Multiple roles of the replication initiation protein Cdt1 during helicase loading in *S. cerevisiae*

by

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ABSTRACT

The faithful transmission of genetic information is critical for the events of cell division and propagation. In eukaryotic cells, chromosomal replication is carefully coordinated with the cell cycle to ensure that the entire genome is replicated exactly once prior to cell division. Underpinning this coordination is the tightly regulated loading and activation of the eukaryotic replicative DNA helicase, the hetero-hexameric Mcm2-7 complex. As cells enter G1 phase of the cell cycle, all potential sites of replication initiation are selected by the loading of inactive Mcm2-7 double hexamers. The anti-parallel orientation of the Mcm2-7 hexamers within the double hexamer is proposed to be critical to establish bi-directional sister replisomes upon helicase activation in S phase. Although the proteins involved in helicase loading are known, the mechanism that drives Mcm2-7 double-hexamer formation and loading is unclear.

The replication initiation protein Cdt1 is essential for loading Mcm2-7 onto origin DNA, but its functions during the loading event are unclear. Through analysis of Cdt1 mutations, I identified regions of Cdt1 that are essential for Mcm2-7 helicase binding, origin recruitment, and activation. I found that multiple Cdt1 molecules are recruited to the origin during the helicase-loading process, and disrupting of the assembly of the multi-Cdt1 intermediate prevents Mcm2-7 loading. Finally, I demonstrated that the N-terminal domain of Cdt1, although dispensable for stable helicase loading, is required for subsequent helicase activation and replication initiation.

These findings reveal that Cdt1 performs multiple functions during helicase loading and influences the competence of loaded Mcm2-7 to subsequently become activated. This work provides insight into the process of Mcm2-7 double-hexamer loading and supports a model in which Cdt1 initiates Mcm2-7 double-hexamer formation early in the helicase-loading process.

Thesis Supervisor: Stephen P. Bell
Title: Professor of Biology

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I am greatly indebted to my close friends and family, whose unwavering support and unparalleled spirits inspire me and guide me on a daily basis. Although they are spread across the country, I hold each one of them close to my heart. I am grateful to have them in my life.

Finally, I would like to recognize Grandma Bea and Grandpa Tom. Their unconditional love, patience, intelligence, and guidance have profoundly shaped my life. This thesis is dedicated in loving memory of them.

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Chapter I

Introduction

OVERVIEW

Each time a eukaryotic cell divides, it must ensure that all its genetic information is faithfully transmitted from mother to daughter cell. With a large multi-chromosome genome that can range in size from millions of DNA base pairs (in the unicellular budding yeast *Saccharomyces cerevisiae*) to billions of DNA base pairs (in the multi-cellular human), this feat requires that the cell accurately and precisely duplicate all its chromosomes in a timely fashion prior to chromosome segregation and cell division. Eukaryotic cells tightly regulate how and when genomic duplication occurs. Aberrant genomic over- and under-replication events result in genomic instability, a hallmark of cancer. Therefore, to ensure that its entire genome is replicated exactly once, the eukaryotic cell coordinates chromosomal replication to the cell cycle (Arias & Walter, 2007).

The initiation of DNA replication in all eukaryotes occurs in two temporally distinct phases: helicase loading and helicase activation/replisome assembly (Fig. 1). The separation of these two phases is established by the activity of two kinases, cyclin-dependent kinase (CDK) and Dbf4-dependent kinase (DDK). In G1 phase of the cell cycle, when CDK and DDK activity is low, all potential sites of replication initiation (called origins of replication) are selected by the loading of the eukaryotic replicative helicase, the hexameric Mcm2-7 complex. This process has also been referred to as origin licensing or pre-Replicative Complex (pre-RC) formation. Helicase loading is initiated by the binding of the six-protein Origin Recognition Complex (ORC) to each origin of

Figure 1

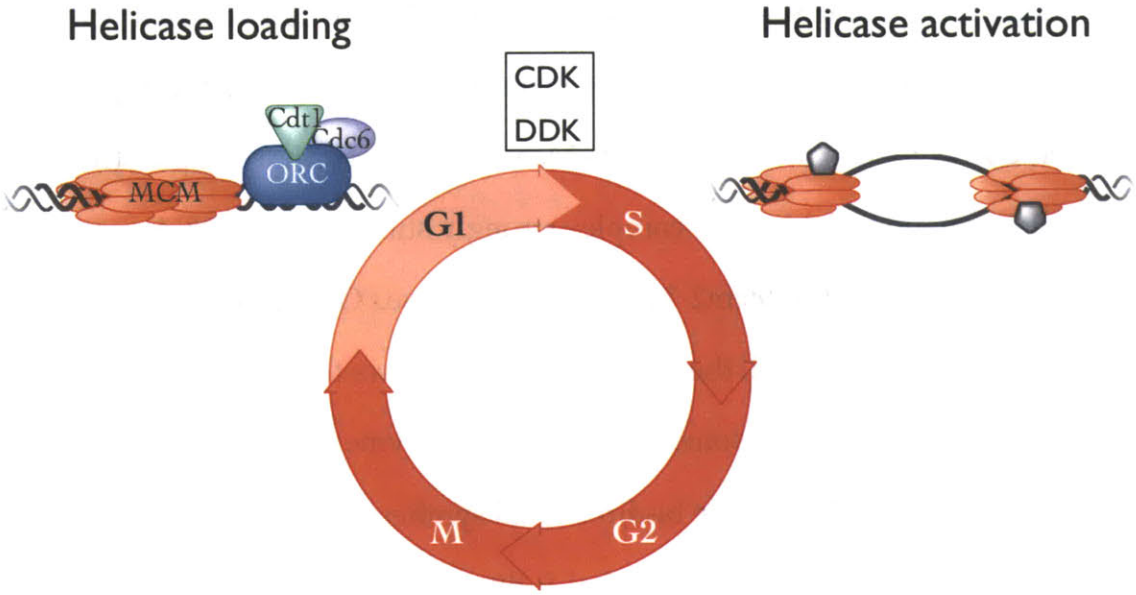

Figure 1. Eukaryotic DNA replication initiation occurs in two temporally distinct phases. In late M and G1 phases of the cell cycle, when CDK activity is low, all potential sites of replication initiation, called origins of replication, are selected by the loading of the Mcm2-7 helicase onto origin DNA. Mcm2-7 is loaded as an inactive double hexamer. An increase in the activity of CDK and DDK triggers the recruitment of helicase-activating factors and replisome components (gray pentagons) to a subset of loaded Mcm2-7 complexes. The activated Mcm2-7 helicase unwinds DNA and initiates bi-directional DNA replication from origins. During S, G2, and early M phases (dark pink), *de novo* helicase loading is prevented via numerous mechanisms in across eukaryotes.

replication (Wyrick et al, 2001). In late M and early G1 phases, ORC recruits Cdc6, Cdt1, and Mcm2-7 to the origin. Together, these proteins load the ring-shaped Mcm2-7 helicase onto origin-proximal DNA as an inactive head-to-head double hexamer encircling dsDNA (Evrin et al, 2009; Remus et al, 2009).

At the onset of S phase, increased CDK and DDK activity results in the activation of loaded Mcm2-7 helicases. Helicase activation is associated with the recruitment of the helicase-activating proteins, Cdc45 and the GINS complex, to the Mcm2-7 helicase to form the Cdc45/Mcm2-7/GINS (CMG) complex (Ilves et al, 2010). Formation of the CMG complex strongly stimulates the DNA unwinding activity of the Mcm2-7 replicative helicase. Once a sufficient length of DNA duplex is unwound, the exposed ssDNA allows the formation of two sister replisomes. Each replisome contains DNA polymerases α, δ, and ε, which function together to catalyze the bi-directional synthesis of new DNA behind the DNA-unwinding CMG complex (Kunkel & Burgers, 2008).

Helicase loading and helicase activation are carefully regulated to ensure that the entire genome is replicated exactly once per cell cycle (Labib, 2010). Helicase loading is restricted to G1 phase, during which time no helicases can be activated due to low levels of CDK and DDK activity. In S phase, elevated levels of CDK and DDK activity trigger helicase activation. Importantly, the environment in S, G2, and M phases is refractory to *de novo* helicase loading, which cannot occur until the next G1 phase.

To effectively control the initiation of replication, eukaryotes load the Mcm2-7 replicative helicase in a tightly regulated, step-wise process (Remus & Diffley, 2009). Although some aspects of the helicase-loading process are well characterized, other aspects remain to be elucidated at the molecular level. For example, it is unclear how and when the Mcm2-7 double hexamer is formed during the loading event, or the process by which the Mcm2-7 ring is opened and closed during its loading. In addition, it remains unknown how the different helicase-loading proteins contribute to this event.

This thesis focuses on the essential contribution of Cdt1 to the event of Mcm2-7 helicase loading. I have used a combination of biochemistry and genetics to determine how Cdt1 facilitates the loading of replication-competent Mcm2-7 double hexamers. I identified regions of Cdt1 that are essential for binding the Mcm2-7 helicase, recruiting the helicase to the origin, and loading the helicase in a conformation that supports subsequent activation by Cdc45 and GINS. Additionally, I found that multiple Cdt1/Mcm2-7 complexes are initially recruited to the origin during the loading process, suggesting that double-hexamer formation depends on Cdt1 and occurs prior to the loading event. In this introduction, I review our current understanding of helicase loading and activation as well as the known roles of Cdt1 during the helicase-loading process. I will begin with a brief overview of origins of replication, the genomic sites of Mcm2-7 loading and replication initiation.

ORIGINS OF REPLICATION

Escherichia coli

Almost half a century ago, Jacob and Brenner proposed the replicon model for the regulation of DNA replication in *Escherichia coli* (Jacob & Brenner, 1963). In this model, a protein (the initiator) recognizes a specific DNA sequence (the replicator) to activate the initiation of replication. The model was subsequently validated in *E. coli* with the discovery of *oriC*, a discrete DNA sequence that conferred replication competence to a non-replicating plasmid (Yasuda & Hirota, 1977), and its cognate initiator protein, DnaA (Fuller & Kornberg, 1983). A member of the AAA+ family of ATPases, DnaA recognizes *oriC* with equal affinity in its ATP- or ADP-bound state, but only ATP-bound DnaA supports DNA melting and replication (Sekimizu et al, 1987). Approximately 250 bp in length, *oriC* contains five 9-bp sequence-specific binding sites for DnaA called DnaA boxes (Fuller et al, 1984). Within *oriC*, three tandem repeats of an AT-rich 13mer sequence function as a DNA Unwinding Element (DUE), at which the melting of the DNA duplex initiates (Bramhill & Kornberg, 1988) (Fig. 2A).

The initiation of bacterial replication occurs in a stepwise manner (Fig. 2B). First, multiple ATP-bound DnaA proteins recognize and bind to the high-affinity DnaA box binding sites within *oriC*. When bound to ATP, DnaA also recognizes a 6-bp low-affinity binding site called the ATP-DnaA box within the replicator (Speck et al, 1999). The oligomerization of multiple *oriC*-bound DnaA

Figure 2

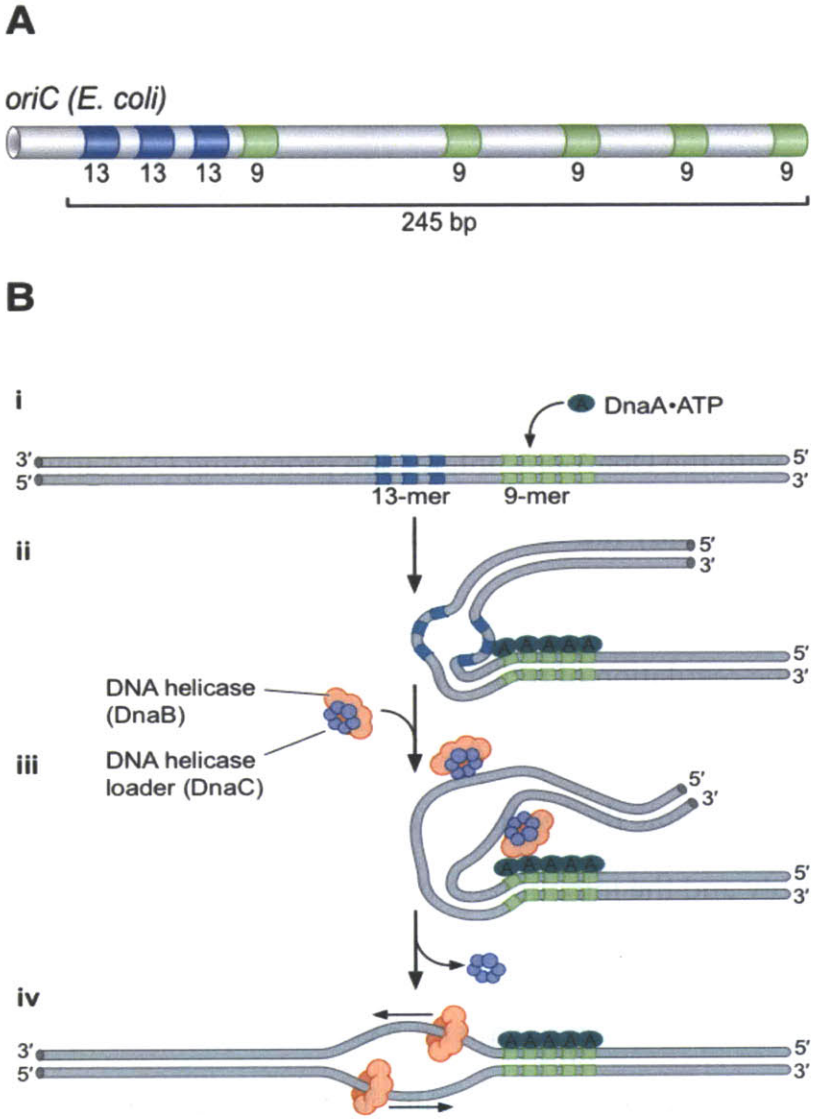

Figure 2. Replication initiation in *E. coli* occurs in a step-wise manner at the *oriC* replicator. (A) The architecture of the *oriC* replicator. Schematic of *oriC* with 9-bp DnaA boxes (in green) and 13mer AT-rich DUEs (in teal) depicted. ATP-DnaA boxes not shown. **(B)** Loading the bacterial replicative helicase DnaB. (i) ATP-bound DnaA binds *oriC*, recognizing 9mer DnaA boxes as well as high affinity ATP-DnaA box. (ii) Oligomerization of multiple *oriC*-bound DnaA lead to binding and origin melting at 13mer DUE sites. (iii) ATP-bound DnaC assists in the sequential loading of two DnaB hexamers onto ssDNA. (iv) Loaded DnaB are active to unwind DNA and proceed bi-directionally on ssDNA in a 5′ to 3′ direction.

leads to the cooperative binding of DnaA at the DUE and the initial melting of the DNA duplex. Next, DnaA cooperatively binds and stabilizes the newly exposed ssDNA within the melted DUE (Speck & Messer, 2001). Finally, the helicase loader, DnaC, assembles two copies of the bacterial replicative helicase, DnaB, onto the stabilized ssDNA template in an ATP-dependent manner (Bramhill & Kornberg, 1988). Replication subsequently initiates from the single *oriC* replicator bi-directionally (Marsh & Worcel, 1977). Not surprisingly, *oriC* overlaps with the single origin of replication in *E. coli* (von Meyenburg et al, 1977), consistent with the DnaA-*oriC* interaction promoting DNA unwinding and replisome formation locally rather than at a distance.

In *E. coli*, the regulation of replication is associated with the control of DnaA activity. Only ATP-bound DnaA can support its multimerization, initiate DNA melting, and promote the loading of active DnaB helicases that drive replisome assembly and the initiation of replication. DnaA activity is negatively regulated following the initiation of replication by numerous means. For example, *oriC* is rapidly sequestered after it has been duplicated to prevent the re-binding of ATP-bound DnaA. The hemimethylated state of recently replicated DNA is rapidly detected by the SeqA protein, whose tight binding precludes DnaA activity at newly replicated daughter origins (Kaguni, 2006). Additionally, in a process known as Regulatory Inactivation of DnaA (RIDA), the β-clamp of the DNA polymerase III holoenzyme and the regulatory protein Hda for a complex that stimulates the ATP hydrolysis by DnaA, rendering it unable to direct a new round of replication (Kaguni, 2006).

In validating the bacterial replicon model, these studies demonstrated the multi-step process of DNA replication. Even before nucleotide incorporation, multiple biochemically discernable events occur at the origin of replication to support subsequent replisome assembly. This paradigm provided a useful foundation for the early studies of eukaryotic DNA replication, despite distinct differences in the regulation of helicase loading and activation.

Saccharomyces cerevisiae

Unlike their prokaryotic counterparts, eukaryotes typically have much larger genomes that are spread across multiple linear chromosomes. To accommodate these differences, eukaryotic cells initiate chromosomal replication from multiple genomic sites to support the timely and complete replication of their genomes. The first eukaryotic replicators were identified in a screen for DNA sequences that confer autonomous replication upon episomal elements in the yeast *Saccharomyces cerevisiae* (Hsiao & Carbon, 1979; Struhl et al, 1979). These Autonomously Replicating Sequences (or ARSs) were later demonstrated to direct the replication of linear chromosomal DNA (Stinchcomb et al, 1979) and, as in prokaryotes, overlap with origins of replication (Brewer & Fangman, 1987; Huberman et al, 1987). More recently, approximately 350 origins have been mapped within the *S. cerevisiae* genome (Nieduszynski et al, 2007) by genome-wide applications like density transfer coupled with microarrays (Raghuraman et al, 2001), chromatin immunoprecipitation (ChIP) coupled with microarrays (ChIP-chip) (Wyrick et al, 2001; Xu et al, 2006), and ChIP coupled with high-throughput sequencing (ChIP-seq) (Eaton et al, 2010).

Structural analyses of several yeast origins reveal a common modular origin structure (Fig. 3A). Each origin contains a 11-bp T-rich element called the ARS Consensus Sequence (ACS) that is essential, but not sufficient, for origin function. The ACS constitutes half of a bi-partite binding site for the eukaryotic initiator protein, ORC (see below). Consistent with the ACS being insufficient for determining origins, the yeast genome contains approximately 10- to 100-fold fewer *bona fide* origins than predicted ACS matches in the genome, demonstrating that other elements contribute to origin function (Eaton et al, 2010). These sequences are found downstream (3') of the T-rich ACS strand and are referred to as B-elements. In addition to the ACS, each yeast origin that has been tested contains two or more B-elements, which are collectively essential, but individually dispensable for origin function (Bell, 1995; Marahrens & Stillman, 1992). B-elements are uniformly located on one side of the asymmetric ACS and conserved in function despite poor sequence conservation amongst one another.

The most well characterized origin in *S. cerevisiae* is *ARS1*, which contains three B-elements named B1, B2, and B3 (Marahrens & Stillman, 1992). The B1 element constitutes the second half of the bi-partite binding site for ORC (Rao et al, 1994; Rowley et al, 1995). The B2 element, a 9 out of 11 match to the ACS in opposite relative orientation, is important for helicase loading but its precise function remains unclear. One possibility is that it acts as a second binding site for ORC, recruiting two ORC molecules to the origin simultaneously (Evrin et al, 2009; Gambus et al, 2011; Remus et al, 2009). However, *in vitro* DNase protection reactions of *ARS1* with purified ORC (Bell & Stillman, 1992) does not support

Figure 3

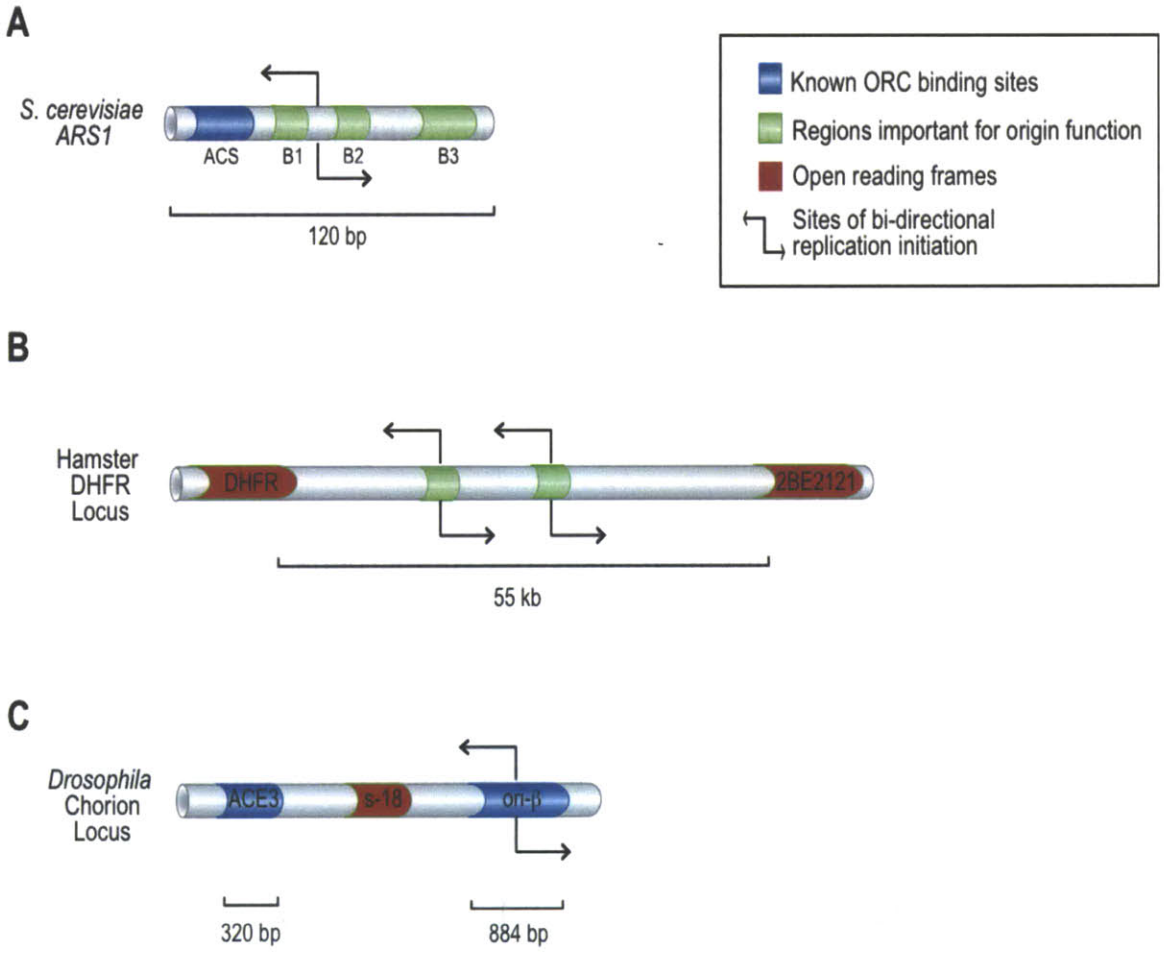

Figure 3. Architecture of eukaryotic replicators. (A) *ARS1* origin of *S. cerevisiae*. **(B)** DHFR initiation zone of Chinese hamster cells. Replication initiates from multiple sites within the 55-kb intergenic locus, but only the two most prominently used sites are depicted. **(C)** *Drosophila* chorion amplicon, *DAFC-66D*.

this possibility. The simultaneous binding of ORC at the ACS and B2 elements has only been detected *in vitro* with Orc1-5 complexes that lack the Orc6 subunit (Wilmes & Bell, 2002), which is not essential for DNA binding (Lee & Bell, 1997). Finally, the B3 element functions as the binding site for Abf1, a transcription factor (Diffley & Stillman, 1988). In combination with ORC and the local A-rich bias in nucleotide composition, Abf1 supports the maintenance of an asymmetrically positioned nucleosome free region (NFR) located downstream of the T-rich ACS strand (Eaton et al, 2010; Venditti et al, 1994). NFRs may be important in providing sufficient space to load the replicative helicase (Eaton et al, 2010).

