containing less than the maximum of six DnaC monomers per DnaB hexamer (Galletto et al. 2003).

DnaC controls the activity of DnaB.

DnaC like DnaA is a member of the AAA+ family of ATPases. Despite the presence in DnaC of motifs shared by AAA+ family members and the high affinity of other AAA+ proteins for ATP, DnaC binds weakly to this nucleotide; the Kd is about 8 μM (Davey et al. 2002; Galletto et al. 2003; Biswas et al. 2004). By itself, DnaC is also a feeble ATPase. The influence of ATP on DnaC function has been an enigma, but new evidence suggests that its ATPase activity is activated at a specific step of the initiation process (see below). Stimulated by ATP, DnaC also interacts weakly with single-stranded DNA (Davey et al. 2002; Learn et al. 1997; Mott et al. 2008), supporting the idea that this activity may be involved in helicase loading. Because DnaC complexed to DnaB inhibits its ATPase and helicase activities (Allen and Kornberg 1991; Wahle et al.1989a; Wahle et al. 1989b; Mott et al. 2008; Davey et al. 2002), DnaC must dissociate from DnaB for helicase activation to occur.

DnaA unwinds a region near the left border of oriC*.*

Assembled at *oriC*, DnaA bound to ATP induces limited unwinding of *oriC* (Bramhill and Kornberg 1988; Sekimizu et al. 1987, Figure 1). The DNA that becomes single-stranded corresponds to the region containing the M and R 13-mers based on its potassium permanganate sensitivity (Gille and Messer, 1991). In contrast, the amount of single-stranded

DNA attributed to a supercoiled *oriC* plasmid of 6.6 kb is about 400 nucleotides (Baker et al. 1987; Sekimizu et al. 1988). This estimate is based on the level of single-stranded DNA produced by DnaB in the absence of DNA gyrase after DnaB has loaded at *oriC* and begun to act as a DNA helicase. Were DNA gyrase present, it would relieve the positive supercoils brought about by the additional unwinding by DnaB. These observations suggest that DnaA is able to confine some of this single-stranded character in a supercoiled DNA to this AT-rich region. Recent studies suggest a biochemical mechanism of unwinding that requires the assembly of a DnaA oligomer as a right-handed filament (Duderstadt et al. 2011; Duderstadt et al. 2010; Erzberger et al. 2006; Ozaki and Katayama 2011; Ozaki et al. 2008). This helical structure appears to bind via interactions between the negatively charged single-stranded DNA and positively charged and hydrophobic amino acids that line the interior of the filament.

Helicase loading at oriC *by DnaA.*

DnaA then loads the DnaB-DnaC complex onto this unwound region, which apparently becomes enlarged to include the left 13-mer and additional sequences beyond it (Figure 1; Davey et al., 2002). Footprinting studies suggest that the DnaB-DnaC complex protects a region on the top strand near the left border of *oriC*. On the bottom strand, the area protected is to the left of DnaA box R1 that overlaps the right 13-mer. Quantitative analysis of this protein complex assembled at *oriC* supports the conclusion from footprinting studies that two DnaB-DnaC complexes are bound (Carr and Kaguni 2001; Fang et al. 1999).

Helicase loading appears to involve two regions of DnaA. One region is within domain 3 based on studies of a monoclonal antibody that interferes with the interaction between DnaA and DnaB measured in solid phase binding assays (Marszalek and Kaguni 1994; Sutton et al. 1998). This antibody recognizes an epitope within residues 111-148 of DnaA. Deletion analysis localized the interacting region to amino acids 135-148 (Seitz et al. 2000). A region within domain 1 is also needed because an alanine substitution for phenylalanine 46 abrogates the

interaction between DnaA and DnaB (Keyamura et al. 2009). On the basis that DnaC from Aquifex aeolicus can interact with DnaA, and like DnaA forms a helical filament as determined by X-ray crystallography, a specific proposal is that DnaA oligomerized at *oriC* loads one DnaB-DnaC complex on the bottom strand via the interaction between DnaA molecules at the left end of the DnaA filament and DnaB in the DnaB-DnaC complex (Mott et al. 2008). The second DnaB-DnaC complex loads on the top strand through the interaction between the left end of the DnaA oligomer and DnaC complexed to DnaB . During loading, it is interesting to consider that one of the interfaces between DnaB protomers must open in this ring-shaped protein in order for the helicase to encircle the single-stranded DNA.

After DnaA loads one DnaB-DnaC complex on each of the separated strands of *oriC*, DnaC must then dissociate to turn on the helicase. Of interest, recent studies suggest that three DnaC monomers are bound to each DnaB hexamer (Makowska-Grzyska and Kaguni 2010), whereas earlier experiments suggested that the DnaB-DnaC complex contains six DnaC monomers per DnaB (Kobori and Kornberg 1982; Lanka and Schuster 1983; Wahle et al. 1989a; Wickner and Hurwitz 1975). Perhaps, the different forms of the DnaB-DnaC complex respectively function at the initiation stage of DNA replication, and in restarting stalled or collapsed replication forks.