Metazoa

In contrast to their unicellular eukaryotic counterparts, metazoa possess replicators that are not defined by a specific sequence. The apparent lack of sequence-specific replication initiation has hindered the identification of discrete origins of replication. Embryos of *Xenopus laevis* and *Drosophila melanogaster*, for example, rapidly duplicate their DNA content without any preference for sequence (Harland & Laskey, 1980; Sasaki et al, 1999). Metazoan replication origins, however, are commonly found to encompass broad zones in which replication can initiate. Even as *Xenopus* embryos develop to late blastula stage, replication initiation becomes circumscribed to broad initiation zones, coincident with transcriptional changes (Danis et al, 2004). Another well characterized initiation zone is the 55-kb intergenic region between the convergently transcripted DHFR and 2BE2121 genes in Chinese hamster cells (Looney & Hamlin, 1987). Within this zone, multiple loci serve as inefficient initiation sites,

although two distinct initiation sites are prominently used (Fig. 3B) (Leu & Hamlin, 1989).

A few instances of highly localized, sequence-specific replication are known in metazoa. For example, in the well-characterized chorion amplicon of *Drosophila* follicle cells, *DAFC-66D*, the 320-bp ACE3 and 884-bp ori-β elements are necessary and sufficient to initiate the amplification of the chorion gene loci (Delidakis & Kafatos, 1989; Handeli et al, 1989; Orr-Weaver et al, 1989). The ACE3 element acts as a replicator (Austin et al, 1999), stimulating replication initiation at ori-β in *cis* (Fig. 3C) (Lu et al, 2001). More recently, in the amplicon *DAFC-62D*, an intragenic origin, ori-62, was defined. The second round of amplification at the ori-62 origin uniquely requires transcription through the *yellow-g2* gene within which it resides (Xie & Orr-Weaver, 2008).

Although the replicon model is too simplistic to fully explain the regulation of DNA replication in metazoa, the key events that govern replication initiation are remarkably conserved from bacteria to metazoa. Despite ill-defined replicators, metazoa also utilize the same initiator protein, ORC, which is conserved across all eukaryotes studied to date. The mechanism by which metazoan ORC selects origins in a sequence-independent manner is unclear, but ORC binding in metazoa is influenced by negative supercoiling (Remus et al, 2004), AT-richness (Vashee et al, 2003), chromatin structure (Aggarwal & Calvi, 2004), and decreased nucleosome occupancy (Eaton et al, 2011). Analogous to DnaA, origin-bound ORC stimulates the initiation of replication locally in a

tightly regulated, multi-step process. Yet unlike DnaA, binding of ORC to origins is not sufficient to drive DNA unwinding or the initiation of replication in eukaryotes. Instead, eukaryotic cells temporally separate the event of helicase loading from the events of helicase activation and polymerase assembly.

HELICASE LOADING

The first event in the replication of the eukaryotic genome is the selection of potential origins of replication, a process also referred to as origin licensing. During this process, the Mcm2-7 helicase is loaded onto all potential origins in a tightly regulated, step-wise event. The helicase-loading event results in loaded, inactive Mcm2-7 double hexamers that can only be activated to unwind DNA upon entry into S phase. Below, I will review what is known about the mechanism and regulation of Mcm2-7 helicase loading in eukaryotes. I begin with an introduction to the eukaryotic replicative helicase, Mcm2-7.

The Mcm2-7 replicative helicase

The eukaryotic replicative helicase, the Mcm2-7 (Mini-Chromosome Maintenance) complex, consists of six highly related protein subunits. The *MCM* genes encoding each polypeptide subunit were first identified in screens for plasmid maintenance or cell cycle progression defects. The *MCM2*, *MCM3*, and *MCM5* genes were identified in *S. cerevisiae* in a genetic screen for mutations that resulted in the defective maintenance of mitotically stable plasmids (mini-chromosomes) (Maine et al, 1984). The *MCM6* gene was isolated in an analogous study in *Schizosaccharomyces pombe* (Takahashi et al, 1994). *MCM4* and *MCM7*

were identified in independent screens for cell cycle division mutants (Hennessy et al, 1991; Moir et al, 1982). Consistent with their role in a fundamental cellular process, each of the six genes is essential for viability (Schwacha & Bell, 2001).

Conserved among archaea and eukaryotes, the *MCM* genes encode AAA+ (ATPases associated with a variety of cellular activities) superfamily proteins (Iyer et al, 2004) that assemble into hexameric, ring-shaped structures (Pape et al, 2003). In archaea, a single Mcm protein oligomerizes to form a homo-hexameric structure (Pape et al, 2003). In eukaryotes, however, the six paralogous Mcm proteins (Mcm2-7) assemble into a hetero-hexameric ring-like structure (Remus et al, 2009). As AAA+ ATPases, the Mcm proteins form ATPase active sites at their subunit interfaces. One Mcm subunit binds the nucleotide (via a Walker A motif) and contributes to hydrolysis (via a Walker B motif) activity in *cis*, while an adjacent subunit contributes to nucleotide hydrolysis in *trans* (via a catalytic arginine finger motif). The unique hetero-hexameric structure of the Mcm2-7 complex results in the formation of six unique ATPase sites, all of which are essential for viability (Schwacha & Bell, 2001). The distinct ATPase activities of each active site contributed to the initial elucidation of the subunit arrangement within Mcm2-7 (Davey et al, 2003). The subunits within the Mcm2-7 complex proceed in the following order: Mcm2, Mcm5, Mcm3, Mcm7, Mcm4, Mcm6 (Fig. 4). Mutational analyses of *S. cerevisiae* Mcm2-7 ATPase sites coupled with *in vitro* helicase assays demonstrate that the Mcm3/7, Mcm7/4, and Mcm4/6 active sites are uniquely involved in helicase activity while the other three active sites play unknown but essential roles (Bochman et al, 2008; Bochman & Schwacha, 2008;

Figure 4

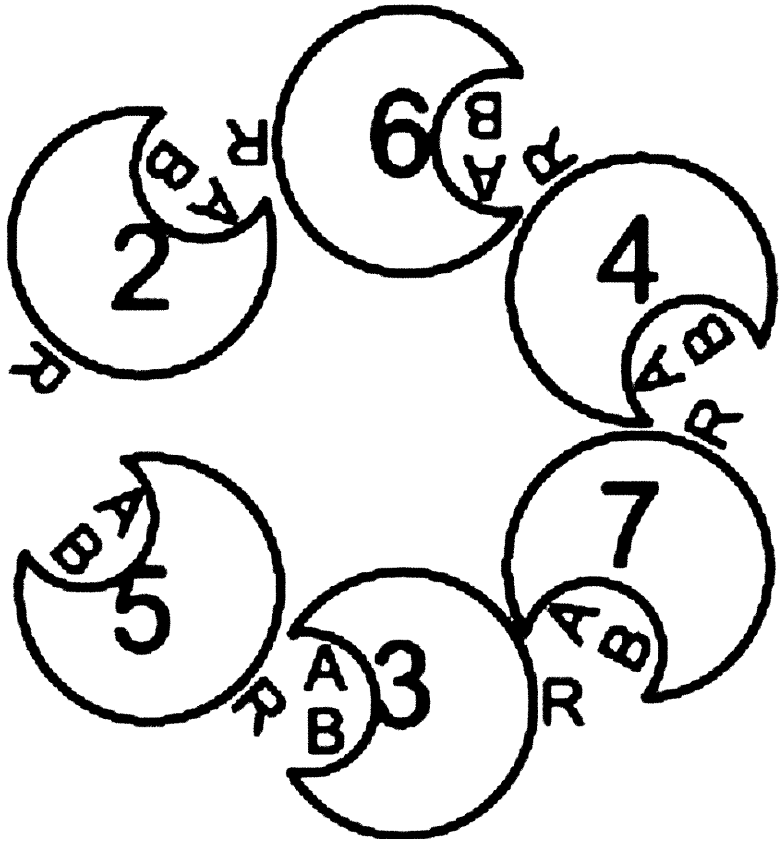

Figure 4. Subunit organization of the hexameric Mcm2-7 helicase. Relative subunit organization with distinct ATPase active sites is depicted. Walker A motif, "A"; Walker B motif, "B"; and catalytic arginine finger, "R". Discontinuity between the Mcm2 and Mcm5 subunits is depicted (see text below). (Bochman et al, 2008)

Schwacha & Bell, 2001). Notably, activation of Mcm2-7 by the formation of a CMG complex could result in an altered relative contribution of the six ATPase sites towards helicase activity. Consistent with this possibility, *in vitro* helicase assays testing the *Drosophila* CMG complex show that the Mcm2/5, Mcm6/2, and Mcm5/3 active sites are involved in helicase activity while the Mcm4/6 active site is dispensable (Ilves et al, 2010). Alternatively, the function of each Mcm2-7 ATPase site might not be conserved between organisms.

Structural studies of the archaeal Mcm protein provided the first insight into the organization of eukaryotic Mcm2-7 proteins. Each Mcm protein possesses a prominently conserved C-terminal AAA+ ATPase domain and a more divergent N-terminal oligonucleotide-binding (OB)-like fold domain. In the context of a homo-hexamer, both the N-terminal and C-terminal domains appear to make contacts with adjacent subunits to form a bi-lobed ring-like structure with six-fold symmetry (Pape et al, 2003). In solution, the archaeal MCM homo-hexamer is capable of forming stable MCM double hexamers in which two MCM homo-hexamers interact via their N-terminal domains (Fletcher et al, 2005; Gomez-Llorente et al, 2005) (Fig. 5A). Similar to the archaeal Mcm protein, the eukaryotic Mcm2-7 proteins appear to adopt an analogous bi-lobed structure despite consisting of six different Mcm subunits (Costa et al, 2011; Remus et al, 2009) (Fig. 5B). Unlike in the archaeal MCM hexamer, however, the hetero-hexameric Mcm2-7 complex is not capable of forming stable Mcm2-7 double hexamers in solution. Biochemical studies suggest that Mcm2-7 only forms a stable head-to-head double hexamer upon loading onto DNA in G1

Figure 5

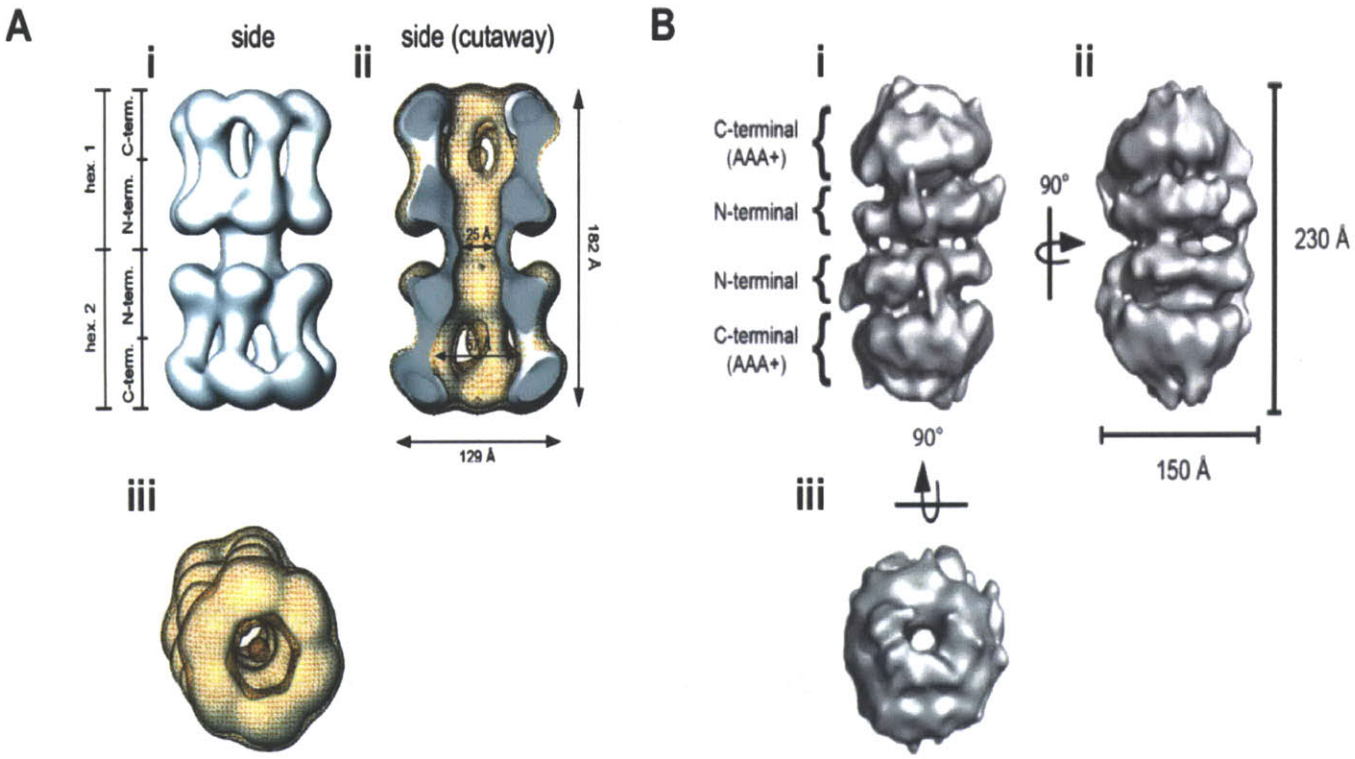

Figure 5. Structures of the archaeal MCM and eukaryotic Mcm2-7 double hexamer. **(A)** 25-Å resolution, three-dimensional electron microscopy reconstruction of the MCM double hexamer from *Methanobacterium thermoautotrophicum*. (i) Side view of the head-to-head MCM double hexamer. (ii) Cut-away side view. (iii) Bottom view. **(B)** 30-Å resolution, three-dimensional electron microscopy reconstruction of the loaded Mcm2-7 double hexamer from *S. cerevisiae*. (i) Side view of the head-to-head Mcm2-7 double hexamer. (ii) Side view, 90° rotation. (iii) Bottom view. (Gomez-Llorente et al, 2005; Remus et al, 2009)

phase (Evrin et al, 2009; Gambus et al, 2011; Remus et al, 2009). The loaded Mcm2-7 double hexamer encircles dsDNA and, although inactive, is able to slide along the DNA helix in an energy-independent manner *in vitro* (Evrin et al, 2009; Remus et al, 2009).

The Mcm2-Mcm5 "gate" hypothesis

To have Mcm2-7 encircle dsDNA, the Mcm2-7 ring would have to be closed (and possibly opened) around DNA. Biochemical and structural studies strongly implicate that the Mcm2-Mcm5 interface acts as a putative Mcm2-Mcm5 "gate," which can be opened and closed to facilitate the deposition of Mcm2-7 around DNA (Bochman & Schwacha, 2008; Costa et al, 2011; Davey et al, 2003). Although the Mcm2-7 complex assembles into a ring-like structure in solution (Bochman & Schwacha, 2007), these studies demonstrate that a discontinuity between the Mcm2 and Mcm5 subunits exists. The presence of a gap in the Mcm2-7 hexamer was originally hypothesized based on *in vitro* subunit association and ATPase studies suggesting that Mcm2 and Mcm5 are adjacent Mcm2-7 subunits but do not interact or exhibit ATPase activity (Davey et al, 2003). The hypothesis was extended and further supported by studies suggesting that the affinity of Mcm2-7 to bind a circular ssDNA substrate was dependent on the presence of an ATP-dependent Mcm2-Mcm5 gate (Bochman & Schwacha, 2008). Most recently, structural studies of *Drosophila* Mcm2-7 show that in solution equilibrium the helicase exists in two distinct forms: a planar, notched conformation and a spiral, lock-washer conformation, both of which exhibit a persistent discontinuity between the Mcm2-Mcm5 interface (Fig. 6A)

Figure 6

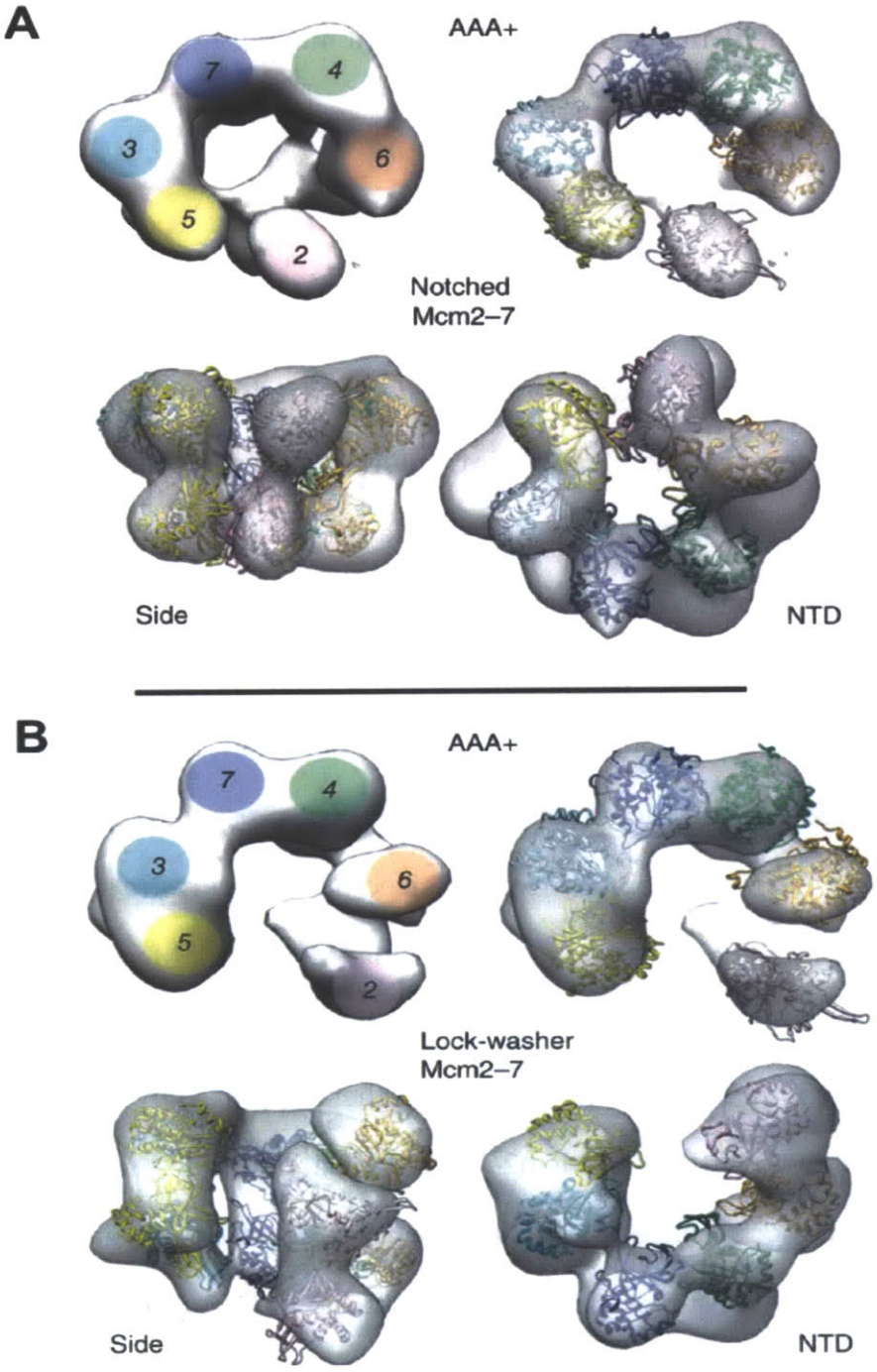

Figure 6. Single-particle electron microscopy reconstructions of *Drosophila* Mcm2-7. **(A)** 3D reconstruction of the planar, notched Mcm2-7 complex viewed. (*top left*) View from C-terminal face. (*top right*) Slab view from C-terminal face. (*bottom left*) Side view. (*bottom right*) View from N-terminal face. **(B)** 3D reconstruction of the spiral, lock-washer conformation of the Mcm2-7 complex. Views as in *A*. (Costa et al, 2011)

Figure 6

C

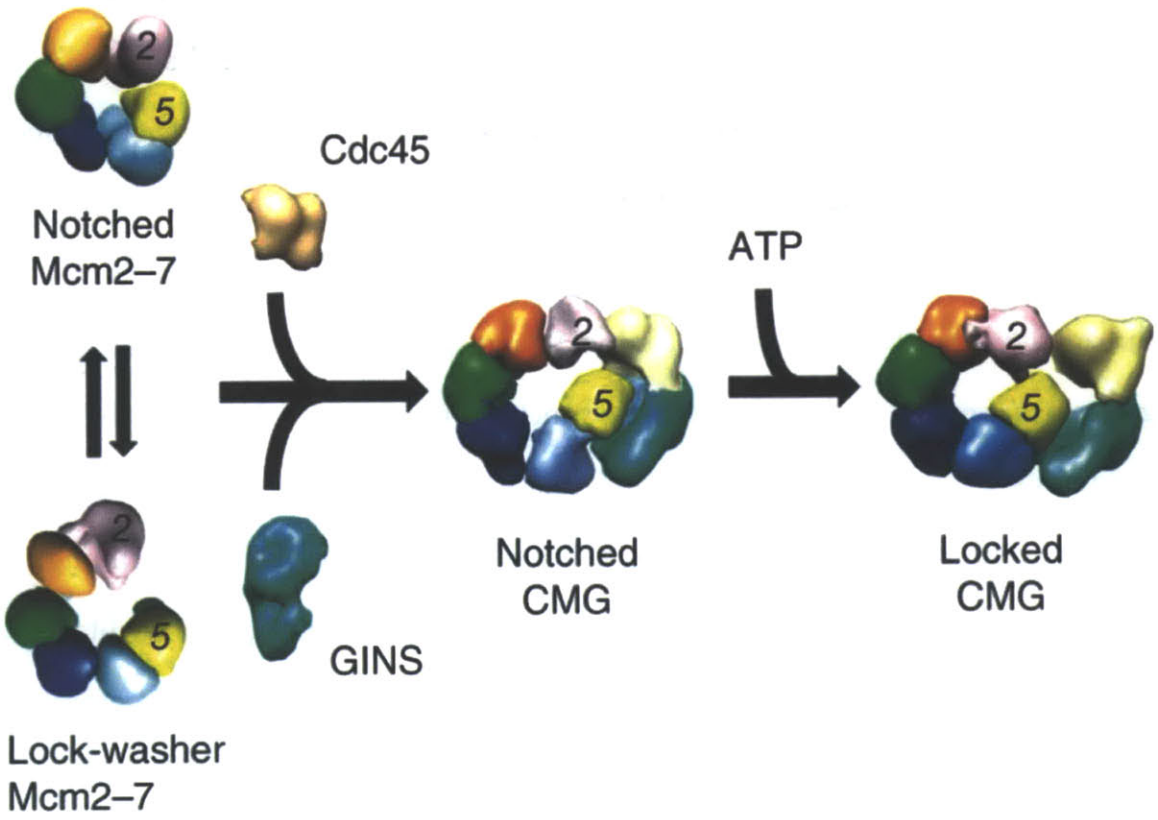

Figure 6. Closure of the Mcm2-Mcm5 gate is stimulated by nucleotide and binding of helicase-activating factors. (C) The discontinuity at the Mcm2-Mcm5 interface of the *Drosophila* Mcm2-7 is bridged and closed by the binding of the helicase-activating proteins, Cdc45 and GINS, in the presence of nucleotide. (Costa et al, 2011)

(Costa et al, 2011). These studies suggest that closure of the Mcm2-Mcm5 gate is influenced by nucleotide binding at the Mcm2/5 active site as well as the association of helicase-activating factors (Costa et al, 2011) (Fig. 6B).

The propensity of Mcm2-7 to adopt an open-gate conformation may circumvent the requirement of an initial ring-opening event during helicase loading. However, loaded Mcm2-7 double hexamers appear topologically linked around DNA in a closed conformation by single-particle electron microscopy (Remus et al, 2009), suggesting that a gate-closing event occurs after dsDNA is threaded into the Mcm2-7 central cavity. It is unclear what drives this initial gate-closure event. Notably, if Mcm2-7 functions as a canonical helicase that encircles ssDNA upon its activation in S phase, additional gate-opening and –closing events must also occur during helicase activation (see below). Although recent structural studies show that closure of the Mcm2-Mcm5 gate is facilitated by the binding of the helicase-activating factors Cdc45 and GINS (CMG complex formation) in S phase (Costa et al, 2011) the gate-opening mechanism is unclear.

The role of ATP hydrolysis during helicase loading

The Mcm2-7 replicative helicase is loaded around dsDNA in a step-wise manner that requires sequential ATP binding and hydrolysis events (Fig. 7). Because Mcm2-7 has no intrinsic affinity for DNA, it is recruited to origins via interaction with origin-bound ORC and other helicase-loading factors. ORC, a hetero-hexameric complex consisting of the Orc1-6 proteins, binds to origin DNA in an ATP-dependent manner *in vitro* (Bell & Stillman, 1992). Although five of

Figure 7

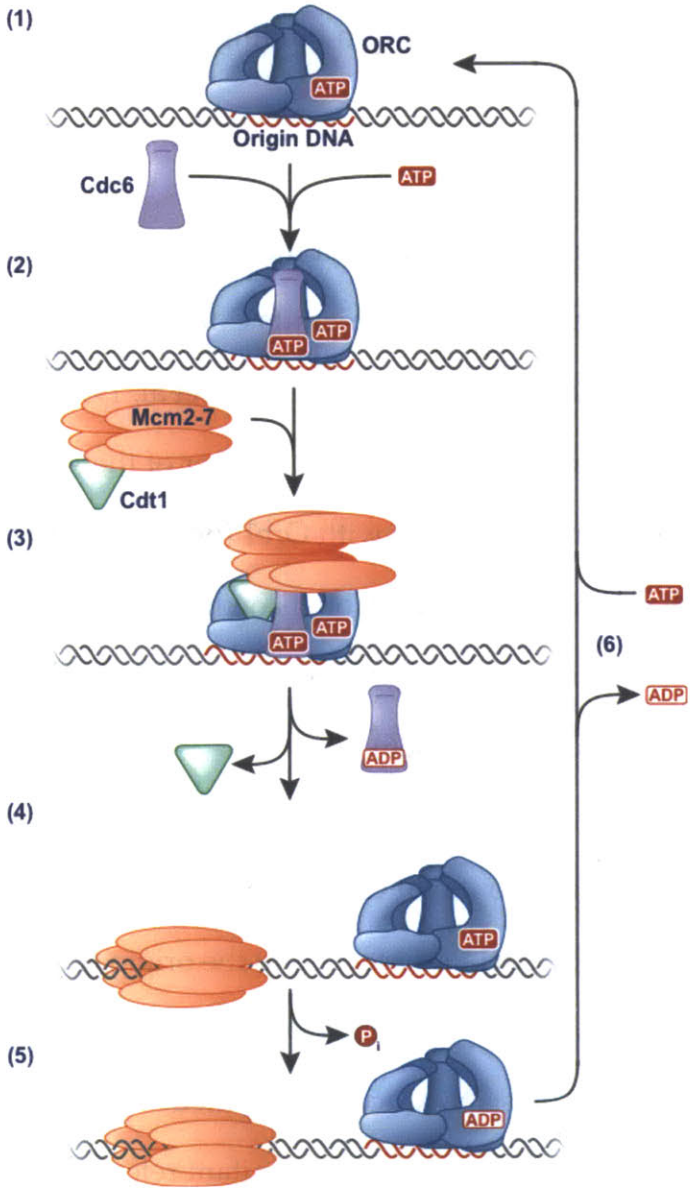

Figure 7. Sequential ATP hydrolysis events are required for Mcm2-7 loading at origin DNA. (1) ATP-bound ORC binds origin DNA in a sequence-specific manner. (2) Cdc6 binds ATP in an ORC-dependent manner and is recruited to the origin. (3) The ternary origin-bound ORC/Cdc6 complex recruits Cdt1 and the Mcm2-7 helicase. (4) ATP hydrolysis by Cdc6 results in the loading of Mcm2-7 and the release of Cdt1 from the origin. (5) ATP hydrolysis by ORC completes the cycle and allows for additional rounds of Mcm2-7 loading at the same origin.

the six ORC subunits (Orc1-5) are members of the AAA+ superfamily, mutational analyses of ORC reveal that only Orc1 and Orc5 bind ATP. Only ATP binding by Orc1 is essential for the origin-binding activity of ORC *in vitro* (Klemm et al, 1997).

ORC is bound to origins throughout the cell cycle in *S. cerevisiae* (Aparicio et al, 1997; Liang & Stillman, 1997) and recruits Cdc6 to origins at the M-to-G1-phase transition when CDK activity is lost (Cocker et al, 1996; Wang et al, 1999). Cdc6, like Orc1-5 and Mcm2-7 proteins, is a member of the AAA+ superfamily and shares strong sequence similarity with Orc1 (Bell et al, 1995). Interestingly, Cdc6 neither binds nor hydrolyzes ATP on its own. Cdc6 ATP binding and hydrolysis activities are revealed only in the context of its association with origin-bound ORC (Randell et al, 2006).

Together with origin-bound ORC, ATP-bound Cdc6 facilitates the subsequent recruitment of Cdt1 and Mcm2-7 to the origin (Randell et al, 2006; Remus et al, 2009). The origin association of Cdt1 is short-lived and can only be observed when ATP hydrolysis by Cdc6 is prevented. ATP hydrolysis by Cdc6 is required for Mcm2-7 loading and the concomitant release of Cdt1 from the origin (Randell et al, 2006). The process by which Cdc6 ATP hydrolysis promotes Mcm2-7 loading and Cdt1 release is unclear. However, it is likely that ATP hydrolysis by Cdc6 supports the threading of dsDNA into the central channel of Mcm2-7, presumably via the Mcm2-Mcm5 gate, and may also facilitate subsequent gate closure.

Finally, the loading of additional Mcm2-7 complexes at origins of replication is regulated by Orc1 ATP hydrolysis (Bowers et al, 2004). Orc1 ATP hydrolysis is mediated in *trans* by a predicted arginine finger motif in the adjacent Orc4 subunit. Biochemical studies using ORC that is able to bind but not hydrolyze ATP demonstrate that Orc1 ATP hydrolysis is critical to reset the pre-RC helicase-loading machinery for additional rounds of Mcm2-7 loading (Bowers et al, 2004). The molecular mechanism by which ORC ATP hydrolysis facilitates multiple rounds of Mcm2-7 loading is not well understood. One possibility is that ORC changes conformation during the Mcm2-7 loading reaction and that re-establishment of the starting conformation requires ATP hydrolysis by ORC, release of ORC from DNA, and the rebinding of ATP-bound ORC to DNA (Bowers et al, 2004; Tsakraklides & Bell, 2010).

The over-representation of loaded Mcm2-7 complexes compared to chromatin-bound ORC has been observed from yeast (Bowers et al, 2004; Donovan et al, 1997; Lei et al, 1996) to metazoa (Edwards et al, 2002; Mahbubani et al, 1997), with Mcm2-7 being more abundant than ORC by a four- to 50-fold molar excess. Although multiple Mcm2-7 complexes are loaded per origin, only a subset of loaded Mcm2-7 are eventually activated for initiation (Edwards et al, 2002). Studies in *Xenopus* and human cells suggest that the excess Mcm2-7 might be critical to support replication initiation from dormant origins when replicative stress threatens to compromise complete genome replication (Ge et al, 2007; Woodward et al, 2006).

Interaction between helicase-loading components

The step-wise process of helicase loading requires the coordinated interplay of the helicase-loading components. The initial nucleotide-dependent binding of ORC at origin DNA, for example, results in a change in the overall structure of origin DNA. DNase I footprinting of purified ORC bound to the *ARS1* origin reveals three sites of enhanced cleavage that are approximately 10 bp apart, suggesting that DNA might wrap around ORC upon its binding (Bell & Stillman, 1992). Consistent with this finding, atomic force microscopy of *Drosophila* ORC bound to the ACE3 chorion gene replicator suggests that ORC wraps approximately 130 bp of linear DNA upon its binding (Clarey et al, 2008).

The initial origin recruitment of Cdc6 by ORC also leads to detectable changes. The ternary origin-bound ORC/Cdc6 complex results in a larger DNase I footprint that extends from the ACS to the B2 element of *ARS1*, suggesting a Cdc6-induced conformational change in ORC or an ORC-dependent association of Cdc6 with DNA (Speck et al, 2005). The DNase I footprint also reveals a significant reduction of enhanced cleavage sites upon formation of the ternary origin-bound ORC/Cdc6 complex, indicating that DNA wrapping could be minimal by this step in helicase loading or that Cdc6 protects the sites of cleavage (Speck et al, 2005).

The recruitment of Cdc6 to the origin requires Orc1 (Klemm & Bell, 2001; Wang et al, 1999) and is proposed to support the formation of an ORC/Cdc6 ring-like structure, reminiscent of other AAA+ assemblies (Speck et al, 2005). As mentioned above, the ATP binding and hydrolysis activities of Cdc6 are revealed only when associated with origin-bound ORC. Additionally, biochemical studies

suggest that the origin recruitment of Cdc6 exposes the Orc6 subunit (Mizushima et al, 2000), which could be significant for the subsequent recruitment of Cdt1 and the Mcm2-7 helicase to the origin (see below).

The recruitment of Cdt1 to the origin depends on the presence of the ternary origin-bound ORC/Cdc6 complex (Remus et al, 2009). The Orc6 protein is essential for Cdt1 recruitment and makes direct protein-protein interactions with Cdt1 via two distinct binding sites (Chen et al, 2007). To support iterative rounds of Mcm2-7 loading, Cdt1 must also be released from the origin (Chen et al, 2007). The release of Cdt1 from the origin is concomitant with ATP hydrolysis by Cdc6. Consistent with this observation, evidence in *S. pombe* suggests that Cdt1 and Cdc6 interact directly (Nishitani et al, 2000).

The Mcm2-7 helicase associates with the origin-bound ORC/Cdc6 complex with similar kinetics to Cdt1 *in vitro* (Randell et al, 2006). Recent evidence suggests that once loaded, Mcm2-7 occupies space downstream of the ACS, on the B-element side of origins in yeast (Eaton et al, 2010). The B-side of origins is generally nucleosome free, which could provide sufficient space for the Mcm2-7 helicase to be loaded (Eaton et al, 2010).

THE REPLICATION INITIATION PROTEIN CDT1

Cdt1 is essential for loading the Mcm2-7 helicase and is the central focus of this thesis. Below, the replication initiation protein Cdt1 will be discussed in more detail.

Cdt1 and its orthologs

The *CDT1* (Cdc10-dependent transcript 1) gene was first identified in *S. pombe* as a target of the G1/S transcription factor, Cdc10 (Hofmann & Beach, 1994). Cells without Cdt1 are impaired for the initiation of replication and inappropriately accumulate in G1 phase (Nishitani et al, 2000), consistent with the essential role of Cdt1 in helicase loading. Subsequently, orthologs of Cdt1 were identified in *Xenopus* (Maiorano et al, 2000), *Drosophila* (Whittaker et al, 2000), human (Wohlschlegel et al, 2000), and *S. cerevisiae* cells (Devault et al, 2002; Tanaka & Diffley, 2002). The initial identification of a *S. cerevisiae* Cdt1 ortholog was hindered by its low sequence similarity (~10%) to all other characterized orthologs (Tanaka & Diffley, 2002).

Although the three-dimensional structure of full-length Cdt1 is unknown, structural and biochemical studies of metazoan Cdt1 indicate that the protein can be divided into three domains: a poorly conserved N-terminal domain with DNA binding activity (Yanagi et al, 2002); a central domain, conserved across metazoa, that binds the metazoan Cdt1 inhibitor, geminin (see below) (Ferenbach et al, 2005; Lee et al, 2004); and a highly conserved C-terminal domain that is important for Mcm2-7 binding (Jee et al, 2010; Khayrutdinov et al, 2009) (Fig. 8A and 8B). The central and C-terminal domains of Cdt1 both adopt structurally similar winged helix domain (WHD) folds (Fig. 8A) despite sharing poor sequence similarity (<10%) (Jee et al, 2010; Khayrutdinov et al, 2009; Lee et al, 2004). The WHD fold is frequently found in DNA binding proteins, however,

Figure 8

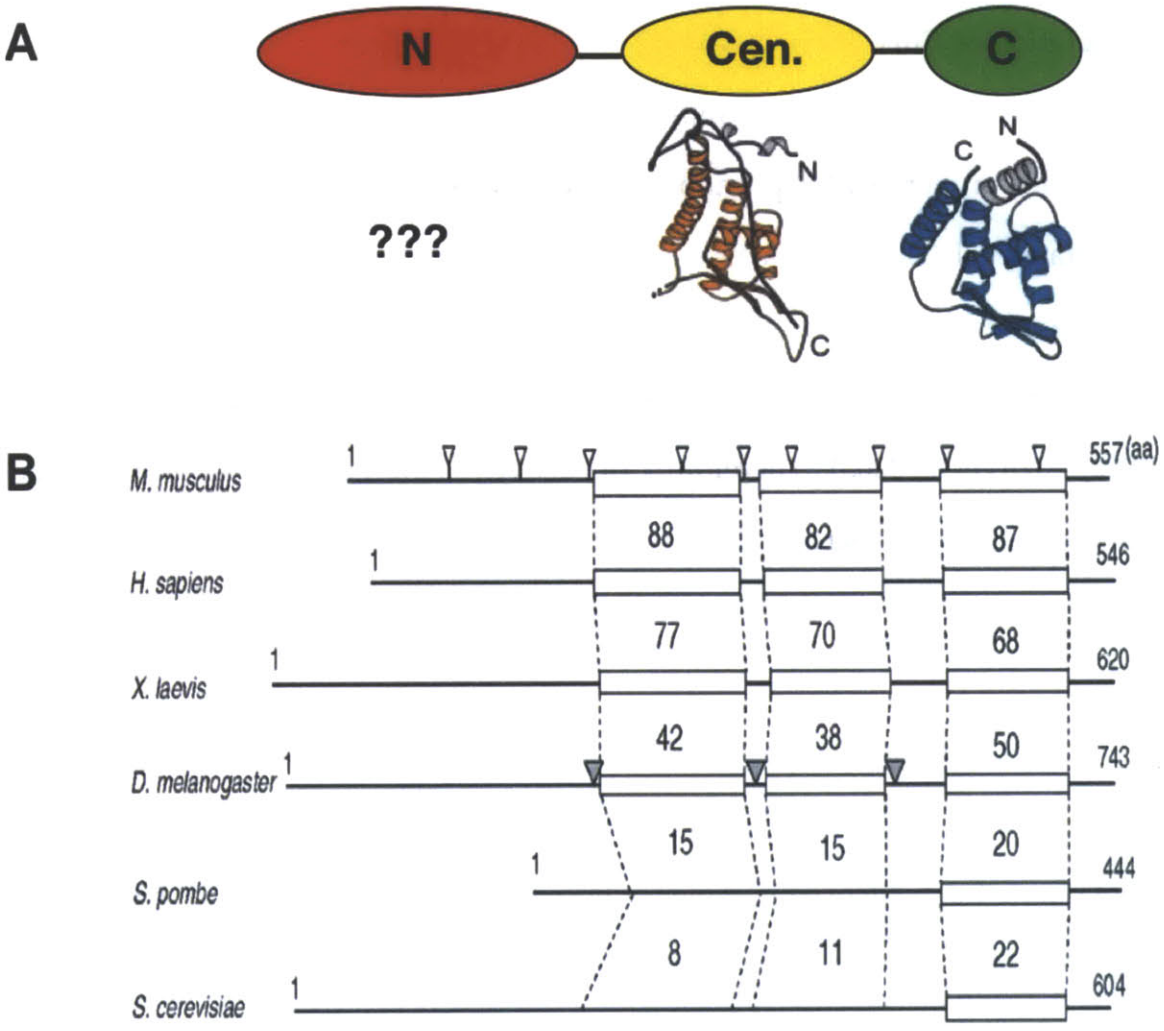

Figure 8. Domain organization, structure, and sequence conservation of Cdt1. (A) (*top*) Domain organization of Cdt1. (*bottom*) Corresponding Cdt1 domain structures. No N-terminal domain structure is available. The structures of the central and C-terminal domains of mouse Cdt1 are depicted in ribbon view. Both adopt similar winged-helix domain (WHD) folds. (B) Sequence conservation of Cdt1 across eukaryotes. Sequences of high conservation are shown by open boxes, and the sequence conservation of each region (relative to mouse Cdt1) is indicated. Open arrowheads indicate exon-intron boundaries for mouse Cdt1, closed arrowheads indicate large insertions observed in *Drosophila* Cdt1.

there is no evidence for DNA binding by the central or C-terminal domains of Cdt1.

Regulation of Cdt1

Cdt1 is essential for Mcm2-7 loading in G1 phase, and its regulation is critical for proper DNA replication and genomic stability (Maiorano et al, 2000; Nishitani et al, 2000; Whittaker et al, 2000). Over-expression of Cdt1 outside of G1 phase results in over-replication in some cells (Li & Blow, 2005; Zhu et al, 2004), which can ultimately lead to cell death and tumorigenesis (Arentson et al, 2002; Vaziri et al, 2003). Therefore, Cdt1 activity is uniformly down-regulated at the onset of S phase across all eukaryotes. Interestingly, the mechanisms by which Cdt1 is cell cycle regulated vary markedly from yeast to human.

In *S. cerevisiae*, Cdt1 is regulated by nuclear localization. Budding yeast Cdt1 interacts with Mcm2-7 prior to their origin recruitment to form a heptameric Cdt1/Mcm2-7 complex, whose formation is necessary for the nuclear import of either component protein (Tanaka & Diffley, 2002). At the onset of S phase, CDK modification of an NLS-NES transport module on Mcm2-7 results in the nuclear export of soluble, but not chromatin-bound, Mcm2-7 and any associated Cdt1 (Liku et al, 2005; Nguyen et al, 2000). During the cell cycle, Cdt1 protein levels do not appreciably change, suggesting that in *S. cerevisiae* nuclear localization is the principal regulatory mechanism of Cdt1 activity.

In *S. pombe* and higher eukaryotes, Cdt1 is regulated at the level of transcription and protein degradation. Cdt1 is a constitutively nuclear protein

whose levels fluctuate during the cell cycle, peaking between mitotic exit and the end of G1 phase (Nishitani et al, 2000; Nishitani et al, 2001). During S and G2 phases, Cdt1 is recognized for proteolysis by two distinct E3 ubiquitin ligases: SCF-Skp2 and DDB1-Cul4 (Nishitani et al, 2006). CDK phosphorylation of Cdt1 targets it for SCF-Skp2-mediated ubiquitination and proteosomal degradation (Liu et al, 2004; Sugimoto et al, 2004). In addition, binding of Cdt1 by PCNA targets Cdt1 for DDB1-Cul4-mediated ubiquitination and subsequent degradation (Arias & Walter, 2006; Senga et al, 2006). This second pathway drives the proteolysis of Cdt1 in a CDK-independent, but replication-dependent manner. The two independent pathways are used to varying extent in different organisms. For example, whereas humans trigger Cdt1 proteolysis by both pathways (Nishitani et al, 2006), *C. elegans* appears to predominantly utilize the PCNA-dependent DDB1-Cul4-mediated pathway (Kim & Kipreos, 2007).