ATP or ATPγS bound to DnaC supports the DnaA-dependent loading of the DnaB-DnaC complex at *oriC* (Davey et al. 2002; Makowska-Grzyska and Kaguni 2010). That DnaC remains bound to DnaB in the presence of either ATP or the analogue suggests that the act of helicase loading does not stimulate the hydrolysis of ATP bound to DnaC or its release from DnaB (Makowska-Grzyska and Kaguni 2010). With ATP and other ribonucleotides, the inclusion of primase (DnaG), which interacts with the N-terminal domain of DnaB as it forms primers for DNA replication, induces the release of DnaC from the C-terminal (large) domain of DnaB. These observations suggest that the interaction of primase with DnaB while it synthesizes a primer leads to a conformational change in DnaB that leads to the release of DnaC. Other

results with mutant forms of DnaC that are speculated to be defective in ATP hydrolysis suggest that the interaction of primase with DnaB during primer synthesis stimulates the hydrolysis of ATP bound by DnaC that leads to activation of DnaB. This step of helicase activation is comparable in function to the activation of the Mcm 2-7 complex at a eukaryotic replication origin (Labib 2010).

Helicase Loading in Eukaryotic Cells

Origin licensing, pre-RC formation and helicase loading.

Early studies of eukaryotic DNA replication revealed that the events of replication initiation were separated into separate cell cycle stages. Mammalian cell fusion studies suggested the existence of positive factors in S phase cells that causes G1 cells to initiate DNA replication (Johnson and Rao 1970). Studies of cycling *Xenopus* egg extracts suggested the existence of a “licensing factor” that only gained access to the DNA after nuclear envelope breakdown (during mitosis) and was consumed during S phase (Blow and Laskey 1988). Finally, in vivo footprinting studies at *S. cerevisiae* origins showed a distinct and more extensive protection pattern during G1 (called the pre-replicative complex or pre-RC) than in S, G2 and M phase (Diffley et al. 1994).

We now know that replication origin licensing and pre-RC formation are both related to the helicase loading event that occurs in G1 cells (Bell and Dutta 2002; Sclafani and Holzen 2007). The “licensing factor” that is excluded from the nucleus is probably not identical among different cell types (Arias and Walter 2007) but origin licensing is clearly equivalent to helicase loading. The pre-RC footprint that is associated with *S. cerevisiae* origins during G1 is due to binding of helicase loading factors to the origin DNA (Perkins and Diffley 1998; Speck et al. 2005). Much of this footprint can be reproduced by ORC and Cdc6, however, at least part of the footprint is dependent on helicase loading. For this reason, the use of pre-RC formation has become synonymous with helicase loading. As they are more descriptive of the actual events,

we will refer to the G1 events at origins as helicase loading and the events that occur in S phase as helicase activation and replisome assembly.

Mcm2-7 Helicase

The eukaryotic replicative DNA helicase is the Mcm2-7 complex. Each Mcm2-7 complex contains one copy of the six essential and related Mcm2, Mcm3, Mcm4, Mcm5, Mcm6 and Mcm7 proteins (reviewed in Bochman and Schwacha, 2009). Although high-resolution structural data is not available, electron microscopy and subunit interaction studies indicate that the Mcm2-7 proteins interact in a defined order (Davey et al. 2003; Bochman et al. 2008; Yu et al. 2004) to form either a ring (Remus et al. 2009) or a gapped ring (Costa et al. 2011) with a positively charged central channel (Fig. 2). The C-terminal half of each Mcm2-7 subunit contains a conserved AAA+ domain that includes insertions specific to the Mcm2-7 proteins (Iyer et al. 2004) that are predicted to form beta-hairpins that may interact with the single-stranded DNA during unwinding (Bochman and Schwacha 2009). As with other AAA+ multimers, the interface between subunits in the Mcm2-7 complex forms an ATPase active site. The N-terminal half of each subunit contains an OB-fold related motif that is found in many ssDNA binding proteins and several of the subunits also contain a Zn-finger motif. Although the AAA+ domain is highly conserved between Mcm2-7 subunits the characteristic N- and C-terminal extensions allow homologs for each of the six Mcm2-7 proteins to be readily identified in all eukaryotes studied.

Helicase loading proteins

Eukaryotic helicase loading is directed by three additional proteins: the origin recognition complex (ORC), Cdc6 and Cdt1. ORC is a heterohexamer with five of the six subunits showing homology to AAA+ proteins, although only two of these subunits (Orc1 and Orc5) are known to bind ATP (Klemm et al. 1997). Four ORC subunits (Orc1, 2, 4 and 5) also contain potential