In metazoans, regulation of Cdt1 is believed to be the primary means by which re-replication is prevented. Therefore, metazoan cells additionally regulate Cdt1 function through the Cdt1 inhibitor, geminin (Wohlschlegel et al, 2000). Structural studies indicate that geminin forms a homodimer with a central coiled-coil domain that interacts directly with Cdt1 (Lee et al, 2004; Saxena et al, 2004). The binding of geminin to Cdt1 sterically obstructs Mcm2-7 binding as well as the recruitment and loading of Mcm2-7 at origins of replication (Lee et al, 2004). Consistent with its inhibitory role, geminin accumulates during S, G2, and M phases and is absent during G1 phase when Cdt1 activity is required for helicase loading (McGarry & Kirschner, 1998).

The role of Cdt1 in helicase loading

During G1 phase, when Mcm2-7 loading is permitted, the replication initiation protein Cdt1 plays a transient yet essential role in the helicase-loading process. In *S. cerevisiae*, Cdt1 physically associates with the Mcm2-7 helicase prior to their origin recruitment, and formation of the hetero-heptameric Cdt1/Mcm2-7 complex is essential for the nuclear import of either component (Tanaka & Diffley, 2002). After nuclear import, Cdt1 is initially recruited to the origin by associating with the origin-bound ORC/Cdc6 complex (Randell et al, 2006; Remus et al, 2009). The interaction of Cdt1 with the ternary complex is mediated, at least in part, by a direct interaction with Orc6, which possesses two distinct and essential Cdt1 binding sites (Chen & Bell, 2011; Chen et al, 2007). The Mcm2-7 helicase arrives at the origin at the same time as Cdt1 *in vitro* (Randell et al, 2006), raising the possibility that Cdt1 not only assists in the nuclear import of Mcm2-7 but also in the origin recruitment of the helicase. In metazoa, Cdt1 does not form a stable complex with Mcm2-7, but their interaction is still critical for helicase loading (Ferenbach et al, 2005; Yanagi et al, 2002).

After the origin recruitment of Cdt1 and Mcm2-7, ATP hydrolysis by Cdc6 results in the loading of a head-to-head Mcm2-7 double hexamer around dsDNA (Evrin et al, 2009; Remus et al, 2009) and the release of Cdt1 from the origin (Randell et al, 2006). The molecular details of this step in the helicase-loading event are poorly understood. If Cdt1 plays an additional role in helicase loading at this step, Cdt1 might be essential for opening or closing the Mcm2-7 ring during helicase loading, orienting Mcm2-7 for proper loading, facilitating Mcm2-7 loading by an alternative mechanism, or any combination of the above. A more

detailed analysis of Cdt1 is necessary to discern the scope of its contribution to Mcm2-7 helicase loading, and this is the focus of this thesis.

Other roles of Cdt1

Multiple lines of evidence suggest that Cdt1 plays additional roles in helicase loading. In human cells, robust helicase loading depends on a Cdt1-dependent activity of HBO1 (Human acetylase Binding to Orc1), a histone H4-specific acetylase (Miotto & Struhl, 2008). HBO1 binds directly to Cdt1 during G1 phase and stimulates helicase loading by acetylating histone H4 lysine 5, lysine 8, and lysine 12 residues at origins *in vivo* (Miotto & Struhl, 2008; Miotto & Struhl, 2010). HBO1 activity is down-regulated at the onset of S phase by geminin binding directly to the Cdt1/HBO1 complex (Miotto & Struhl, 2010). Vertebrates additionally regulate helicase loading via the Cdt1 activator, MCM9 (Lutzmann & Mechali, 2008). MCM9 binds to origins in an ORC- and Cdt1-dependent manner and prevents modest geminin levels from inhibiting Cdt1 function during helicase loading (Lutzmann & Mechali, 2008).

HELICASE ACTIVATION AND POLYMERASE ASSEMBLY

The Mcm2-7 helicase is initially loaded onto origin DNA as an inactive head-to-head double hexamer. At the onset of S phase, a subset of loaded Mcm2-7 double hexamers is activated by the recruitment of two helicase-activating factors, Cdc45 and GINS. In *S. cerevisiae*, the recruitment of both Cdc45 and GINS depends on the recruitment and function of three proteins: Sld2, Sld3, and Dpb11. The Cdc45/Mcm2-7/GINS (CMG) complex is active for DNA

unwinding. Consistent with the CMG complex functioning as a replicative "unwindsome," Cdc45, Mcm2-7, and GINS all have been shown to travel with the replication fork *in vivo* (Aparicio et al, 1997; Calzada et al, 2005; Kanemaki & Labib, 2006; Pacek et al, 2006). DNA replication initiates with the recruitment of an additional protein, Mcm10, and the three replicative DNA polymerases (Pols) α, δ, and ε.

Forming the CMG complex

Like helicase loading, the activation of the Mcm2-7 helicase is a tightly regulated, multi-step process (Fig. 9). At the G1-to-S-phase transition, the increased activity of two kinases, DDK and CDK, act to promote the formation of the CMG complex. DDK acts prior to CDK (Jares & Blow, 2000; Walter, 2000) and targets the Mcm4 and Mcm6 subunits of loaded Mcm2-7 complexes (Francis et al, 2009; Randell et al, 2010; Sheu & Stillman, 2006; Sheu & Stillman, 2010). Phosphorylation by DDK alleviates an inhibitory activity of the N-terminal domain of Mcm4 (Sheu & Stillman, 2010), but the importance of Mcm6 modification by DDK is less clear. Following DDK modification, loaded Mcm2-7 complexes recruit the helicase-activating factor, Cdc45, and Sld3 (Masai et al, 2006; Yabuuchi et al, 2006; Zou & Stillman, 2000). The recruitment of Cdc45 to the Mcm2-7 complex is dependent on Sld3, which forms a complex with Cdc45 and directly binds the Mcm2-7 complex (Bruck & Kaplan, 2011a). The mechanism by which DDK supports Cdc45 and Sld3 recruitment is poorly understood but might be the result of a conformational change in Mcm2-7 or direct binding of Cdc45 or Sld3 to phosphorylated Mcm2 or Mcm6 proteins

Figure 9

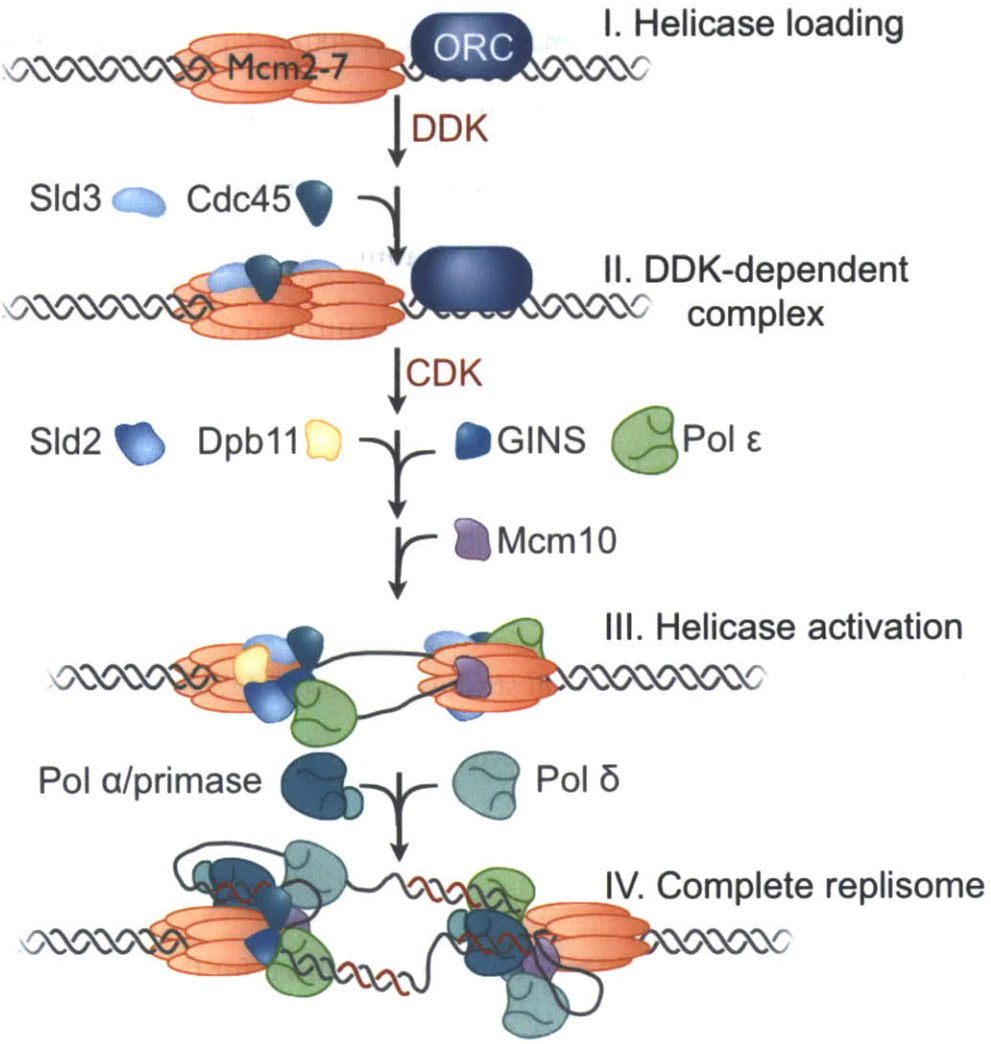

Figure 9. Step-wise process of Mcm2-7 helicase activation and replisome assembly. At the G1-to-S phase transition, DDK activity supports the recruitment of Cdc45 and Sld3 proteins to loaded Mcm2-7 complexes at the origin. CDK activity results in the origin recruitment of Sld2, Dpb11, GINS, and Pol ε to form the active CMG helicase complex. *In vitro* studies in *S. cerevisiae* indicate that Mcm10 subsequently associates with the origin (pictured), although the temporal association of Mcm10 with the origin is unclear and may not be conserved across eukaryotes. Sld2, Dpb11, and Sld3 are released from the origin during helicase activation. Sufficient ssDNA allows the assembly of the lagging-strand polymerases, Pol α/primase and Pol δ (pictured), and other accessory replisome factors like PCNA to form the complete replisome.

(Fletcher et al, 2003).

Following the action of DDK, CDK modifies its two essential targets, Sld2 and Sld3 (Tanaka et al, 2007; Zegerman & Diffley, 2007). Upon phosphorylation, both Sld2 and Sld3 have increased affinity for the BRCT phospho-protein binding domain of Dpb11. Dpb11/Sld2 also weakly interact with GINS and DNA Pol ε independently of origin DNA (Muramatsu et al, 2010). Therefore, CDK phosphorylation results in the collective recruitment of GINS, DNA Pol ε, Dpb11, and Sld2 to loaded Mcm2-7 complexes through interactions between Dpb11 and the helicase-bound Sld3 protein (Heller et al, in prep.). Once recruited to the loaded Mcm2-7 complex, GINS interacts tightly with Mcm2-7 and Cdc45 to form the activated CMG helicase complex (Costa et al, 2011; Ilves et al, 2010; Moyer et al, 2006). Structural studies in *Drosophila* suggest helicase activation is achieved by closure of the Mcm2-Mcm5 gate upon Cdc45 and GINS association (Fig. 6C).

Although Sld2, Sld3, and Dpb11 are each required for the formation of the CMG complex, all three proteins are released prior to replisome formation and do not travel away from origins with replication forks (Araki, 2010; Kanemaki & Labib, 2006). Recent biochemical studies suggest that Sld3 release might be facilitated by competition for Mcm2-7 binding by GINS (Bruck & Kaplan, 2011a) and high affinity for newly generated ssDNA at the origin (Bruck & Kaplan, 2011b), but the events that facilitate the release of Sld2 and Dpb11 remain unclear.

Initiation of replication

The conserved replication initiation factor, Mcm10, is another protein known to be required for the initiation of replication. *In vivo* studies of *S. cerevisiae* Mcm10 demonstrate that it associates with origins in G1 phase and subsequently becomes an essential component of the replication fork in S phase (Ricke & Bielinsky, 2004). Consistent with reports in *Xenopus* (Wohlschlegel et al, 2002), the origin association of *S. cerevisiae* Mcm10 in late G1 phase requires the presence of origin-bound Mcm2-7 (Ricke & Bielinsky, 2004). Interestingly, the precise function of Mcm10 in replication initiation might not be conserved across organisms. In *Xenopus* and *S. pombe*, Mcm10 is required for the origin association of Cdc45 (Gregan et al, 2003; Wohlschlegel et al, 2002). However, *in vitro* studies in *S. cerevisiae* suggest that Mcm10 is dispensable for Cdc45 origin association and acts at a later step in the initiation process to recruit the lagging-strand DNA polymerases (α and δ) (Heller et al, in prep.). Consistent with a role in polymerase recruitment, other studies in yeast and human cells demonstrate that Mcm10 recruits and stabilizes Pol α (Ricke & Bielinsky, 2004; Zhu et al, 2007). Although not necessary for Mcm10 recruitment, the origin recruitment of Pol α and Pol δ require substantial DNA unwinding (Heller et al, in prep.). In addition to the recruitment of DNA polymerases α and δ, the association of accessory replisome factors such as PCNA, Ctf4, and Tof1 (Gambus et al, 2009) is required to establish the complete replisome.

Although the activated CMG complex is part of the replisome, it is unclear how the CMG complex acts to unwind DNA ahead of the replication fork. If the loaded Mcm2-7 double hexamer functions as a single unit, the mechanism by which loaded Mcm2-7 unwinds DNA could be analogous to that of the SV40 large T antigen helicase, which has been proposed to pump dsDNA towards its hexamer-hexamer interface to separate the DNA duplex (Fig. 10A) (Smelkova & Borowiec, 1997). Alternatively, if single Mcm2-7 hexamers can unwind dsDNA, the mechanism of helicase action might be more analogous to *E. coli* DnaB, which functions as a canonical single hexameric unit that encircles and translocates along ssDNA, sterically excluding one DNA strand from its central channel (Fig. 10B).

DNA synthesis is initiated bi-directionally from each origin by two sister replisomes. *In vitro* biochemical studies in *Xenopus* suggest that once established the pair of replisomes travel in opposite directions and function autonomously (Yardimci et al, 2010), favoring a model in which sister Mcm2-7 hexamers uncouple upon their activation.

Polymerase assembly and replisome formation

The eukaryotic replisome contains three distinct DNA polymerases—Pol α, Pol δ, and Pol ε—that coordinate genomic duplication at each replication fork (Kunkel & Burgers, 2008). Pol α is the only polymerase with primase activity and synthesizes the RNA/DNA primers required for the initiation of leading and lagging strand synthesis. A growing body of evidence demonstrates that the

Figure 10

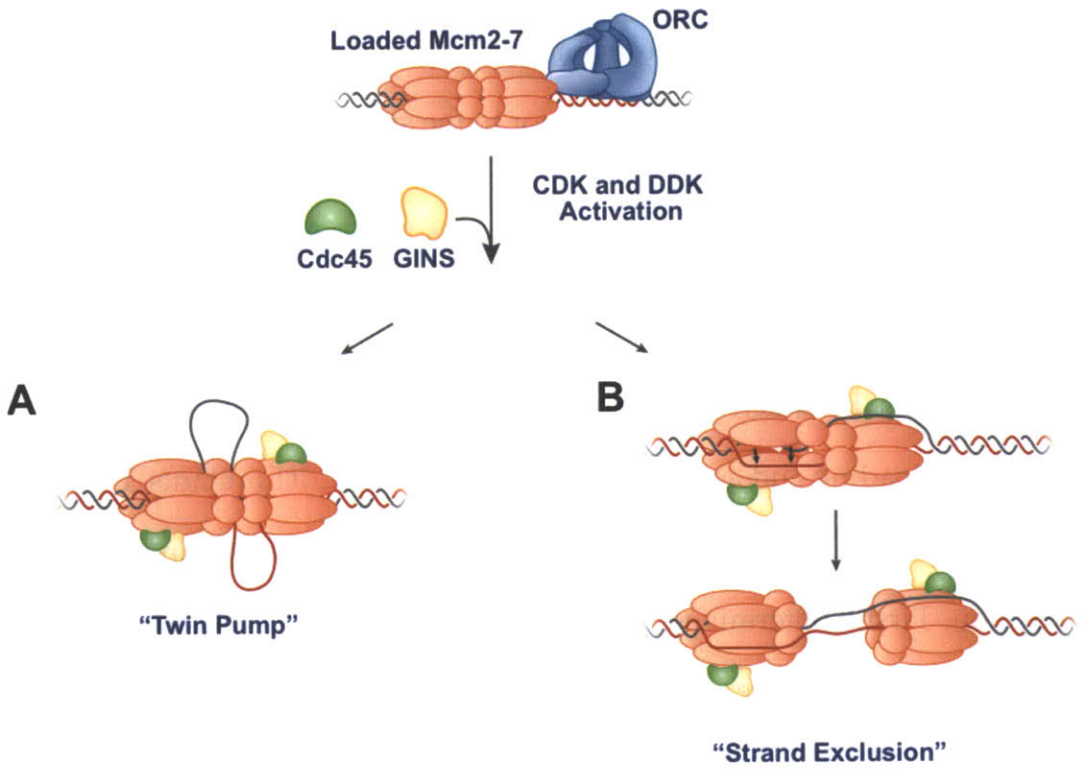

Figure 10. Two models for helicase action during replication elongation. (A) "Twin pump" model, analogous to the proposed mechanism of action of SV40 large T antigen helicase, in which the simultaneous translocation of the Mcm2-7 double hexamer on dsDNA drives DNA unwinding and the extrusion of ssDNA at the hexamer-hexamer interface. **(B)** "Strand exclusion" model, analogous to the mechanism of action of the DnaB helicase, in which the Mcm2-7 double hexamer re-arranges its conformation upon activation to encircle and translocate along ssDNA, unwinding DNA by steric exclusion of the unengaged strand from the central Mcm2-7 channel. (Figures adapted from Takara & Bell, 2009)

majority of leading and lagging strand synthesis is catalyzed by Pol ε and Pol δ, respectively (Kunkel & Burgers, 2008; Nick McElhinny et al, 2008; Pursell et al, 2007). *In vitro* studies of replication initiation in yeast suggest that Pol ε is recruited prior to Pol α and Pol δ (Muramatsu et al, 2010). This temporal order of polymerase recruitment would ensure that the leading strand DNA polymerase is already present at the replication fork prior to the synthesis of the first RNA/DNA primer by Pol α/primase.

THESIS SUMMARY

In this thesis, I characterize the role of the replication initiation protein Cdt1 in helicase loading and activation. Using a series of Cdt1 mutants, I elucidated the contribution of different Cdt1 regions to helicase binding, loading, and activation. First, I determined that Cdt1 binds the Mcm2-7 helicase via a C-terminal Mcm2-7 binding site. Second, I demonstrated that the initial origin recruitment of Cdt1 requires its central domain as well as its direct interaction with Mcm2-7. Third, I showed that multiple Cdt1 molecules are recruited to the origin during the helicase-loading event, and that formation of a multi-Cdt1 helicase-loading intermediate is required for Mcm2-7 loading. Finally, I provided evidence that the N-terminal domain of Cdt1 is required for ensuring that loaded Mcm2-7 complexes are competent for helicase activation and replication initiation. Together, these data demonstrate that despite its transient presence during helicase loading, Cdt1 plays multiple essential roles that ensure the proper loading of replication-competent Mcm2-7 complexes during G1 phase.

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Chapter II

Multiple roles of the replication initiation protein Cdt1 during helicase loading in *S. cerevisiae*

SUMMARY

Eukaryotic origins of replication are selected by the loading of an anti-parallel head-to-head double hexamer of Mcm2-7 around the origin DNA. The mechanism that drives formation and loading of the Mcm2-7 double hexamer remains unknown but requires the origin recognition complex (ORC), Cdc6 and Cdt1. Through analysis of Cdt1 mutations, we identify multiple essential domains of Cdt1 that performs multiple functions during helicase loading. The C-terminal domain of Cdt1 interacts with Mcm2-7, and we find that neither protein can be recruited to the origin in the absence of this interaction. Two central regions of Cdt1 mediate Cdt1/Mcm2-7 origin association and are required for subsequent Mcm2-7 loading. We find that two Cdt1 molecules are initially recruited by ORC and Cdc6 to the origin during pre-RC formation. Importantly, mutations that disrupt formation of this multi-Cdt1 intermediate prevent helicase loading. Although not required for stable Mcm2-7 loading, the N-terminal domain of Cdt1 is required for subsequent Cdc45 association and DNA replication. Our data support a model in which recruitment of two Cdt1 molecules initiates double-hexamer formation early in the helicase-loading process and demonstrate that, in addition to being required for Mcm2-7 loading, Cdt1 influences the competence of loaded Mcm2-7 to participate in subsequent helicase activation.

INTRODUCTION

Eukaryotic cells duplicate their genome in a timely fashion by initiating DNA replication from multiple genomic sites known as origins of replication. At each origin, DNA replication commences bi-directionally via a pair of replication forks. The establishment of bi-directional replication forks requires the essential DNA unwinding activity of the replicative DNA helicase, Mcm2-7. In addition to this essential function, the loading of the Mcm2-7 helicase onto DNA (in G1 phase) and its subsequent activation (in S phase) are tightly regulated to ensure once-and-only-once initiation from eukaryotic origins of replication (Remus & Diffley, 2009).

Helicase loading, also referred to as pre-replicative complex (pre-RC) formation or origin licensing, is nucleated by a DNA-bound origin recognition complex (ORC). In *Saccharomyces cerevisiae*, origins of replication called autonomously replicating sequences (ARSs) are first recognized and bound by the hetero-hexameric ORC (Bell & Dutta, 2002). As cells enter G1, ORC recruits Cdc6 followed by a complex of Cdt1 and the Mcm2-7 helicase. These proteins load Mcm2-7 in an inactive form onto all potential origins of replication (Remus & Diffley, 2009). During this process, Cdt1 transiently interacts with the origin-bound ORC/Cdc6 complex but is released after ATP hydrolysis by Cdc6 and Mcm2-7 loading (Randell et al, 2006). Notably, the Mcm2-7 helicase is loaded as a head-to-head double hexamer with dsDNA running through a central channel, although only hexameric Mcm2-7 complexes are observed in solution (Evrin et al, 2009; Gambus et al, 2011; Remus et al, 2009). The stable association of only

Mcm2-7 double hexamers with DNA upon loading suggests that two Mcm2-7 hexamers are loaded in a coordinated process (Remus et al, 2009). The anti-parallel orientation of the Mcm2-7 hexamers within the double hexamer is proposed to be critical to establish bi-directional replication forks.

Because both the origin of replication and ORC lack obvious symmetry, it is unclear how they direct the assembly of the symmetric Mcm2-7 double hexamer. One possibility is that two ORC molecules bind the origin in opposite orientations to coordinately load the head-to-head double hexamer. Another possibility is that one ORC molecule sequentially recruits and loads Mcm2-7 hexamers in opposite orientations onto origin-proximal DNA. A third possibility is that a single ORC molecule is necessary to direct the formation of the double hexamer by simultaneously recruiting two Mcm2-7 molecules to the origin.

At the onset of S phase, elevated levels of Dbf4-dependent kinase (DDK) and S-phase cyclin-dependent kinase (CDK) activity activate a subset of loaded Mcm2-7 double hexamers to initiate DNA unwinding and support replisome assembly. In *S. cerevisiae*, phosphorylation of the Mcm4 and Mcm6 subunits by DDK is required to alleviate the inhibitory function of the Mcm4 N-terminus (Randell et al, 2010; Sheu & Stillman, 2010) and to recruit the helicase-activating protein Cdc45 to the origin (Labib, 2010). Phosphorylation of the replication initiation factors Sld2 and Sld3 by CDK is required to recruit a second helicase-activating protein, the GINS complex (Tanaka et al, 2007; Zegerman & Diffley, 2007).

Although Cdt1 is essential for the loading of Mcm2-7 onto DNA, its function(s) during the helicase-loading process are unknown. In *S. cerevisiae*, the only well-defined role of Cdt1 in helicase loading is its formation of a heptameric complex with Mcm2-7, which is necessary for the nuclear import of either component (Tanaka & Diffley, 2002). A role for Cdt1 during the initial recruitment of Mcm2-7 to the origin is supported by direct interactions between Cdt1 and other pre-RC components such as the Orc2 and Orc6 proteins (Asano et al, 2007; Chen & Bell, 2011; Chen et al, 2007). Cdt1 also could support Mcm2-7 loading by positioning the recruited helicase in the proper orientation for loading or assisting in the opening or closing of the Mcm2-7 ring that is necessary to load the donut-shaped Mcm2-7 around DNA. A clearer understanding of the contribution of Cdt1 to helicase loading would provide insight into the requirements of loading the Mcm2-7 double hexamer.

In this study, we investigate the role of Cdt1 function in helicase loading. Using a series of Cdt1 mutants, we identified regions of Cdt1 that contribute to Mcm2-7 helicase binding, origin recruitment, and loading. We show that the formation of the Cdt1 / Mcm2-7 complex is required for the recruitment of both proteins to the origin DNA. Additionally, we show that two molecules of Cdt1 are initially recruited to the origin, and Cdt1 mutations that allow recruitment of only one Cdt1 prevent helicase loading. Intriguingly, we show that the N-terminus of Cdt1 is dispensable for its Mcm2-7 binding, origin recruitment, and loading functions but is required for the resulting loaded Mcm2-7 helicases to initiate DNA replication. Our findings demonstrate that Cdt1 plays numerous roles during the helicase-loading process, facilitating the origin recruitment of a pair of Mcm2-7

complexes for subsequent loading and ensuring that the loaded Mcm2-7 complexes are capable of initiating replication.

RESULTS

To investigate ScCdt1 function, we constructed a series of N- and C-terminal deletions based on structure-based profile-profile alignment (HHpred) and secondary structure prediction (Jpred3) tools. These analyses predicted three domains for ScCdt1: an N-terminal domain (a.a. 11-272), and a central (a.a. 310-435) and C-terminal domain (a.a. 500-602), both of which are predicted to adopt a WHD fold as observed for metazoan Cdt1 (Jee et al, 2010; Khayrutdinov et al, 2009; Lee et al, 2004). Inter-domain regions separate the N-terminal from the central domain (IDR1) and the central from the C-terminal domain (IDR2) (Fig. 1).

In vivo Complementation Analysis of Cdt1 Deletion Mutants

We first investigated the regions of ScCdt1 that are required for its function *in vivo*. We observed that all three domains of Cdt1 were indispensable *in vivo*. Only the smallest C-terminal deletion mutant (ΔC17 Cdt1) complemented a *CDT1* deletion (Fig. 1A and 1B).

The N-terminal domain of human Cdt1 (HsCdt1) contains a nuclear localization signal (NLS) that is critical for its nuclear import and function (Nishitani et al, 2004). Consistent with Cdt1 nuclear localization being mediated

Figure 1

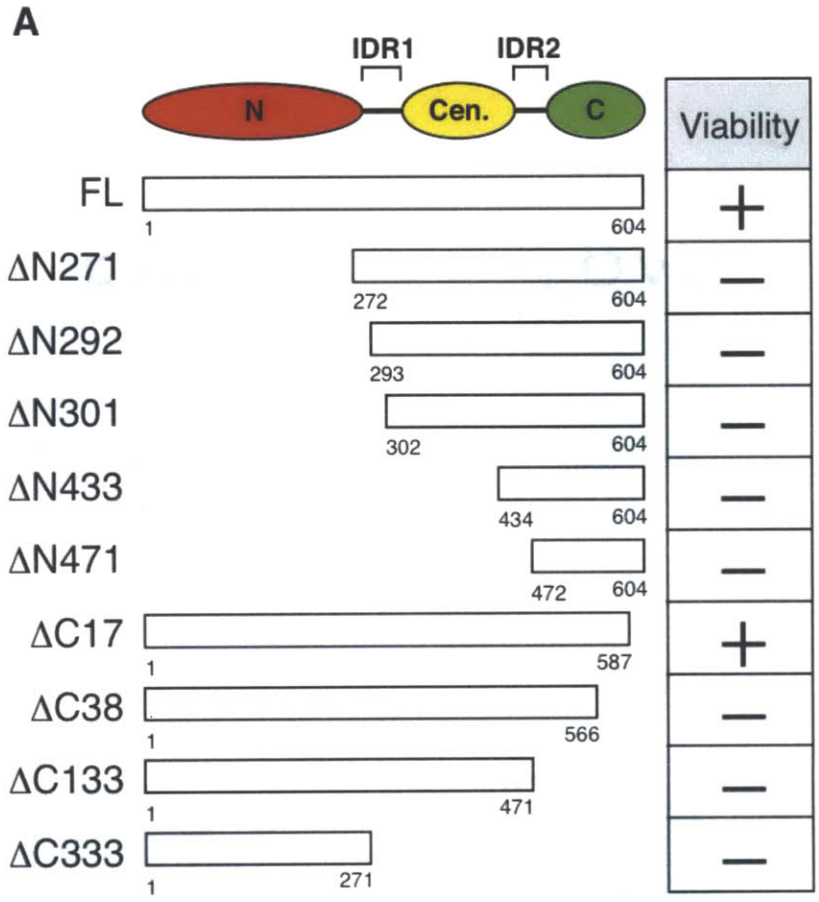

Figure 1. ***In vivo*** **complementation analysis of ScCdt1 deletion mutants. (A)** *Top,* Diagram of ScCdt1 structural domains predicted by HHpred analysis. ScCdt1 is predicted to contain three discrete domains (N-terminal, central, and C-terminal) and two inter-domain regions (IDR1, IDR2). *Left,* Schematic representation of N- and C-terminal Cdt1 deletion constructs. Endpoints of each construct are indicated. *Right,* Complementation analysis of Cdt1 mutants. Each Cdt1 mutant was placed under its native promoter and tested for complementation in a yeast strain lacking Cdt1.

Figure 1

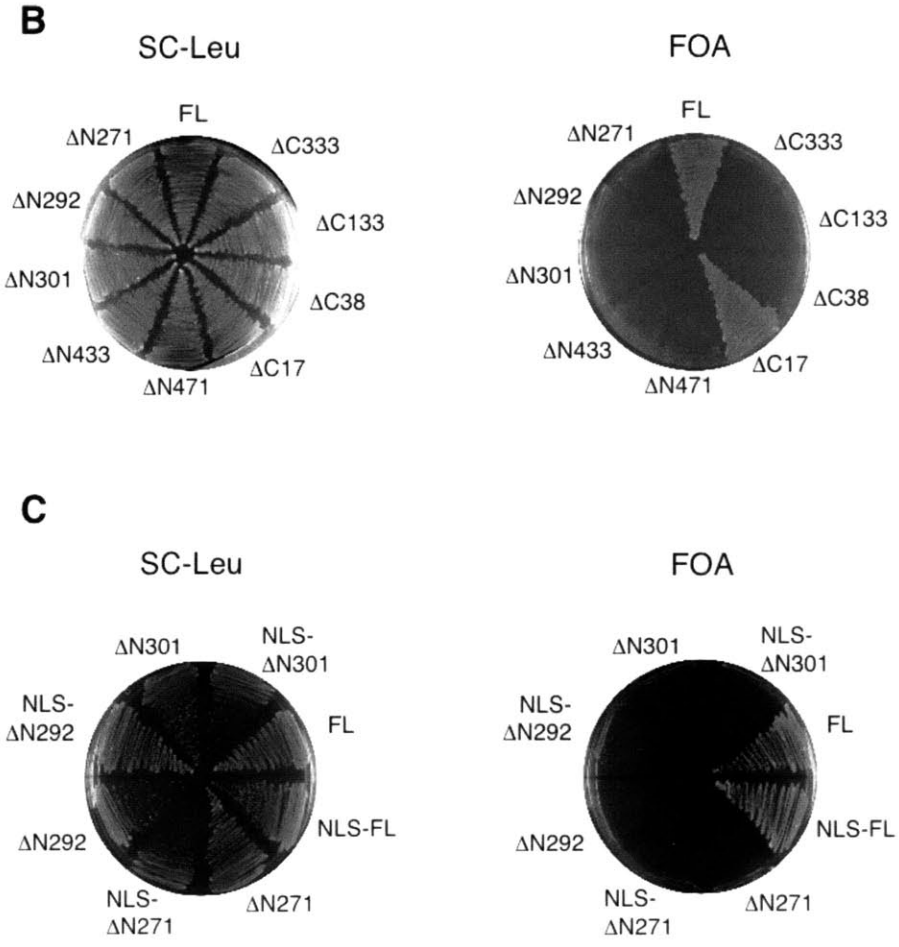

Figure 1. ***In vivo*** **complementation analysis of ScCdt1 deletion mutants. (B)** *Top,* Each Cdt1 construct was placed under its native promoter and transformed into a Cdt1 swapper strain (yTJT73). Complementation was scored as the ability of the Cdt1 construct to support yTJT73 growth on 5-FOA. **(C)** *Bottom,* Full-length and N-terminal domain Cdt1 deletion constructs were N-terminally tagged with the SV-40 NLS, placed under control of the native promoter, and transformed into a Cdt1 swapper strain (yTJT73). Complementation was scored as the ability of the Cdt1 construct to support yTJT73 growth on 5-FOA.

by binding to Mcm2-7 (Tanaka & Diffley, 2002), we did not identify an NLS motif within the ScCdt1 coding region. Nevertheless, we asked whether the addition of the SV-40 NLS to the N-terminal deletion mutants restored complementation. In all cases, this modification did not change the ability of the mutant to complement a *CDT1* deletion (Fig. 1C).

Origin Recruitment of Cdt1 Requires an Inter-Domain Region and Central Domain

The nuclear accumulation of Cdt1 requires its interaction with the Mcm2-7 helicase, and neither protein is competent for nuclear entry alone (Tanaka & Diffley, 2002). Although the C-terminus of metazoan Cdt1 is critical for its interaction with Mcm2-7 (Ferenbach et al, 2005; Teer & Dutta, 2008; Yanagi et al, 2002; You & Masai, 2008), a Mcm2-7 binding site in ScCdt1 has not been identified. To identify this region in ScCdt1, we generated yeast strains that over-expressed all six Mcm proteins and each Cdt1 deletion mutant with a C-terminal FLAG epitope. Cdt1 was immunoprecipitated from G1 phase-arrested extracts, and all immunoprecipitated proteins were examined by silver staining (Fig. 2). Full-length Cdt1 and each N-terminal deletion co-precipitated approximately equimolar amounts of Mcm2-7. In contrast, only the smallest C-terminal truncation mutant (ΔC17) co-precipitated detectable Mcm2-7. These data demonstrate that the Mcm2-7 binding domain is contained within the C-terminal 133 residues of ScCdt1.

Figure 2

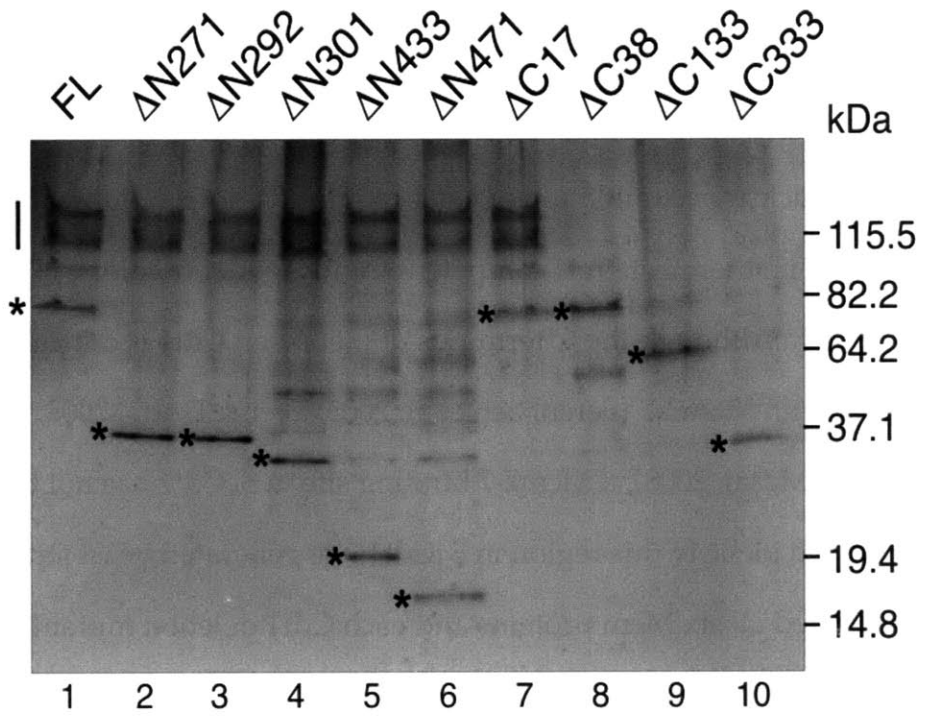

Figure 2. The C-terminal domain of Cdt1 binds Mcm2-7. FLAG immunoprecipitations of G1-arrested strains over-expressing all six Mcm proteins and the indicated FLAG-tagged Cdt1 constructs were analyzed by 4-20% SDS-PAGE separation followed by silver staining. When present, bars indicate Mcm2-7 proteins and the Cdt1 protein is indicated by the *.

To identify regions of ScCdt1 that are required for the initial recruitment of the Cdt1 to the origin, we used a reconstituted helicase-loading assay (Evrin et al, 2009; Remus et al, 2009; Seki & Diffley, 2000; Tsakraklides & Bell, 2010) to test each Cdt1 deletion that bound Mcm2-7. To detect association of Cdt1, we added ATPγS to the assays to arrest the helicase-loading process prior to ATP hydrolysis by Cdc6 (Randell et al, 2006). Under these conditions, ScCdt1 and Mcm2-7 stably associate with origin DNA, but Mcm2-7 loading is inhibited. Deletion of the entire N-terminal domain (ΔN271) or the C-terminal 17 residues of Cdt1 did not affect Cdt1 origin recruitment (Fig. 3A, compare lanes 1, 2, and 7). In contrast, deletion of the central domain of Cdt1 (ΔN433 and ΔN471) eliminated Cdt1 and Mcm2-7 recruitment (Fig. 3A, lanes 5 and 6). Notably, two N-terminal deletions that removed the N-terminal domain and IDR1 (ΔN292 and ΔN301) reduced Cdt1 origin association by approximately half (Fig. 3B). These data suggest that both IDR1 and the central domain contribute to origin recruitment of Cdt1.

Cdt1/Mcm2-7 Complex Formation is Necessary for Their Recruitment to the Origin

To test the three C-terminal deletion mutants that were unable to bind the Mcm2-7 complex, we used an extract-based helicase-loading assay (Randell et al, 2006). We immunodepleted endogenous Cdt1 from a G1 phase-arrested extract. Immunodepletion of Cdt1 did not result in co-depletion of Mcm2-7 (Fig. 4A), presumably due to excess Mcm2-7 in the extract. Consistent with previous studies (Randell et al, 2006), the Cdt1-depleted pre-RC assembly extract

Figure 3

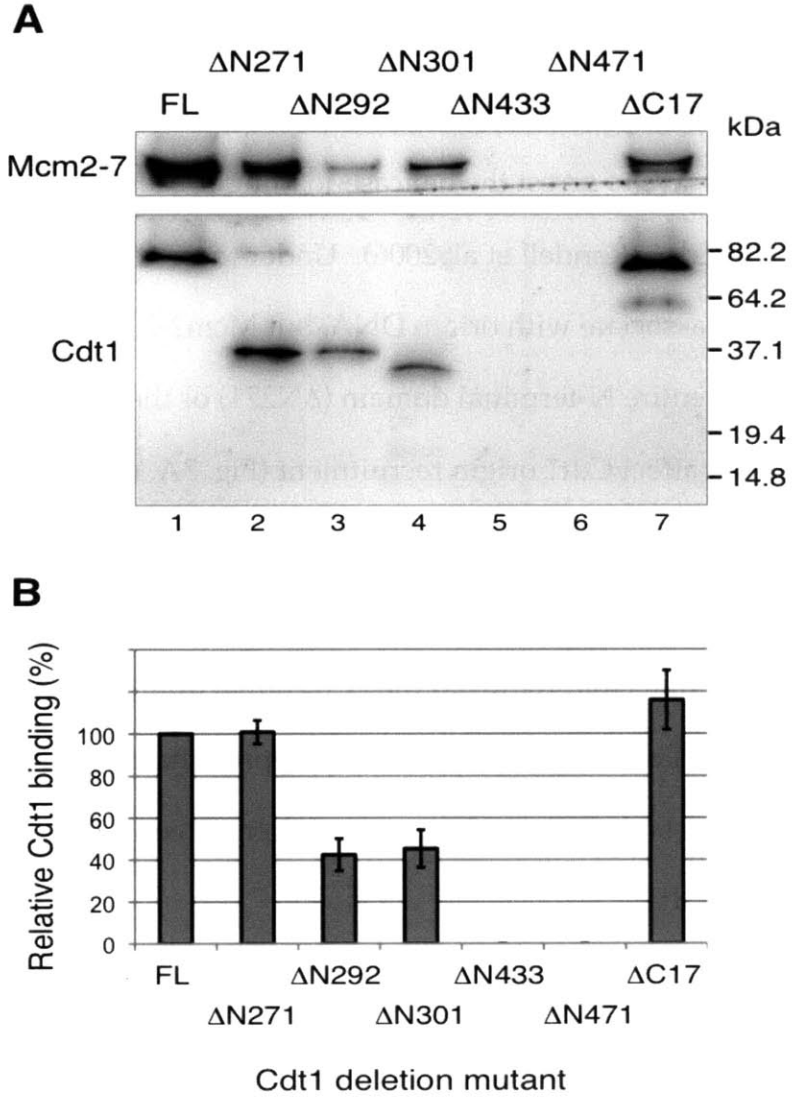

Figure 3. IDR1 and the central domain of Cdt1 are required for full Cdt1 origin recruitment. (A) Reconstituted Cdt1 recruitment assays were performed on 4 pmol 1039-bp *ARS1* DNA using 12 pmol purified ORC, Cdc6, and indicated Cdt1/Mcm2-7 in the presence of ATPγS. Assembly was allowed to proceed for 20 min at 25°C and origin-associated proteins were analyzed by 4-20% SDS-PAGE separation followed by immunoblotting with anti-Mcm2-7 (UM174) and anti-FLAG (Sigma) antibodies for Cdt1. Note that the use of a 4-20% SDS-PAGE gel results in the apparent detection of only one or two broad Mcm2-7 bands by immunoblot analysis. **(B)** Quantitation of Cdt1 origin association, expressed as a percentage relative to full-length Cdt1 origin recruitment. Error bars represent the standard deviation of three independent experiments.

Figure 4

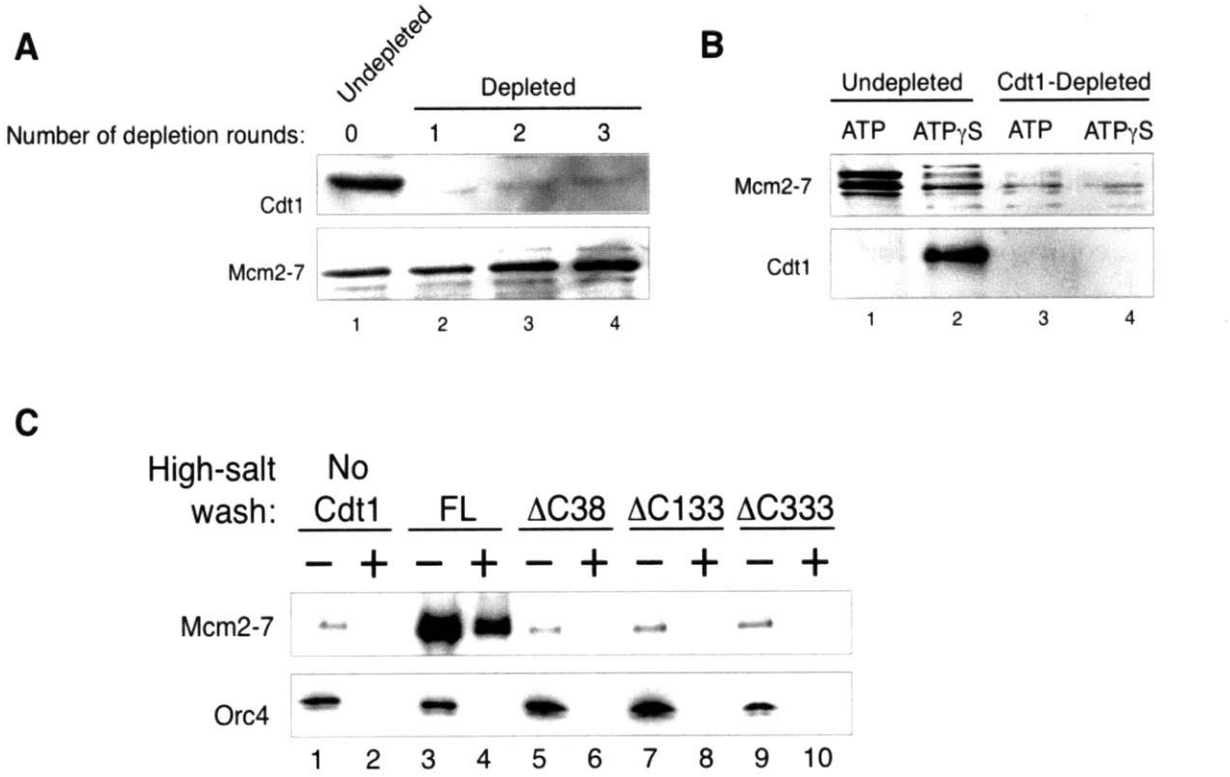

Figure 4. Cdt1 deletion mutants defective for Mcm2-7 interaction are unable to support Mcm2-7 origin association and loading *in vitro*. (A) Cdt1-specific immunodepletion from WCE. Cdt1 was immunodepleted from G1-arrested WCE competent for helicase loading (ySC17) by three successive rounds of incubation with beads coupled to anti-Cdt1 antibody (HM5352). All detectable Cdt1 was removed after the first round of Cdt1 depletion and Mcm2-7 was not co-depleted in the process. **(B)** Cdt1-depleted WCE does not support Mcm2-7 loading. Helicase-loading assays were performed with Cdt1-depleted and untreated ySC17 WCEs in the presence of ATP or ATPγS using 1 pmol *ARS1* DNA and H/buffer containing 300 mM KGlut. DNA was released from beads following three low-salt washes and DNA-associated proteins were analyzed by 8% SDS-PAGE followed by immunoblotting with anti-Mcm2-7 (UM174) and anti-Cdt1 (HM5352). **(C)** Extract-based Cdt1 recruitment assays were performed on 1 pmol *ARS1* DNA using Cdt1-depleted ySC17 extract supplemented with 3 pmol of the indicated purified Cdt1 in the presence of ATP. Assembled pre-RCs were washed with H/300 buffer containing 300 mM KGlut (low-salt wash) or 500 mM NaCl (high-salt wash) to distinguish loaded Mcm2-7 complexes. DNA-protein complexes were analyzed by 4-20% SDS-PAGE separation followed by immunoblotting with anti-Mcm2-7 (UM174) and anti-Orc4 (SB6) antibodies.

supported neither origin recruitment of Cdt1 in the presence of ATPγS nor Mcm2-7 loading in the presence of ATP (Fig. 4B).