winged-helix domains at their C-termini. The smallest ORC subunit, Orc6, is related to TFIIB in metazoans (Shixuan Liu et al. 2011), although fungal Orc6 lacks this similarity. Like Mcm2-7 and ORC, Cdc6 is an AAA+ protein in the same initiator clade as the AAA+ ORC subunits (Iyer et al. 2004). In addition, the C-terminus of Cdc6 folds into a winged helix domain (J Liu et al. 2000). Like Orc6, Cdt1 is poorly conserved across eukaryotes, however, all Cdt1 proteins carry a pair of winged-helix domains in their C-terminal half (Jee et al. 2010; Khayrutdinov et al. 2009; Changwook Lee et al. 2004) and the most C-terminal winged helix binds to Mcm2-7 (Yanagi et al. 2002; Teer and Dutta 2008; You and Masai 2008; Jee et al. 2010). Interestingly, structural studies suggest that the winged-helix domains associated with ORC, Cdc6 and Cdt1 are related (Jee et al. 2010; Khayrutdinov et al. 2009). Mcm8 and Mcm9 are Mcm2-7-related proteins found in metazoan cells, but are not found to associate with any of the Mcm2-7 proteins either in or out of the Mcm2-7 complex (Lutzmann and Méchali 2008; Maiorano et al. 2005). Although these proteins have been implicated in helicase loading (Volkening and Hoffmann 2005; Lutzmann and Méchali 2008) this view remains controversial (Blanton et al. 2005; Hartford et al. 2011) and a direct mechanistic involvement in helicase loading has not been demonstrated.

Initial recruitment of the helicase to the origin DNA

Before helicase loading, ORC, Cdc6, Cdt1 and Mcm2-7 are recruited to the origin DNA. This complex is short-lived and is only detected *in vitro* when Cdc6 ATP hydrolysis is inhibited (Randell et al. 2006). *In vivo*, ChIP assays only detect ORC and Mcm2-7 at specific origins (Aparicio et al. 1997; Tanaka et al. 1997), presumably due to the rapid release of Cdc6 and Cdt1 after helicase loading.

Origin recognition

The first step of helicase loading is the binding of ORC to origin DNA. In the yeast *S. cerevisiae* ORC recognizes a conserved sequence within the origin of replication and this event

is ATP-dependent (Bell and Stillman 1992). This sequence is not sufficient to direct ORC binding *in vivo*. An important additional determinant is an asymmetric nucleosome-free region surrounding the consensus sequence (Eaton et al. 2010). The determinants for ORC binding in other organisms are less clear. The Orc4 subunit of *S. pombe* ORC includes an AT-hook domain that directs binding to AT-rich DNA (Chuang and Kelly 1999; Lee et al. 2001; Kong and DePamphilis 2001). Although ORC from metazoan cells does not exhibit sequence-specific DNA binding (Vashee et al. 2003; Remus et al. 2004), ORC is localized to specific sites along metazoan chromosomes, indicating the existence of other determinants for ORC localization (Karnani et al. 2010; MacAlpine et al. 2010). Genome-wide studies of metazoan ORC binding support a number of determinants including nucleosome-depleted regions, promoter proximity and dynamic nucleosomes (reviewed in (Ding and MacAlpine 2011). In addition, ORC DNA binding studies show that ORC prefers to bind negatively supercoiled DNA (Houchens et al. 2008; Remus et al. 2004), which may be related to the wrapping of DNA around ORC (Clarey et al. 2006; Clarey et al. 2008). Finally, human ORC binds a protein called ORCA or LRWD1 that interacts with modified nucleosomes that are associated with heterochromatin, suggesting an alternative method to localize ORC to compacted chromatin (Shen et al. 2010; Vermeulen et al. 2010; Bartke et al. 2010).

Interaction of Cdc6, Cdt1 and Mcm2-7

As cells enter G1 phase, ORC recruits Cdc6, Cdt1 and Mcm2-7 to the origin DNA. Biochemical studies support a model in which ORC first interacts with Cdc6 and this complex then recruits Cdt1 and Mcm2-7. Loss of Cdt1 does not prevent Cdc6 chromatin association *in vivo* or origin recruitment *in vitro* (Randell et al. 2006; Nishitani et al. 2000; Maiorano et al. 2000; Tsuyama et al. 2005; Remus et al. 2009). In contrast, elimination of Cdc6 interferes with Cdt1 origin binding *in vitro* (Remus et al. 2009; Randell et al. 2006). Studies in *Xenopus* extracts have shown that Cdt1 associates with chromatin in the absence of Cdc6 (Gillespie et al. 2001;

Tsuyama et al. 2005), however, only Cdt1 associated in the presence of Cdc6 is able to contribute to Mcm2-7 loading (Tsuyama et al. 2005). Consistent with a robust interaction between Cdc6 and ORC, a complex between the proteins has been structurally characterized (Speck et al. 2005) and origin-bound ORC stimulates Cdc6 ATP hydrolysis (Randell et al. 2006).

Mcm2-7 and Cdt1 are recruited to the origin as a complex. The C-terminal winged-helix domain of Cdt1 binds Mcm2-7 (Zhang et al. 2010; Yanagi et al. 2002; Teer and Dutta 2008; You and Masai 2008; Ferenbach et al. 2005; Takara and Bell 2011). Cdt1 mutants lacking this region prevent or dominantly inhibit Mcm2-7 loading (Zhang et al. 2010; Ferenbach et al. 2005; Takara and Bell 2011). In *S. cerevisiae* cells Cdt1 and Mcm2-7 are imported to the nucleus as a complex (Tanaka and Diffley 2002) and formation of this complex is necessary for the recruitment of either protein to the origin (Takara and Bell 2011). The primary Cdt1 binding site on Mcm2-7 is the C-terminus of Mcm6 and structural studies suggest that this region also folds into a winged-helix domain (Wei et al. 2010; Yanagi et al. 2002).

Although short-lived, the helicase loading intermediate composed of ORC, Cdc6, Cdt1 and Mcm2-7 suggests how a single asymmetric ORC protein can direct the assembly of a bidirectional replication fork. Binding of Cdt1/Mcm2-7 to the origin requires the smallest ORC subunit, Orc6, which includes two binding sites for Cdt1 (Chen et al. 2007). Analysis of the protein complex formed at the origin prior to helicase loading (when Cdc6 ATP hydrolysis is inhibited) indicates that multiple Cdt1 proteins and their associated Mcm2-7 complexes are initially recruited to each ORC/Cdc6 complex (Takara and Bell 2011). It is most likely that this complex contains two Cdt1 molecules, one bound to each of the two Cdt1 binding sites on Orc6. Importantly, interfering with the formation of this multi-Cdt1 intermediate prevents subsequent

helicase loading and it is likely that this intermediate facilitates the simultaneous loading of a Mcm2-7 double hexamer (see below).

How Cdc6 contributes to Cdt1 and Mcm2-7 recruitment is less well understood. It is possible that there are direct interactions between Cdc6 and Cdt1 and/or Mcm2-7. Alternatively, Cdc6 could alter the conformation of ORC to allow Cdt1 and Mcm2-7 to bind. The latter hypothesis would explain why ORC does not bind either Cdt1 or Mcm2-7 in the absence of Cdc6 (Takara and Bell, 2011) but isolated Orc6 can bind Cdt1 (Chen et al. 2007).

Loading of recruited helicases

After recruitment of the helicase loading factors and Mcm2-7 to the origin, loading of the recruited Mcm2-7 requires ATP hydrolysis and involves significant changes of both the proteins associated with the origin and the structure of the Mcm2-7 ring. Loaded Mcm2-7 complexes form double-hexameric pairs that encircle dsDNA. Thus, helicase loading necessarily requires the establishment of strong interactions between the N-termini of two Mcm2-7 complexes and opening and closing of the Mcm2-7 ring to allow DNA access to the Mcm2-7 central channel. Finally, to ensure the proper regulation of replication initiation, as initially loaded, the Mcm2-7 complexes are inactive for unwinding the parental duplex.

Structure of loaded Mcm2-7.

The reconstitution of Mcm2-7 loading using four purified proteins has led to important advances in the understanding of the architecture of loaded Mcm2-7. Electron microscopy (EM) of loaded Mcm2-7 shows head-to-head “double hexamers” with the N-termini of the Mcm2-7 subunits mediating the interactions (Remus et al. 2009; Evrin et al. 2009). This structure is similar to the double hexamers observed for the homohexameric archaea Mcm complexes (Brewster and Chen 2010). Unlike the archaea homologs, Mcm2-7 double hexamers are only

detected after loading. Mcm2-7 double hexamers survive treatment with DNase and gel filtration, indicating that once formed these complexes are very stable (Evrin et al. 2009; Gambus et al. 2011). Based on structural studies of the archaea Mcm complex, these interactions likely involve the Zn-finger domains in the N-termini of Mcm2-7 subunits (Fletcher et al. 2003). The inter-Mcm2-7 contacts within the double hexamer are unknown but must be largely heterotypic due to their head-to-head interaction. A lack of loaded single hexamers and the presence of multiple Cdt1/Mcm2-7 complexes prior to Mcm2-7 loading supports a model in which both hexamers are loaded in a concerted fashion (Remus et al. 2009; Takara and Bell 2011). Importantly, both EM and topological linkage studies indicate that loaded Mcm2-7 complexes encircle and slide non-directionally on dsDNA (Evrin et al. 2009; Remus et al. 2009). Thus, loaded Mcm2-7 is topologically linked to the DNA but is neither active as a helicase nor tightly engaged with the DNA.

Role of ATP during helicase loading.

ATP binding and hydrolysis plays critical roles during helicase loading. As described above, ATP binding but not hydrolysis is required for the initial recruitment of the helicase to the origin (Randell et al. 2006; Gillespie et al. 2001). ATP hydrolysis by Cdc6 and ORC plays distinct and ordered roles in helicase loading. Cdc6 is activated to bind and hydrolyze ATP when it associates with origin-bound ORC and this hydrolysis event is required to observe Mcm2-7 loading (Perkins and Diffley 1998; Randell et al. 2006). ORC ATP hydrolysis is also essential but functions after Cdc6 hydrolysis and appears to regulate repeated Mcm2-7 loading (Bowers et al. 2004; Randell et al. 2006). One likely role for ORC ATP hydrolysis is to reset its activity for another cycle of helicase loading by driving the release of the helicase loading factors from the origin (Tsakraklides and Bell 2010). Whether ORC ATP hydrolysis is required for the loading of each double hexamer or for repeated rounds of double hexamer loading remains unclear. Overall, the coordinated and ordered set of ATP hydrolysis reactions ensures

that Cdc6 only acts on Cdt1-Mcm2-7 when bound to ORC at the origin and a new round of loading cannot occur until the previous round is complete.