Although addition of full-length Cdt1 restored Mcm2-7 origin association and loading to the Cdt1-depleted assembly extract, Cdt1 deletion mutants compromised for Mcm2-7 binding (ΔC38, ΔC133, ΔC333) could not support either function in the assay described above despite the presence of Mcm2-7 (Fig. 4C). Loaded Mcm2-7 complexes were biochemically distinguished from associated Mcm2-7 complexes by their resistance to high-salt wash, which removes ORC and all the proteins that depend on it for their origin association (Bowers et al, 2004; Donovan et al, 1997; Rowles et al, 1999). These data suggest that, in addition to possessing an intact IDR1 and central domain, Cdt1 must be in a complex with Mcm2-7 to support helicase recruitment and loading.

To test directly whether Cdt1/Mcm2-7 complex formation was critical for the recruitment of either component to the origin, we used the reconstituted Cdt1 recruitment assay to allow separate addition of Mcm2-7 and Cdt1 (Fig. 5A). We found that neither Cdt1 nor Mcm2-7 could robustly associate with origin DNA in the absence of the other (Fig. 5B, lanes 2-3). Addition of both Cdt1 and Mcm2-7 to the reaction did not restore Cdt1 or Mcm2-7 recruitment (Fig. 5B, lane 4). In contrast, if Cdt1 and Mcm2-7 were pre-incubated for 30 minutes and then added to the helicase-loading assay, Cdt1 and Mcm2-7 recruitment was rescued (Fig. 5B, lane 5). Importantly, when Cdt1 is immunoprecipitated from Cdt1 and Mcm2-7 mixtures with or without a 30-minute pre-incubation, the extent of

Figure 5

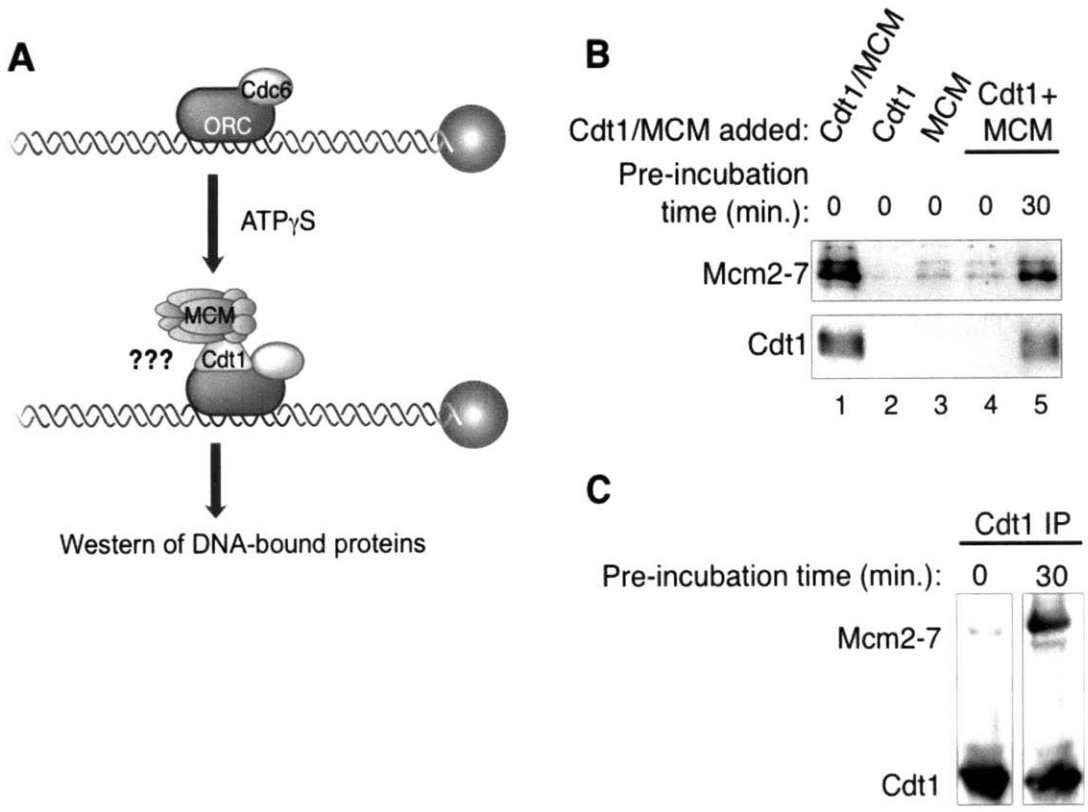

Figure 5. Cdt1/Mcm2-7 complex formation is required for recruitment of either component to the origin. (A) Experimental outline for *B*. ORC and Cdc6 were incubated with *ARS1* DNA in the presence of ATPγS and supplemented with purified Cdt1/Mcm2-7 complex, Cdt1, Mcm2-7, or Cdt1 and Mcm2-7 that was or was not pre-incubated with each other in H/300 buffer for 30 min at 25°C prior to their addition. Assays were performed using 1 pmol *ARS1* DNA and 4 pmol of each pre-RC component. **(B)** Pre-incubation of Cdt1 and Mcm2-7 supports their origin recruitment. The experiment was performed as described in *A*. DNA-protein complexes were isolated and analyzed by immunoblot with anti-Mcm2-7 (UM174) and anti-Cdt1 (HM5352) antibodies. **(C)** Pre-incubation of Cdt1 and Mcm2-7 results in Cdt1/Mcm2-7 complex formation. 8 pmol of purified Cdt1 and Mcm2-7 were incubated in H/300 buffer for 0 or 30 min at 25°C. After the indicated pre-incubation time, half of the Cdt1 and Mcm2-7 pre-incubation mixture was incubated with anti-Cdt1 (HM5352) antisera coupled to GammaBind G Sepharose beads for 20 min at 4°C. The other half was utilized as descripted in *A*. Immunoprecipitated fractions were collected following low-salt wash, TCA precipitated, and analyzed by immunoblot as in *B*.

Cdt1/Mcm2-7 complex formation correlated with the extent of origin recruitment of the two proteins (Fig. 5C). These data indicate that Cdt1/Mcm2-7 complex formation promotes the robust association of either component with the origin.

Multiple Cdt1 Molecules Assemble at the Origin to Load the Mcm2-7 Double Hexamer

The above recruitment studies demonstrate that Cdt1 plays a critical role in escorting the Mcm2-7 helicase to the origin. If IDR1 and the central domain of Cdt1 constitute one ORC/Cdc6 binding site, a single Mcm2-7 hexamer would be initially recruited to the origin. Alternatively, if IDR1 and the central domain of Cdt1 function as two independent ORC/Cdc6 binding sites, this would allow the initial recruitment of two Mcm2-7 hexamers to the origin. Consistent with the latter model, the Orc6 protein contains two essential, independent Cdt1 binding sites (Chen & Bell, 2011; Chen et al, 2007), and deletion of IDR1 of Cdt1 could inhibit the ability of Cdt1 to bind one of these two sites on Orc6. Consistent with this, deletion of the N-terminal domain and IDR1 decreased Cdt1 origin recruitment by approximately half, whereas further deletion of the central domain of Cdt1 eliminated Cdt1/Mcm2-7 origin recruitment completely (Fig. 3B). To distinguish between the two models, we asked if more than one Cdt1 molecule is recruited to the origin.

To determine whether one or multiple Cdt1 molecules assemble at the origin during helicase loading, we performed reconstituted Cdt1 recruitment assays with a mixture of untagged and Myc-tagged Cdt1 in complex with Mcm2-7. Tagged and untagged Cdt1 were equally functional for Mcm2-7 interaction,

origin recruitment, and helicase loading on the *ARS1* origin-containing DNA template (Fig. 6). After performing the assembly reaction in the presence of ATPγS, we released the DNA-bound intermediates from the beads and performed an immunoprecipitation using anti-Myc antibody (Fig. 7A). Consistent with the formation of a multi-Cdt1 ATPγS-stabilized intermediate, untagged Cdt1 was present in the anti-Myc immunoprecipitate when both tagged and untagged Cdt1 were used in the recruitment assay (Fig. 7B, lanes 3-5). Anti-Myc immunoprecipitation of helicase-loading intermediates also co-precipitated ORC and Mcm proteins (Fig. 7B). Importantly, in the absence of origin DNA only Mcm proteins were associated with tagged Cdt1 (Fig. 7B, lanes 1 and 2), indicating that co-immunoprecipitation required formation of the ATPγS-stabilized intermediate and was not the result of interactions in solution. Thus, multiple molecules of Cdt1 are recruited to the origin.

The recruitment of multiple Cdt1 molecules to the origin could be achieved by two distinct means: (1) a single origin-bound ORC/Cdc6 complex interacting with multiple Cdt1 molecules or (2) one Cdt1 interacting with multiple ORC/Cdc6 complexes bound to a single origin. Although the *ARS1* origin contains only one high-affinity ORC binding site, the B2 element of *ARS1* has the potential to accommodate a second ORC molecule in opposite orientation (Wilmes & Bell, 2002). To test if a second ORC molecule bound to B2 mediated the formation of a multi-Cdt1 helicase-loading intermediate, we performed the same IP experiment above using an *ARS1* template lacking B2. As in the experiment using wild-type *ARS1* template, untagged Cdt1 was co-precipitated

Figure 6

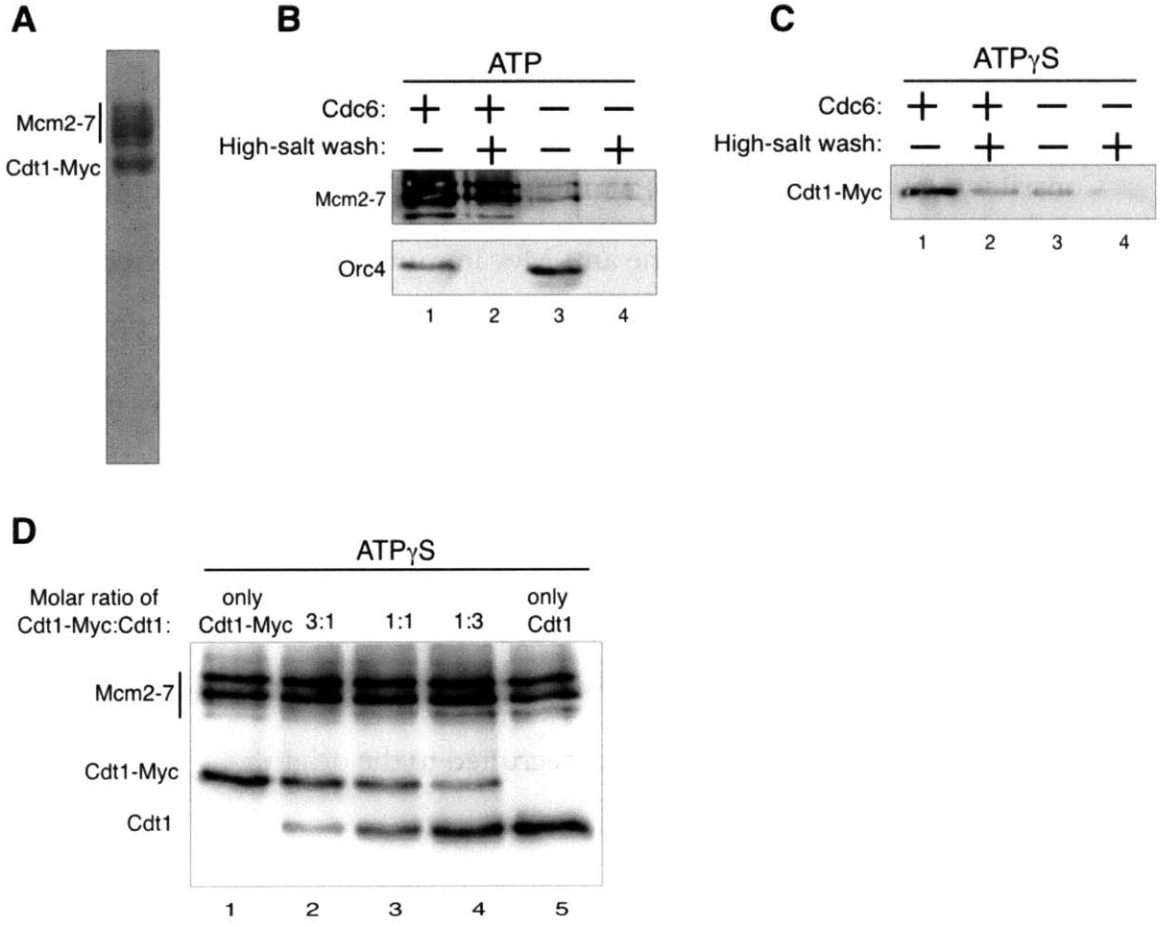

Figure 6. Myc-tagged Cdt1/Mcm2-7 complexes are capable of supporting Mcm2-7 helicase loading. (A) Purified Myc-tagged Cdt1/Mcm2-7 complexes. G1-arrested whole cell extract was generated from a strain over-expressing all six Mcm proteins and Cdt1 tagged with 4.5xMyc and 1xFLAG at its C-terminus. Tagged Cdt1/Mcm2-7 complexes were purified by single-step FLAG purification. Mcm2-7 co-purified with tagged Cdt1 as analyzed by Coomassie staining. **(B)** Reconstituted helicase-loading assays were performed on 1 pmol *ARS1* DNA using 4 pmol of ORC, Cdc6 and tagged Cdt1/Mcm2-7 in the presence of ATP. Cdc6 was omitted from the reaction mixture in lanes 3 and 4. DNA-protein complexes were analyzed by 8% SDS-PAGE followed by immunoblotting with anti-Mcm2-7 (UM174) and anti-Orc4 (SB6) antibodies. **(C)** Reconstituted Cdt1 recruitment assays were performed as described in *B*, except that the reactions were supplied with ATPγS instead of ATP. Anti-Cdt1 (HM5352) antibody was used to detect Cdt1 by immunoblot. **(D)** Tagged and untagged Cdt1 exhibit equivalent levels of incorporation into the pre-RC. Fully reconstituted Cdt1 recruitment assays were performed on 2 pmol *ARS1* DNA using 6 pmol purified ORC, Cdc6, and indicated molar ratio of tagged and untagged Cdt1/Mcm2-7 (6 pmol total) in the presence of ATPγS and H buffer containing 300 mM KGlut (H/300). Assembled complexes were washed, DNA-protein complexes were released from beads, and samples were separated by 7% SDS-PAGE and analyzed by immunoblot with anti-Mcm2-7 (UM174) and anti-Cdt1 (HM5352) antibodies.

Figure 7

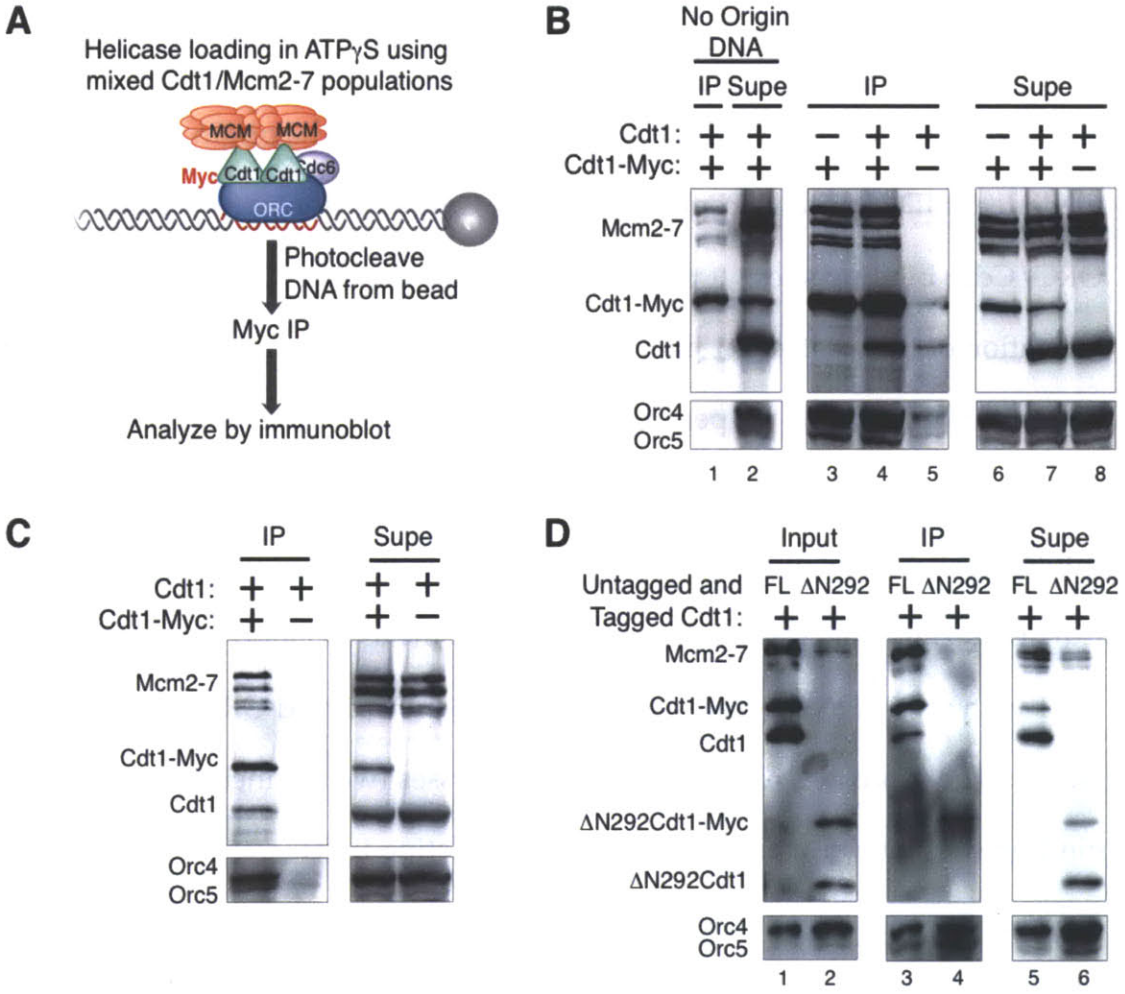

Figure 7. Multiple Cdt1 molecules associate with the origin to promote Mcm2-7 loading. (A) Experimental outline for *B*. Fully reconstituted Cdt1 recruitment assays were performed using purified ORC, Cdc6, and Cdt1/Mcm2-7 in the presence of ATPγS. When tagged and untagged Cdt1/Mcm2-7 species were mixed, equimolar amounts of each species were used. Assembled complexes were washed, DNA and associated proteins were released from beads, and incubated with anti-Myc antibody coupled to GammaBind G Sepharose beads. Immunoprecipitated (IP) fractions were separated from supernatant fractions, and both fractions were TCA precipitated and analyzed by immunoblotting with anti-Mcm2-7 (UM174), anti-Cdt1 (HM5352), and anti-ORC (1108) antibodies. **(B)** Helicase loading occurs via a multi-Cdt1 helicase-loading intermediate. The experiment was performed as outlined in *A*. The "no-origin DNA" control was performed with 8 pmol of all pre-RC components in the absence of *ARS1* DNA. **(C)** Untagged Cdt1 is co-immunoprecipitated in the absence of an intact *ARS1* B2 element. Experiment was performed as described in *A*, except a 1-kb linear *ARS1* B2- template was used instead of the wild-type DNA version. **(D)** IDR1 is essential for formation of a multi-Cdt1 intermediate. The experiment was performed as described in *A*, except that tagged and untagged ΔN292 Cdt1/Mcm2-7 were also tested.

with tagged Cdt1 (Fig. 7C). Thus, binding of ORC to the B2 element is not necessary to form a multi-Cdt1 helicase-loading intermediate.

Recruitment of two Cdt1/Mcm2-7 molecules to the origin could be mediated if IDR1 and the central domain of Cdt1 function as two independent ORC/Cdc6 binding sites. If this is the case, then deleting IDR1 should eliminate formation of the multi-Cdt1 intermediate and co-precipitation of untagged Cdt1. To test this possibility, we performed the immunoprecipitation assay using tagged and untagged IDR1 deletion mutant ΔN292 Cdt1/Mcm2-7. In contrast to full-length Cdt1, we did not observe the co-precipitation of untagged ΔN292 Cdt1 (Fig. 7D). This was not due to a defect in the association of tagged ΔN292 Cdt1 with origin DNA because this protein co-precipitated ORC (Fig. 7D, lanes 3 and 4). Therefore, we suggest that Cdt1 possesses two distinct ORC/Cdc6 binding sites, one of which is disrupted by deleting IDR1.