Mcm2-7 gate function and implications for loading.

The Mcm2-7 ring structure must be opened during origin loading to provide access to the central DNA binding channel. Binding of purified Mcm2-7 to ssDNA circles suggests the interface between Mcm2 and Mcm5 acts as a “gate” to the central channel (Bochman and Schwacha 2008) and EM studies of purified *Drosophila* Mcm2-7 support this conclusion (Costa et al. 2011). Although ATP binding is thought to close the Mcm2/5 gate, it is likely that some combination of Cdt1 binding and Cdc6 ATP hydrolysis modulates Mcm2-7 ring opening during helicase loading. Because Cdt1 is released from Mcm2-7 after loading (Randell et al. 2006), it is tempting to speculate that Cdt1 binding opens the Mcm2/5 gate and Cdc6 ATP hydrolysis separates Cdt1 from Mcm2-7 leaving behind a closed form of the Mcm2-7 ring. The role of Mcm2-7 ATP binding and hydrolysis during helicase loading is uncertain. Mutations in the Mcm6 and Mcm7 ATP binding sites show normal chromatin association but are defective for replication initiation (Ying and Gautier 2005). It is unclear, however, whether the observed chromatin association reflects loading or just Mcm2-7 recruitment. In addition, the distinct functions of six Mcm2-7 ATPase active sites (reviewed in Bochman and Schwacha 2009) suggest that ATP bound to these other sites could be required for helicase loading.

Regulation and dynamics of helicase loading.

Helicase loading is tightly regulated during the cell cycle to ensure that no origin of replication can initiate DNA replication more than once per cell cycle (Chapter 23 and reviewed in Arias and Walter 2007). This regulation is mediated by the inhibition of helicase loading outside of G1 phase. The mechanisms of inhibition are diverse and vary between organisms. In budding yeast, inhibition is primarily mediated by CDK phosphorylation of helicase loading

proteins leading to Cdc6 degradation (Drury et al. 2000), Mcm2-7 nuclear export (Labib et al. 1999; Nguyen et al. 2000) and the inhibition of Cdt1/Mcm2-7 binding to ORC (Chen and Bell 2011). In metazoan organisms, Cdt1 is a primary target for inhibition. Cdt1 is inhibited by geminin binding outside of G1 (Wohlschlegel et al. 2000; McGarry and Kirschner 1998) and degraded in a PCNA/DNA-dependent (Arias and Walter 2005) or CDK-dependent (Li et al. 2003; Enbo Liu et al. 2004) fashion in S phase. In addition, in many metazoans CDK activity inhibits ORC DNA binding during G2/M phase (reviewed in DePamphilis 2005). Although it is tempting to think that the multiple mechanisms that inhibit helicase loading are redundant, analysis of mutants that are defective for a subset of mechanisms show partial rereplication (Green et al. 2006). Importantly, even limited re-replication is lethal to most dividing cells.

The role of the helicase loading proteins after loading is unclear. *In vitro* studies show that after loading Mcm2-7 no longer requires ORC, Cdc6 or Cdt1 to associate with origin DNA (Randell et al. 2006; Donovan et al. 1997). Consistent with this observation, *in vitro* studies show that the helicase loading proteins are released from the origin upon Mcm2-7 loading (Tsakraklides and Bell 2010; Randell et al. 2006). In contrast to these findings, *in vivo* inactivation of *S. cerevisiae* ORC or Cdc6 in late G1 (after Mcm2-7 loading) results in loss of Mcm2-7 origin association (Aparicio et al. 1997; Semple et al. 2006; Chen et al. 2007). This suggests that either these proteins are required to maintain Mcm2-7 association with the origin DNA or that there is an activity that removes loaded Mcm2-7 from the DNA in G1 cells and the helicase loading proteins are required to restore Mcm2-7 to the origin. In either case, this reliance on helicase loading proteins changes upon entry into S phase, as Mcm2-7 is tightly associated with origins at this stage (unless initiation has occurred) and Cdc6 is degraded. It is noteworthy that studies in mammalian G1 cells suggest that there is little exchange between chromatin associated and free Mcm2-7 (Kuipers et al. 2011), arguing for a model in which

helicase loading proteins stabilize loaded Mcm2-7 rather than the model in which Mcm2-7 release is balanced with new loading.

A model for helicase loading

Based on current knowledge, we propose the following model for the events of eukaryotic helicase loading (Fig. 3). ORC recruitment to origin DNA is directed by a combination of DNA affinity (sequence specific in yeast), local chromatin structure and interaction with other proteins (e.g. ORCA). Upon entry into G1 phase ORC recruits Cdc6. In *S. cerevisiae* cells, we propose that this interaction reveals Cdt1 binding sites on Orc6 that are bound by two Cdt1/Mcm2-7 complexes. The distinct structure of Orc6 in metazoans raises the possibility that the initial recruitment is mediated by different interactions in these organisms. The connection of these events to Mcm2-7 ring opening is unclear, however, it seems most likely that the Mcm2/5 gate is opened at some point during these events. Alternatively the ring could be opened before recruitment (e.g. after Cdt1 binds to Mcm2-7), although this would risk Mcm2-7 interacting with non-origin DNA. Assuming two Mcm2-7 complexes are loaded coordinately, we suggest that the Mcm2/5 gates of the two helicases are adjacent to one another to form a single continuous opening/gate. By analogy to the function of the sliding clamp loader function (Kelch et al. 2011), we speculate that the Mcm2-7 complexes can encircle the DNA at this stage but remain open. The next stage of loading is triggered by Cdc6 ATP hydrolysis. This event leads to the release of Cdc6 and Cdt1 from the helicase and this event is coupled to the closing of the Mcm2-5 gate with the DNA enclosed within the Mcm2-7 central channel. Finally, we propose that ATP hydrolysis by ORC leads to the release of the loaded helicases from ORC to prepare for a new round of loading after ADP->ATP exchange.

Once loaded the Mcm2-7 complex awaits activation. Biochemical studies show that this activation requires the association of two helicase activating proteins (Cdc45 and GINS) with

Mcm2-7 (Ilves et al. 2010). Association of these proteins is triggered by the action of two kinases: S-phase cyclin dependent kinases (S-CDK) and the Dbf4-dependent Cdc7 kinase (DDK). DDK phosphorylates Mcm2-7 (Randell et al. 2010; Sheu and Stillman 2006; 2010). As a result of Mcm2-7 phosphorylation, Cdc45 associates with Mcm2-7 (Heller et al. 2011; Tanaka et al. 2011). Interestingly, this event occurs at a subset of origins prior to entry into S phase (Heller et al. 2011; Tanaka et al. 2011; Aparicio et al. 1999; Kanemaki and Labib 2006). S-CDKs targets two additional proteins, Sld2 and Sld3, causing them to associate with Dpb11 and eventually resulting in the recruitment of the GINS proteins as well as DNA Pol ε (Zegerman and Diffley 2007; Tanaka et al. 2007; Muramatsu et al. 2010). The subsequent activation of DNA unwinding is required for the recruitment of the lagging strand DNA polymerases (DNA Pol α/primase and DNA Pol δ (Heller et al. 2011; Mimura et al. 2000)

Comparison of helicases and helicase loading in E. coli and eukaryotic cells.

There are many similarities between helicase loading at *E. coli* and eukaryotic chromosomal origins that suggest that the fundamental mechanism of these events are related. Both *E. coli* DnaB and eukaryotic Mcm 2-7 are composed of six subunits that assemble into ring-shaped molecules. Second, both events rely an origin recognition proteins (DnaA and ORC) that contact the origin DNA over an extended region. Third, loading of each DNA helicase requires at least one additional helicase loading factor that binds to the helicase. In *E. coli*, DnaC is required for loading and also restrains DnaB helicase activity. In eukaryotes, both Cdc6 and Cdt1 share properties with DnaC. Like DnaC, Cdc6 is a AAA+ protein and is a weak ATPase on its own whereas Cdt1 shares DnaC's association with its cognate helicase. Finally, both helicases must be activated after loading.

There are also a number of important differences between the *E. coli* and eukaryotic helicase loading. First, although similar in overall structure, DnaB and the Mcm2-7 complexes have opposite polarities: DnaB moves on the lagging strand template and Mcm2-7 moves on

the leading strand template. DnaB is a homohexamer that has a RecA-like ATPase domain (Bailey et al. 2007) while Mcm2-7 is a heterohexamer that has AAA+ like ATPase domains (Iyer et al. 2004). Second, while DnaA origin binding leads to local DNA melting, ORC DNA binding does not. Third, two separate single hexamers of DnaB are loaded in opposite orientations around ssDNA after loading, whereas, a head-to head double hexamer of Mcm2-7 is loaded around dsDNA.

These differences are most likely related to the rate of helicase activation after loading. Loading of DnaB around ssDNA favors the rapid activation shortly after loading that is observed at *oriC*. In contrast, the need to restrain helicase activation until S phase in eukaryotic cells suggests a loaded form that is less easily activated. Recent findings indicate that Mcm2-7 surrounds ssDNA and acts as a single hexamer at the replication fork (Fu et al. 2011; Yardimci et al. 2010). This indicates that Mcm2-7 activation requires two major changes in the loaded double hexamer: severing the connections between the Mcm2-7 complexes in the double hexamer and a second opening of the Mcm2-7 ring to expel one of the two ssDNAs prior to extended DNA unwinding. Although we have an initial understanding of how DnaC expulsion leads to activation of DnaB (Makowska-Grzyska and Kaguni 2010), how the helicase activating proteins Cdc45 and GINS and the proteins that direct them to the origin (Sld2, Sld3 and Dpb11) catalyze these events remains unknown.

Although 25 years of research has provided an increasingly detailed understanding of how helicase loading occurs, fundamental questions that remain. How is the opening and closing of the DNA helicase ring controlled? How is the origin DNA positioned correctly to ensure that the closed helicase encircles the correct form of the DNA at the correct position? How are these events regulated by the AAA+ ATPases that are conserved between the different realms of life? These and other questions will continue to drive the replication to study this central event in replication initiation.