Mcm2-7 Helicase Loading Requires Normal Levels of Cdt1 Origin Recruitment

The recruitment of two Cdt1 molecules to the origin by a single ORC molecule suggests a mechanism for the coordinated loading of two Mcm2-7 hexamers (Remus et al, 2009). To address whether Cdt1 functions after the initial recruitment of Mcm2-7 hexamers to the origin, we tested all of the Cdt1 deletions that bound Mcm2-7 for the ability to load Mcm2-7 onto origin DNA. These assays replaced ATPγS with ATP to support nucleotide hydrolysis and helicase loading, and high-salt washes distinguished loaded Mcm2-7 complexes from associated complexes. Both the ΔN271 and ΔC17 Cdt1 mutants, which displayed

no defect in their origin recruitment, supported Mcm2-7 loading *in vitro* (Fig. 8A, lanes 4 and 14), although ΔC17 Cdt1 showed a partial defect (Fig. 8A, compare lanes 2 and 14). In contrast, each of the Cdt1 mutations that was defective for the initial association of Cdt1 / Mcm2-7 was also defective for Mcm2-7 loading. Consistent with their complete loss of origin recruitment, the two Cdt1 deletion mutants lacking the central domain (ΔN433 and ΔN471) showed no Mcm2-7 loading onto origin DNA (Fig. 8A, lanes 9-12). Intriguingly, the two Cdt1 deletion mutants with a disrupted IDR1 (ΔN292 and ΔN301) that showed only a two-fold reduction in their origin recruitment (Fig. 3) exhibited a partial defect in Mcm2-7 association but a complete defect in helicase loading (Fig. 8A, lanes 5-8). These findings indicate that an intact IDR1 and central domain of Cdt1 is not only critical to achieve complete Cdt1 origin recruitment but also to support any degree of Mcm2-7 loading.

Unlike all the other lethal deletion mutations, a ΔN271 Cdt1 deletion mutant supported normal levels of Mcm2-7 loading (Fig. 8B). To test whether ΔN271 Cdt1 supported the loading of topologically linked Mcm2-7 (Remus et al, 2009), we compared residence times of Mcm2-7 complexes loaded by ΔN271 Cdt1 onto either a *ARS1*-containing 3.7-kb DNA circular template or a linear version of the same template. Topologically linked Mcm2-7 showed a shorter half-life of DNA association on the linear DNA template compared to the circular template due to sliding off the end of the linear version (Remus et al, 2009) (Fig. 8C). Mcm2-7 loaded by full-length or ΔN271 Cdt1 each showed similar half-lives

Figure 8

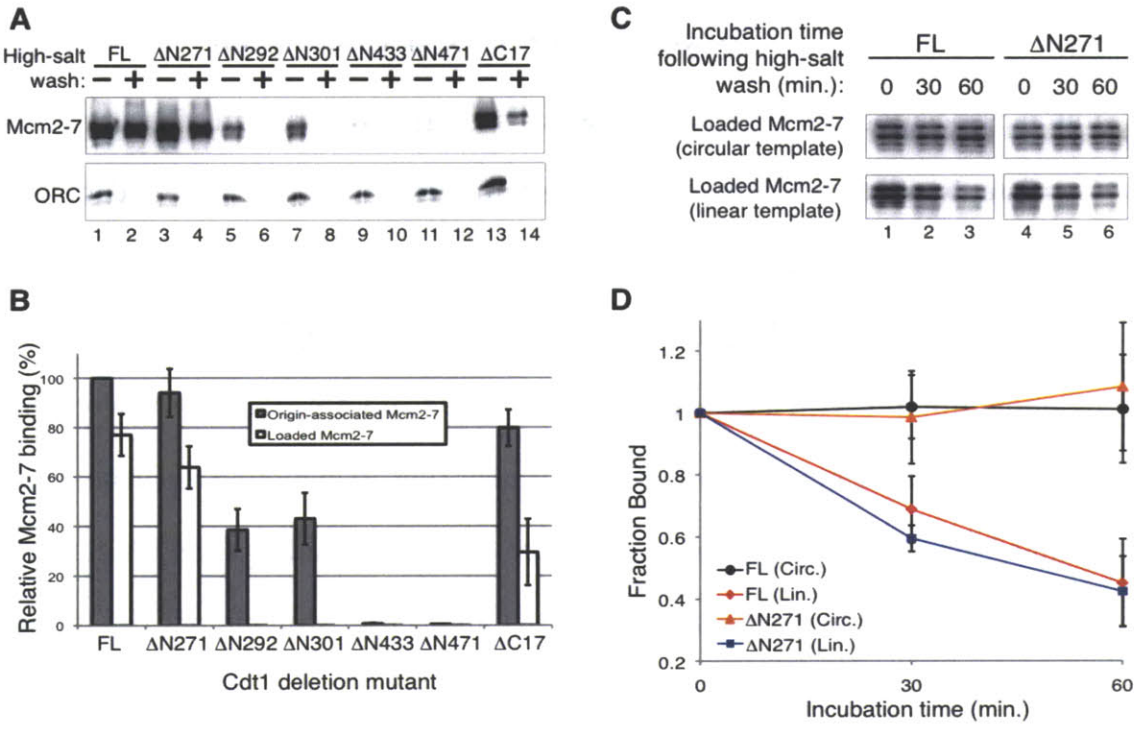

Figure 8. IDR1 and the central domain of Cdt1 are required for Mcm2-7 origin loading. (A) Reconstituted helicase-loading assays were performed on 1 pmol *ARS1* DNA using 4 pmol of ORC, Cdc6 and indicated Cdt1/Mcm2-7 in the presence of ATP. Assembled pre-RCs were washed with H/300 buffer containing 300 mM KGlut (low-salt wash) or 500 mM NaCl (high-salt wash) to distinguish loaded Mcm2-7 complexes. DNA-protein complexes were analyzed by 4-20% SDS-PAGE separation followed by immunoblotting with anti-Mcm2-7 (UM174) and anti-Orc4 (SB6) antibodies. **(B)** Quantitation of Mcm2-7 origin association (low-salt wash) and loading (high-salt wash) for indicated Cdt1 proteins, expressed as a percentage relative to Mcm2-7 origin association levels supported by full-length Cdt1. Error bars represent that standard deviation of three independent experiments. **(C)** Time course analysis of ΔN271 Cdt1-loaded Mcm2-7 and full-length Cdt1-loaded Mcm2-7 complexes on circular and linear DNA templates. Fully reconstituted helicase-loading assays were performed on 175 fmol pUC19 *ARS1* wild-type circular DNA (*top*) using 2 pmol purified ORC, Cdc6, and indicated Cdt1/Mcm2-7 or 500 fmol 1-kb linear *ARS1* DNA (*bottom*) using 1 pmol purified pre-RC components in the presence of ATP and H buffer containing 300 mM KGlut (H/300). Assembled pre-RCs were washed with 500 mM NaCl (high-salt wash) to isolate loaded Mcm2-7 complexes. Loaded Mcm2-7 was incubated in H/300 for the indicated amounts of time at 25°C. DNA-protein complexes were analyzed by 8% SDS-PAGE followed by immunoblotting with anti-Mcm2-7 (UM174) antibody. **(D)** Quantitation of Mcm2-7 Western blot signals. Fraction bound reflects amount of Mcm2-7 at a given time point (30 or 60 min) relative to amount of Mcm2-7 with no incubation time (0 min) for a given DNA template and Cdt1 protein. Error bars represent that standard deviation of three independent experiments.

on both templates, indicating that ΔN271 Cdt1 supported the loading of topologically linked Mcm2-7 helicases.

Mcm2-7 Helicases Loaded by ΔN271 Cdt1 Are Not Competent for Replication Initiation

Our data indicate that the N-terminus of ScCdt1 is dispensable for Mcm2-7 binding, origin recruitment, and topologically linked origin-DNA loading, however, this mutation is unable to complement a *CDT1* deletion (Fig. 1). Therefore, we asked if the N-terminus is required for the downstream steps of replication initiation using a recently developed assay for origin-dependent in vitro replication initiation that supports helicase activation, replisome assembly, and DNA replication initiation and elongation (Heller et al, in prep). This assay recapitulates the G1-to-S phase transition in a three-step process: (1) Mcm2-7 is loaded onto immobilized *ARS1*-containing DNA using a G1 phase-arrested whole cell extract (Bowers et al, 2004); (2) loaded Mcm2-7 is treated with purified DDK; (3) and Mcm2-7 is activated by subsequent incubation with a S-phase extract that is arrested at the non-permissive temperature and poised to initiate replication.

To test the ability of ΔN271 Cdt1 to load replication-competent Mcm2-7, we immunodepleted endogenous Cdt1 from the G1 extract and added back purified Cdt1/Mcm2-7 complex to support helicase loading before exposure to DDK and S-phase extract. Cdt1-depleted G1 extract was unable to support helicase loading (Fig. 9A, lanes 1-2, 5-6) or replication as analyzed by alkaline gel

Figure 9

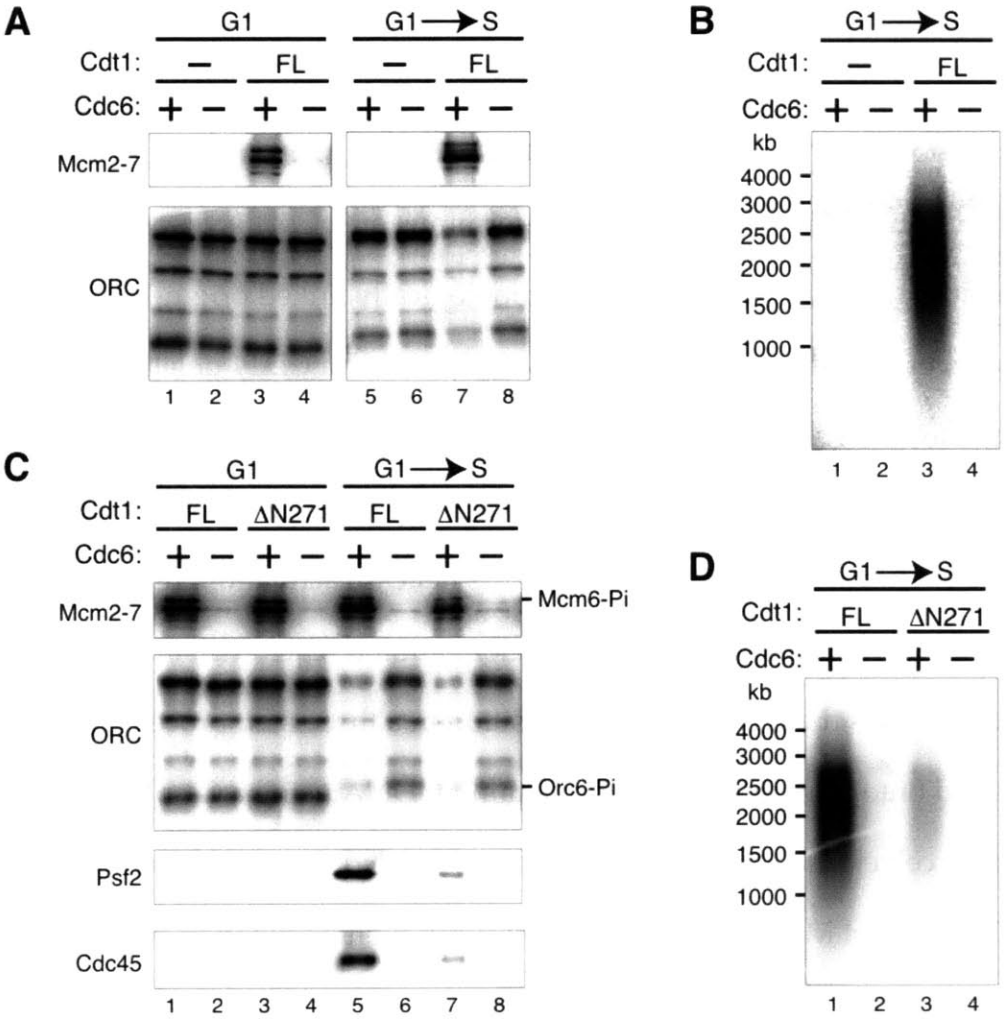

Figure 9. Mcm2-7 Helicases Loaded by ΔN271 Cdt1 Are Not Competent for Replication Initiation. (A) Full-length Cdt1 supports the loading of Mcm2-7 that are competent for replication. *In vitro* replication assays were performed on 175 fmol of bead-coupled pUC19 *ARS1* wild-type DNA. An ORC-overexpressing whole cell extract (F1ORC1) immunodepleted of endogenous Cdt1 was or was not supplied with purified full-length Cdt1/Mcm2-7 and Cdc6 during the helicase-loading reaction step (G1). All reactions were subsequently treated with DDK and exposed to a *cdc7-4* S-phase extract (yRH182) (G→S). Beads were washed and DNA-protein complexes were removed by boiling in Laemmli sample buffer for 5 min. Proteins were analyzed by immunoblotting with anti-Mcm2-7 (UM174) and anti-ORC (1108) antibodies. **(B)** Replication products from the reactions in *A* were analyzed by alkaline gel electrophoresis. **(C)** *In vitro* replication assays were performed as in *A*, except that the helicase-loading reactions were supplied with either full-length or ΔN271 Cdt1. Proteins were analyzed by immunoblotting with anti-Mcm2-7 (UM174), anti-ORC (1108), anti-HA (12CA5), and anti-Myc (9E10) antibodies. yRH182 S-phase extract contains HA-tagged Cdc45 and Myc-tagged Mcm10 and Psf2 proteins. **(D)** Replication products from the reactions in *C* were analyzed by alkaline gel electrophoresis.

(9B, lanes 1-2). Addition of full-length Cdt1/Mcm2-7 to the Cdt1-depleted G1 extract resulted in Cdc6-dependent Mcm2-7 loading and replication initiation (Fig. 9A, lanes 3-4, 7-8; Fig. 9B, lanes 3-4). As expected, addition of ΔN271 Cdt1/Mcm2-7 to the G1 extract restored Cdc6-dependent Mcm2-7 loading at levels comparable to full-length Cdt1 (Fig. 9C, lanes 1-4). Interestingly, when ΔN271 Cdt1-loaded Mcm2-7 complexes were exposed to S-phase extract, Mcm2-7 levels decreased modestly relative to that of full-length Cdt1-loaded Mcm2-7 (Fig. 9C, lanes 5-8) but replication initiation was severely impaired (Fig. 9D, lanes 1-4). Although well characterized targets of both CDK (Orc2 and Orc6) and DDK (Mcm6) were detectably phosphorylated following exposure to S-phase extract (Fig. 9C, lanes 7-8; Fig. 10), Mcm2-7 complexes loaded with ΔN271 Cdt1 were severely impaired for the recruitment of replication initiation factors Psf2 (a GINS subunit) and Cdc45 (Fig. 9C, compare lanes 5 and 7). These data show that the N-terminus of Cdt1 is required to load Mcm2-7 complexes that are competent to interact with helicase activating factors and drive the initiation of replication.

DISCUSSION

Loading of the Mcm2-7 replicative helicase is a highly regulated process that requires the coordinated action of ORC, Cdc6, and Cdt1. This event requires sequential ATP hydrolysis events by ORC and Cdc6, but the contribution of Cdt1 during the loading process is less clear. Here, we examined the function of ScCdt1 during helicase loading. We demonstrate that the C-terminal domain of

Figure 10

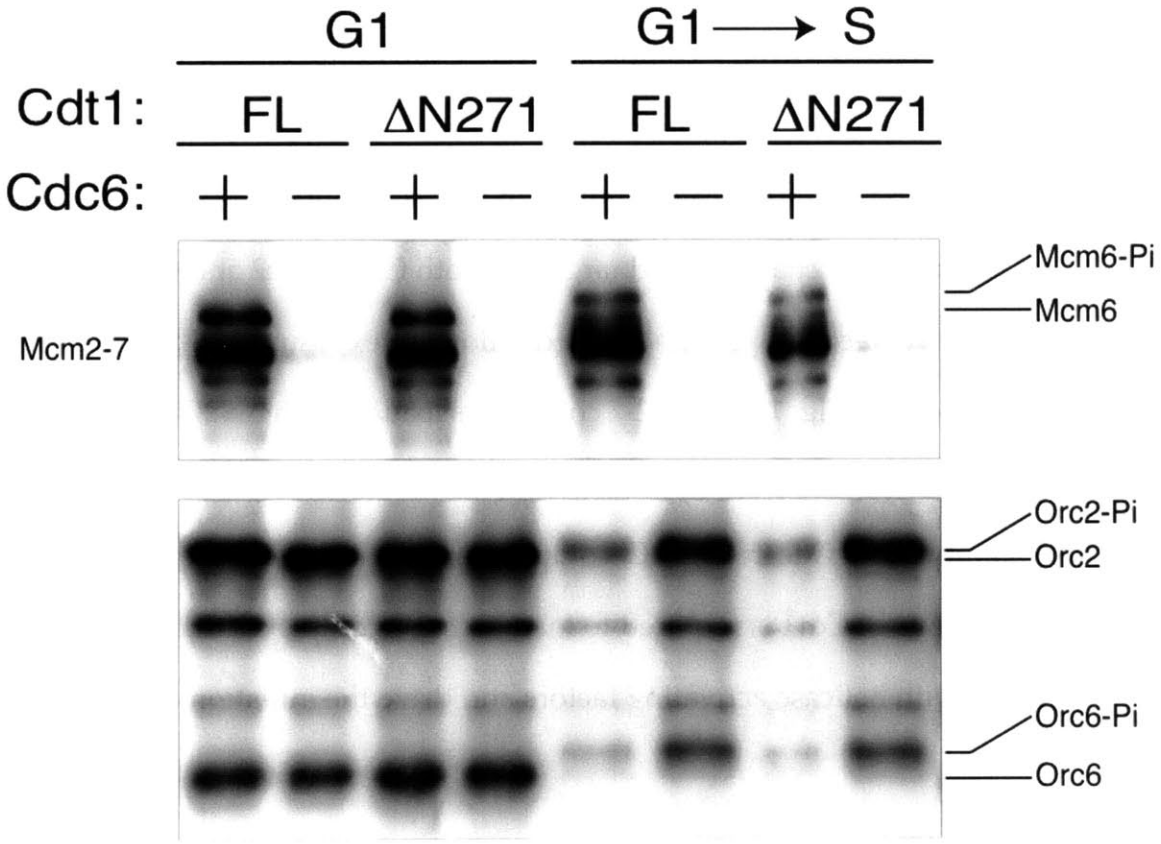

Figure 10. Detection of CDK and DDK phosphorylation of pre-RC components. Enlarged version of Mcm2-7 and ORC immunoblots of Fig. 9C. Bands corresponding to unmodified and modified substrates of CDK (Orc2 and Orc6) and DDK (Mcm6) are noted.

Cdt1 is critical for Mcm2-7 binding, and that Cdt1 must be in a complex with Mcm2-7 for either protein to be recruited to the origin. We identified two distinct sites in the central region of Cdt1 that contribute to Cdt1/Mcm2-7 origin recruitment and are essential for subsequent Mcm2-7 loading (Fig. 11A). Additional studies revealed that two molecules of Cdt1 are recruited to the origin during pre-RC formation, and disrupting the formation of a multi-Cdt1 intermediate prevents Mcm2-7 helicase loading. Finally, we found that the N-terminal domain of Cdt1 is required to establish loaded Mcm2-7 complexes that are competent to recruit helicase-activating proteins and initiate replication. Our findings reveal a multi-faceted role of Cdt1 during helicase loading and support a model in which the formation of a dimeric-Cdt1 helicase-loading intermediate is essential for helicase loading and replication initiation.

In *S. cerevisiae*, the interaction between Cdt1 and the Mcm2-7 helicase is critical for either protein to gain nuclear entry (Tanaka & Diffley, 2002). Our analysis of Mcm2-7 binding by Cdt1 reveals that the C-terminal domain of ScCdt1, predicted to adopt a WHD fold as in metazoan Cdt1 (Jee et al, 2010; Khayrutdinov et al, 2009), contains the Mcm2-7 binding site (a.a. 472-587). We find that residues 566-587, predicted to contain β-strands 2 and 3 (S2 and S3) and Wing 1 (W1) of the C-terminal WHD, define essential residues for helicase binding within the Mcm2-7 binding site, consistent with studies of metazoan Cdt1 that implicate S2 in Mcm2-7 binding (Khayrutdinov et al, 2009) (Fig. 11A).