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Figure legends:

Figure 1. Helicase loading at the *E. coli* replication origin (*oriC*) is a stepwise process. Near the top of the figure, DNA sequence elements in *E. coli oriC* are shown (see Chapter 2 for details). In step 1, DnaA complexed to ATP binds to the DnaA boxes, I- and τ-sites to form a DnaA oligomer. Following the unwinding of the region of *oriC* containing the 13-mers, DnaA then loads a DnaB-DnaC complex on each of the separated strands (step 2). In step 3, primase (DnaG) interacts with the N-terminal region of DnaB. Primer formation by primase leads to the dissociation of DnaC from the C-terminal domain of DnaB, which is necessary to activate DnaB as a DNA helicase (see Chapter 17). Although not shown, the primers shown are then extended by the cellular replicase, DNA polymerase III holoenzyme, for the continuous synthesis of each leading strand at each replication fork (see Chapter 15). A dimer of this DNA polymerase is thought to be at each replication fork, DnaB is proposed to interact with one unit of a dimer of DNA polymerase III holoenzyme to coordinate the unwinding of the parental DNA with DNA synthesis. As this DNA helicase translocates in the 5'-to-3' direction with the replication fork to support its movement, primase occasionally interacts with DnaB to synthesize additional primers. These primers are used by the unit of the DNA polymerase dimer that synthesizes Okazaki fragments.

Figure 2. Structure of the Mcm2-7 complex. A. The Mcm2-7 proteins assemble into a hexameric torroid. Each Mcm2-7 complex includes one copy of the Mcm2-7 proteins that are arranged in the indicated defined order around a central channel. B. Directionality of Mcm2-7 movement. The Mcm2-7 proteins move in a 3'->5' direction along single stranded DNA. Based on analogy with the archaea homologs, the C-terminal AAA+ motif is proximal to the replication fork.

Figure 3. Model for eukaryotic replicative DNA helicase loading. After localization to the origin DNA, ATP-bound ORC recruits Cdc6 bound to ATP. The resulting ORC/Cdc6 complex then recruits two Cdt1/Mcm2-7 complexes via interactions between Cdt1 and Orc6. Although this illustration suggests that the Mcm2-7 complexes have initiated interactions at their N-termini at this stage, it is also possible these interactions only occur during or after helicase loading. The interactions between ORC, Cdc6, Cdt1 and Mcm2-7 are proposed to result in the opening of the Mcm2-7 ring at the Mcm2-5 gate. Cdc6 ATP hydrolysis results in the loading of a Mcm2-7 double hexamer around double stranded origin DNA and the release of Cdt1 from the origin. Whether the DNA enters the Mcm2-7 central channel before (upon initial ring opening) or after (as illustrated) Cdc6 ATP hydrolysis is unknown. ORC ATP hydrolysis is proposed to lead to the release of Cdc6-ADP and loaded Mcm2-7 from ORC. ORC ADP/ATP exchange leads to resetting of the loading machinery allowing a new round of helicase loading to initiate.

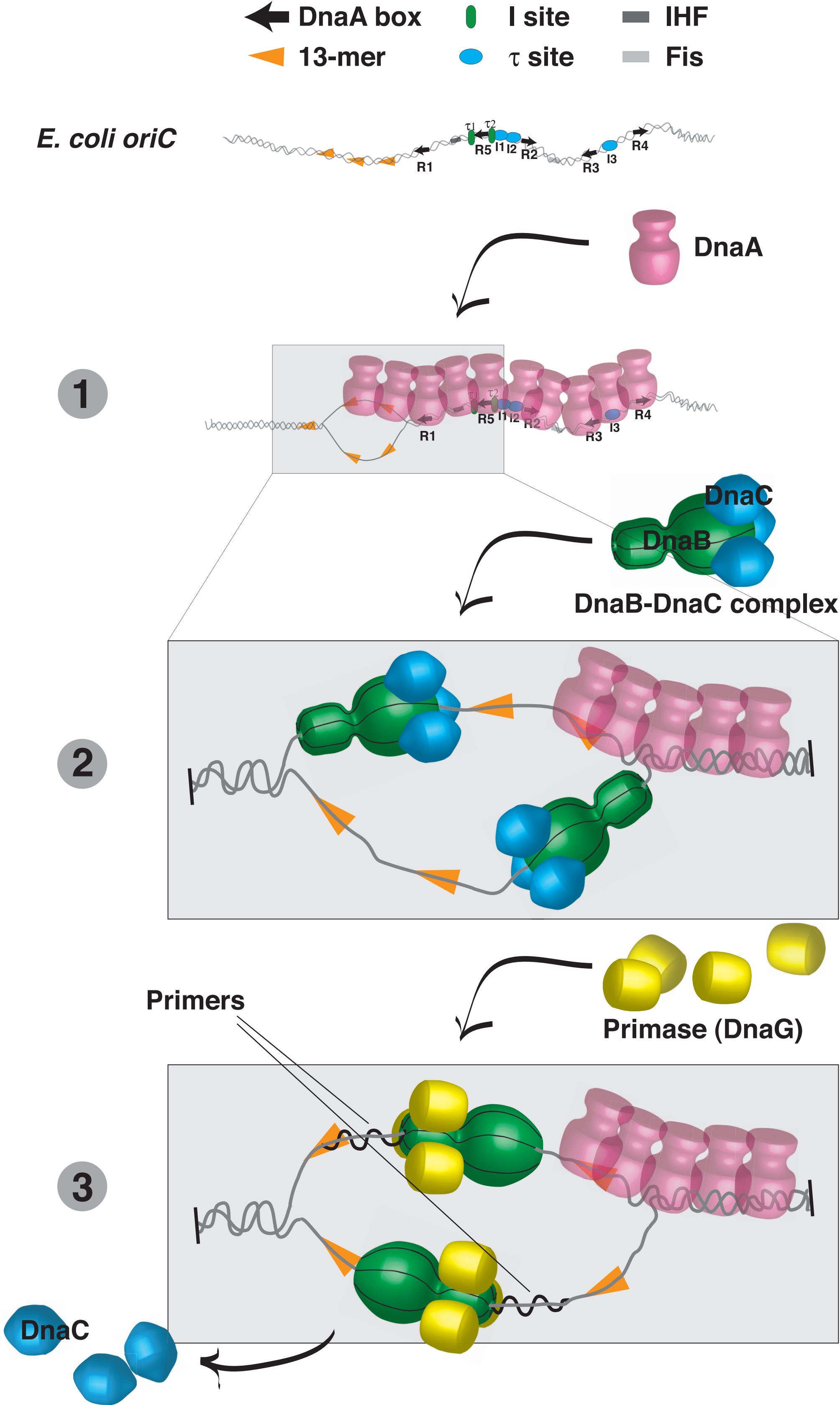
DnaA box
I site
IHF
13-mer
τ site
Fis
E. coli oriC
τ1
τ2
R5
I1
I2
R2
R1
R3
I3
R4
DnaA
1
DnaC
DnaB
DnaB-DnaC complex
2
Primers
Primase (DnaG)
3
DnaC

Fig. 2, Bell and Kaguni

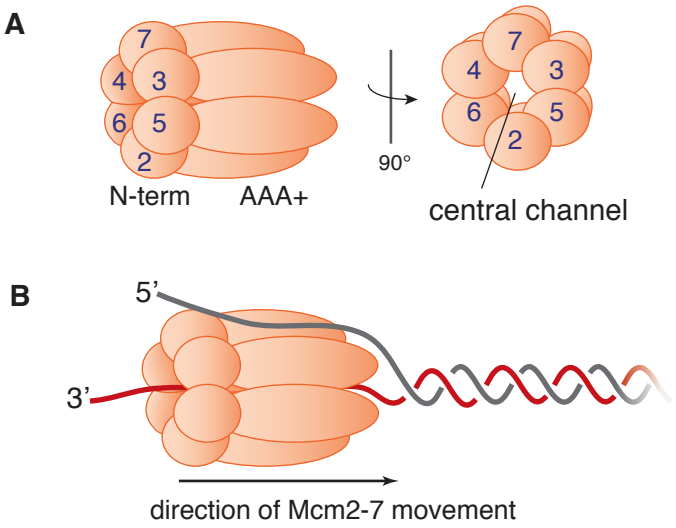
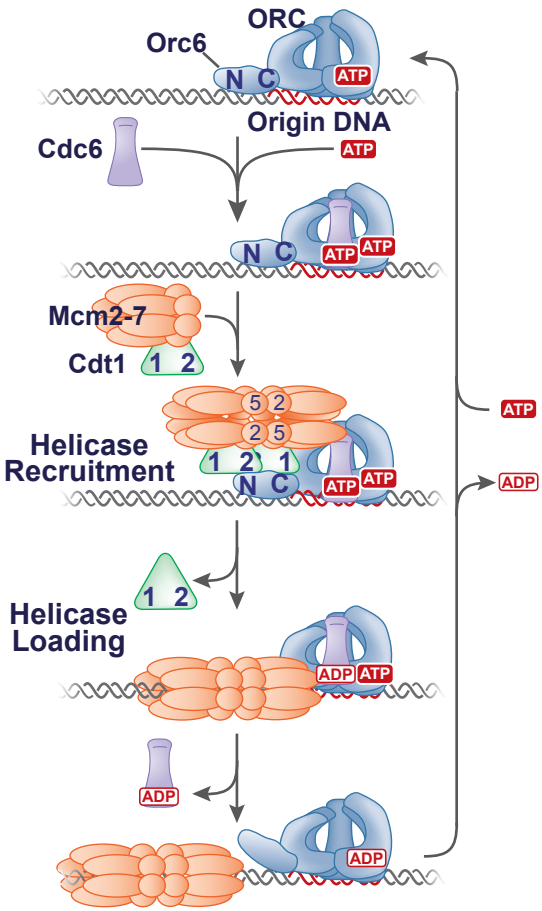
Fig. 3, Bell and Kaguni