Figure 11

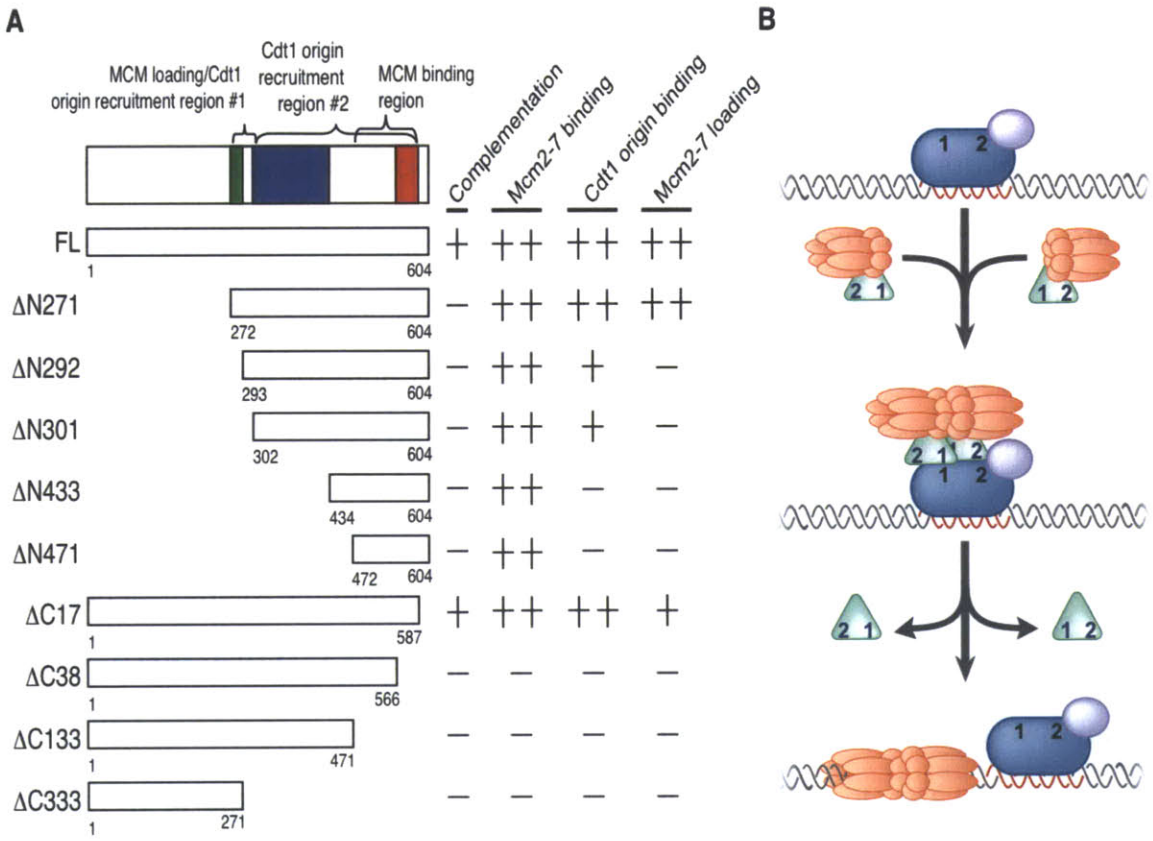

Figure 11. Contribution of Cdt1 during the Mcm2-7 helicase-loading process. (A) Summary of Cdt1 deletion mutant analysis. **(B)** Proposed model of Mcm2-7 helicase loading via a multi-Cdt1 helicase-loading intermediate. A single origin-bound ORC/Cdc6 exposes two Cdt1 binding sites on Orc6 and recruits two Cdt1/Mcm2-7 molecules to the origin. The two Cdt1/Mcm2-7 molecules bind both Orc6 binding sites via its two non-identical origin recruitment regions. At this stage, the two Mcm2-7 hexamers are oppositely oriented with respect to one another and may form a double hexamer as shown. The origin recruitment of two Cdt1/Mcm2-7 complexes leads to ATP hydrolysis by Cdc6 results in the loading of the Mcm2-7 double hexamer and the disassociation of both Cdt1 molecules from the origin.

The requirement for formation of a Cdt1/Mcm2-7 complex to recruit either protein to the origin (Fig. 5) imposes another layer of regulation during helicase loading. It is possible that Cdt1 and Mcm2-7 each have weak ORC/Cdc6 binding sites that are both required for the tight binding required for detection of origin binding in our assays. Alternatively, interaction between Mcm2-7 and Cdt1 may reveal binding sites that are otherwise obscured. Because Cdt1 binds to Orc6 in solution (Chen et al, 2007), it is also possible that interaction of Cdt1/Mcm2-7 with ORC/Cdc6 is required to uncover the Cdt1 binding sites on Orc6.

Origin recruitment of ScCdt1 requires the central domain and IDR1. Disrupting IDR1 (ΔN292/301) consistently reduces Cdt1 origin recruitment by approximately half (Fig. 3) and the same mutation reduces the number of Cdt1 molecules recruited to the origin to only one (Fig. 7D), strongly suggesting that IDR1 and the central domain function as two independent origin recruitment sites. Interestingly, mutants that disrupt IDR1 show a much larger reduction in Mcm2-7 origin recruitment than Cdt1 (Fig. 3 and 7D). This suggests that there are positive interactions between the multiple Cdt1/Mcm2-7 complexes recruited that stabilize Mcm2-7 association. One intriguing possibility is that Mcm2-7 double-hexamer formation is initiated at this earlier step in the helicase-loading process.

Consistent with IDR1 and the central domain functioning as two distinct origin recruitment sites, we demonstrate that multiple Cdt1 molecules are normally recruited to the origin during the helicase-loading process (Fig. 7).

Multiple lines of evidence suggest that a single ORC molecule mediates the formation of the multi-Cdt1 intermediate. First, the multi-Cdt1 pre-RC intermediate can form in the absence of the B2 element of *ARS1* (Fig. 7C), which represents the most likely second ORC binding site at *ARS1*. Second, we observe the immunoprecipitation of only tagged ΔN292 Cdt1 from a helicase-loading intermediate assembled on a wild-type *ARS1* template (Fig. 7D). If two ORC molecules mediate the initial recruitment of Cdt1/Mcm2-7, then untagged ΔN292 Cdt1 bound to one ORC molecule would be expected to co-precipitate via an origin DNA-mediated interaction with the second ORC bound to tagged ΔN292 Cdt1. Third, the Orc6 subunit of ORC, which is required to recruit Cdt1, contains two independent Cdt1 binding sites, both of which are essential for ORC function (Chen & Bell, 2011; Chen et al, 2007).

Based on our data suggesting an additive contribution of IDR1 and the central domain to Cdt1 binding (Fig. 3) as well as previous data showing Orc6 possesses two independent Cdt1 binding sites (Chen et al, 2007), we propose that two Cdt1 molecules are recruited to the origin, each escorting a Mcm2-7 complex, to initiate Mcm2-7 double-hexamer loading during pre-RC formation. In this model, an origin-bound ORC/Cdc6 complex recruits two Cdt1/Mcm2-7 molecules to the origin with the Orc6 protein playing a primary role in these interactions. The origin recruitment of Cdt1 and Mcm2-7 is mediated by IDR1 and the central domain of Cdt1, which we envision act as two distinct Orc6 binding sites that are complementary to the two unrelated Cdt1 binding sites on Orc6. The presence of two independent Cdt1-Orc6 interaction domains has the

potential to recruit and load Mcm2-7 helicases in opposite orientations and facilitate head-to-head double-hexamer formation. In this model, the dual Cdt1 binding sites could initiate double-hexamer formation prior to helicase loading. Loading is completed by Cdc6 ATP hydrolysis, which also evicts Cdt1 from the origin (Fig. 11B).

Disrupting the formation of a multi-Cdt1 helicase-loading intermediate inhibits Mcm2-7 loading. We demonstrate that loss of one putative ORC/Cdc6 binding site (ΔN292) on Cdt1 results in the origin association of only one Cdt1 species (Fig. 7D). This ΔN292 Cdt1 deletion mutant also showed a two-fold reduction in Mcm2-7 association but no helicase loading in the presence of ATP (Fig. 8). Interestingly, a fusion of the C-terminal domain of Orc6 to Cdt1, in which there is one molecule of Cdt1 per ORC complex, also exhibits very inefficient helicase loading (Chen et al, 2007). We suspect the defect in Mcm2-7 loading by the Cdt1-ORC fusion could be due to an inability to recruit two Cdt1/Mcm2-7 complexes to the origin to initiate coordinated helicase loading. Notably, recent work has proposed that preventing the formation of a multi-Cdt1 pre-RC intermediate is likely to be the primary mechanism by which CDK inhibits ORC function outside of G1 phase (Chen & Bell, 2011). By inactivating one of the two Cdt1 binding sites of Orc6, CDK causes a two-fold decrease in the amount of origin-associated Cdt1/Mcm2-7 complexes and inhibits Mcm2-7 loading. These results are consistent with inhibiting helicase loading by preventing the recruitment of one Cdt1/Mcm2-7 complex to the origin and

suggest that CDK regulates ORC function by directly preventing a coordinated helicase-loading process.

Intriguingly, the N-terminal domain of Cdt1 does not appear to influence the function of Cdt1 during the helicase-loading process, but the loaded Mcm2-7 complexes are unable to recruit helicase-activating proteins and are defective for initiating replication (Fig. 9). Why are Mcm2-7 complexes loaded with ΔN271 Cdt1 impaired for the recruitment of helicase-activating factors? One possibility is that ΔN271 Cdt1 can only support the loading of single Mcm2-7 hexamers, which are unable to promote the recruitment of helicase-activating factors when not part of a double-hexameric complex. Interestingly, this model demands that Mcm2-7 resistance to high-salt wash is established at the level of topological linkage around DNA, not double-hexamer formation. Alternatively, Mcm2-7 complexes loaded by ΔN271 Cdt1 could form double hexamers with a conformation that does not support the robust recruitment of downstream helicase-activating factors. However, we do not envision that the ΔN271 Cdt1-loaded Mcm2-7 complexes exhibit greatly different conformations from normally loaded Mcm2-7 complexes for two reasons: (1) ΔN271 Cdt1-loaded Mcm2-7 complexes do not exhibit defects in mobility on DNA (Fig. 8D) and (2) their susceptibility to modification by DDK (Fig. 10) suggests that they are loaded onto DNA in a conformation that facilitates DDK binding and phosphorylation (Francis et al, 2009). One possibility is that Cdc45 and GINS are unable to bind and bridge the discontinuity between the Mcm2 and Mcm5 subunits of ΔN271 Cdt1-loaded Mcm2-7 complexes (Costa et al, 2011). If the opening of the Mcm2-

Mcm5 "gate" is compromised in a ΔN271 Cdt1-loaded Mcm2-7 complex, the gap is not drastic enough to also affect its affinity for DNA (Fig. 8C). Another possibility is that the individual hexamers within the head-to-head Mcm2-7 double hexamer are improperly oriented relative to one another. Intriguingly, this model demands that whereas DDK activity is indifferent to inter-hexamer subunit orientation, the recruitment of helicase activating factors to the double hexamer is sensitive to the orientation of Mcm2-7.

Although our studies demonstrate that Cdt1 plays multiple roles during the helicase-loading process and provide a simple solution to explain how symmetric replication-competent Mcm2-7 double hexamers are loaded from asymmetric origins of replication, many aspects of the Mcm2-7 loading process remain to be elucidated. Does coordinated Mcm2-7 loading entail to concerted or sequential loading of origin-recruited helicases? Are multiple ATP hydrolysis events by Cdc6 required to load the Mcm2-7 double hexamer? What is the stoichiometry of Cdc6 in the helicase loading reaction? The development of new assays that directly monitor the formation of the loaded Mcm2-7 double hexamer with better time resolution will be required to answer these questions.

MATERIALS AND METHODS

Strain Construction

Strains were prepared using standard laboratory methods. Yeast strains used in this study are listed in the Table S1.

Complementation Analysis

Each Cdt1 deletion mutant was placed under the control of the endogenous Cdt1 promoter in a Leu-marked test plasmid (pRS405) and integrated into the genome of yTJT73. Complementation was scored by the ability of the integrated Cdt1 test plasmid to support growth of yTJT73 in the presence of FOA, which selects against the presence of a plasmid including wild-type *CDT1*.

Protein Purification

Purified ORC was prepared from yeast strain F1ORC1 as described previously (Tsakraklides & Bell, 2010). Cdc6 was purified from Escherichia coli as described previously (Randell et al, 2006). Cdt1/Mcm2-7 and Cdt1 complexes were prepared as described previously (Tsakraklides & Bell, 2010), except that yeast cultures were arrested in G1 phase with α-factor for 3 hours before galactose induction for 4.5 hours. Extract was incubated with FLAG resin for 12 hours at 4°C.

Cdt1 Recruitment and Helicase-Loading Assays

Helicase-loading assays for Cdt1 capable of Mcm2-7 binding were performed as previously described (Tsakraklides & Bell, 2010). Cdt1 recruitment assays for Cdt1 capable of Mcm2-7 binding were performed as described above for the helicase-loading assay, except that ATP was replaced with ATPγS and 4 pmol of 1039-bp *ARS1* DNA and 12 pmol purified ORC, Cdc6, and Cdt1/Mcm2-7 were used per reaction. Helicase-loading and Cdt1 recruitment assays for Cdt1 unable to bind Mcm2-7 were performed as described previously (Randell et al, 2006).

Co-Immunoprecipitation Assays

Cdt1-containing helicase-loading intermediates were assembled from purified proteins as described above for the Cdt1 recruitment assay for Cdt1 capable of Mcm2-7 binding, except that 8 pmol of 1039-bp *ARS1* (wild-type or $B2^-$) DNA and 32 pmol of purified ORC, Cdc6, and Cdt1/Mcm2-7 were used per reaction. When tagged and untagged Cdt1/Mcm2-7 species mixed, 16 pmol of tagged Cdt1/Mcm2-7 and 16 pmol of untagged Cdt1/Mcm2-7 were used. Assembled complexes were washed, DNA-protein complexes were released from beads by UV irradiation, and complexes were incubated with anti-Myc antibody coupled to GammaBind G Sepharose beads for 1.5 hr at 4°C in H/300 buffer supplemented with 3 mM ATPγS. Immunoprecipitated (IP) fractions were separated from supernatant fractions by three successive washes with H/300 buffer at 4°C. IP and supernatant fractions were TCA precipitated and analyzed by immunoblotting with anti-Mcm2-7 (UM174), anti-Cdt1 (HM5352), and anti-ORC (1108) antibodies.

In vitro Replication Assays

Replication assays were performed on 3.7-kb pUC19 *ARS1* wild-type containing-plasmids that was biotinylated and coupled to streptavidin-coated magnetic beads (Heller et al, in prep). Each replication reaction was performed using 175 fmol of bead-coupled pUC19 *ARS1*. Helicase loading was performed in a 40-µl reaction volume using standard in vitro helicase-loading conditions (Bowers et al, 2004) with Cdt1-depleted F1ORC1 (Tsakraklides & Bell, 2010) whole cell extract supplied with 1 pmol purified full-length or ΔN271 Cdt1/Mcm2-7 complexes and Cdc6. Endogenous Cdt1 was immunodepleted from F1ORC1 whole cell extract as previously described (Randell et al, 2006). Beads containing DNA-protein complexes were then treated with DDK. DDK phosphorylation reactions were performed as previously described (Francis et al, 2009), except that reactions contained 225 mM KGlut, 3.5 mM MgOAc, 5% glycerol, 1 mM spermine, 3 mM DTT, and 1 mM ATP. DDK-treated reactions were exposed to replication conditions. Each replication reaction was performed in a 40-µl reaction volume containing 25 mM HEPES-KOH (pH 7.6), 20 mM creatine phosphate, 2 mM DTT, 0.04 mg/ml creatine kinase, 225 mM KGlut, 12 mM MgOAc, 3 mM ATP, 200 µM GTP, 200 µM CTP, 200 µM UTP, 40 µM dNTPs, 10 µCi (40 µM) α-^{32}PdCTP, and 750 µg cdc7-4 S-phase whole cell extract (yRH182) (Heller et al, in prep) at 25°C for 150 min. Beads were washed and DNA-protein complexes were removed by boiling in Laemmli sample buffer for 5 min. Proteins were analyzed by immunoblotting with anti-Mcm2-7 (UM174), anti-ORC (1108), anti-HA (12CA5), and anti-Myc (9E10) antibodies. Replication

products were analyzed by alkaline gel electrophoresis. Sample was run on a 0.8% agarose gel at 6 V / cm for 3 hours in running buffer containing 2 mM EDTA and 30 mM NaOH. The gel was dried and autoradiographed (Heller et al, in prep).

SUPPLEMENTAL MATERIAL

Supplemental Table 1. Yeast strains used in this study. All strains are in the W303 background and have the basic genotype *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 lys2::hisG can1-100*, except for yTJT73, which is in the S288C background. yTJT73 was constructed by non-integrative transformation of pRS316-pCdt1-CDT1 into the Mat**a**/α diploid BY4743 (CDT1/CDT1::KanMX) followed by sporulation and selection for **a** mating type, pRS316-pCdt1-CDT1, and G418 resistance.

Strain	Genotype	Source
yTJT1	*bar1::hisG pep4::kanMX his3::pRS403-GAL1,10-MCM2,MCM3 lys2::pRS307-GAL1,10-MCM4,MCM5 trp1::pRS404-GAL1,10-MCM6,MCM7 ura3::pRS306-GAL1,10-CDT1-1xFLAG MATa*	This study
yTJT9	*bar1::hisG pep4::kanMX his3::pRS403-GAL1,10-MCM2,MCM3 lys2::pRS307-GAL1,10-MCM4,MCM5 trp1::pRS404-GAL1,10-MCM6,MCM7 ura3::pRS306-GAL1,10-ΔN271CDT1-1xFLAG MATa*	This study
yTJT67	*bar1::hisG pep4::kanMX his3::pRS403-GAL1,10-MCM2,MCM3 lys2::pRS307-GAL1,10-MCM4,MCM5 trp1::pRS404-GAL1,10-MCM6,MCM7 ura3::pRS306-GAL1,10-ΔN292CDT1-1xFLAG MATa*	This study
yTJT38	*bar1::hisG pep4::kanMX his3::pRS403-GAL1,10-MCM2,MCM3 lys2::pRS307-GAL1,10-MCM4,MCM5 trp1::pRS404-GAL1,10-MCM6,MCM7 ura3::pRS306-GAL1,10-ΔN301CDT1-1xFLAG MATa*	This study
yTJT46	*bar1::hisG pep4::kanMX his3::pRS403-GAL1,10-MCM2,MCM3 lys2::pRS307-GAL1,10-MCM4,MCM5 trp1::pRS404-GAL1,10-MCM6,MCM7 ura3::pRS306-GAL1,10-ΔN433CDT1-1xFLAG MATa*	This study
yTJT11	*bar1::hisG pep4::kanMX his3::pRS403-GAL1,10-MCM2,MCM3 lys2::pRS307-GAL1,10-MCM4,MCM5 trp1::pRS404-GAL1,10-MCM6,MCM7 ura3::pRS306-GAL1,10-ΔN471CDT1-1xFLAG MATa*	This study
yTJT32	*bar1::hisG pep4::kanMX his3::pRS403-GAL1,10-MCM2,MCM3 lys2::pRS307-GAL1,10-MCM4,MCM5 trp1::pRS404-GAL1,10-MCM6,MCM7 ura3::pRS306-GAL1,10-ΔC17CDT1-1xFLAG MATa*	This study
yTJT33	*bar1::hisG pep4::kanMX his3::pRS403-GAL1,10-*	This study

	MCM2,MCM3 lys2::pRS307-GAL1,10-MCM4,MCM5 trp1::pRS404-GAL1,10-MCM6,MCM7 ura3::pRS306-GAL1,10-ΔC38CDT1-1xFLAG MATa	
yTJT14	*bar1::hisG pep4::kanMX his3::pRS403-GAL1,10-MCM2,MCM3 lys2::pRS307-GAL1,10-MCM4,MCM5 trp1::pRS404-GAL1,10-MCM6,MCM7 ura3::pRS306-GAL1,10-ΔC133CDT1-1xFLAG MATa*	This study
yTJT16	*bar1::hisG pep4::kanMX his3::pRS403-GAL1,10-MCM2,MCM3 lys2::pRS307-GAL1,10-MCM4,MCM5 trp1::pRS404-GAL1,10-MCM6,MCM7 ura3::pRS306-GAL1,10-ΔC333CDT1-1xFLAG MATa*	This study
yTJT19	*bar1::hisG pep4::kanMX ura3::pRS306-GAL1,10-CDT1-1xFLAG MATa*	This study
yTJT22	*bar1::hisG pep4::kanMX ura3::pRS306-GAL1,10-ΔN271CDT1-1xFLAG MATa*	This study
yTJT71	*bar1::hisG pep4::kanMX ura3::pRS306-GAL1,10-ΔN292CDT1-1xFLAG MATa*	This study
yTJT48	*bar1::hisG pep4::kanMX ura3::pRS306-GAL1,10-ΔN301CDT1-1xFLAG MATa*	This study
yTJT56	*bar1::hisG pep4::kanMX ura3::pRS306-GAL1,10-ΔN433CDT1-1xFLAG MATa*	This study
yTJT24	*bar1::hisG pep4::kanMX ura3::pRS306-GAL1,10-ΔN471CDT1-1xFLAG MATa*	This study
yTJT34	*bar1::hisG pep4::kanMX ura3::pRS306-GAL1,10-ΔC17CDT1-1xFLAG MATa*	This study
yTJT35	*bar1::hisG pep4::kanMX ura3::pRS306-GAL1,10-ΔC38CDT1-1xFLAG MATa*	This study
yTJT27	*bar1::hisG pep4::kanMX ura3::pRS306-GAL1,10-ΔC133CDT1-1xFLAG MATa*	This study
yTJT29	*bar1::hisG pep4::kanMX ura3::pRS306-GAL1,10-ΔC333CDT1-1xFLAG MATa*	This study
yTJT73	*his3Δ1 leu2Δ0 ura3Δ0 cdt1::kanMX pRS316-pCDT1-CDT1 MATa*	This study
yTJT83	*bar1::hisG pep4::kanMX his3::pRS403-GAL1,10-MCM2,MCM3 lys2::pRS307-GAL1,10-MCM4,MCM5 trp1::pRS404-GAL1,10-MCM6,MCM7 ura3::pRS306-GAL1,10-CDT1-4xMyc-1xFLAG MATa*	This study
yTJT84	*bar1::hisG pep4::kanMX his3::pRS403-GAL1,10-MCM2,MCM3 lys2::pRS307-GAL1,10-MCM4,MCM5 trp1::pRS404-GAL1,10-MCM6,MCM7 ura3::pRS306-GAL1,10-ΔN292CDT1-4xMyc-1xFLAG MATa*	This study
ySC17	*bar1::hisG pep4::kanMX trp1::pRS404-GAL1,10-ORC3,ORC4 lys2::pLys2-GAL1,10-ORC2,ORC5*	Bowers, et al. (2004)

	his3::pRS403-GAL1,10-ORC1,6 ura::pSF322-CDC6-HA MATa	
VTy167	*bar1::hisG (pep4::kanMX)::natMX his3::pRS403-GAL1,10-MCM2,MCM3 lys2::pRS307-GAL1,10-MCM4,MCM5 trp1::pRS404-GAL1,10-MCM6,MCM7 MATa*	Tsakraklides and Bell (2010)
yRH182	*bar1::hisG pep4::Hph KanMX::Psf2-13myc NAT::Mcm10-13myc his3::Cdc45-3HA lys2::HisG leu2::Gal1,10-Cdc45/Dpb11-FLAG lys2::Gal1,10-Sld2-FLAG/Sld3-FLAG*	Heller et al, in prep.

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Chapter III

Discussion

DISCUSSION

Key conclusions

The eukaryotic replicative helicase, Mcm2-7, is loaded onto origin DNA as an inactive double hexamer during G1 phase. The helicase-loading event is a highly regulated, step-wise process that requires the coordinated activity of ORC, Cdc6, and Cdt1. These proteins load the two hexamers within the double hexamer in opposite orientation to support the establishment of bi-directional replication forks following Mcm2-7 activation in S phase (Remus & Diffley, 2009). Although Cdt1 is essential for loading the Mcm2-7 helicase at origins of replication, the contribution of Cdt1 during the loading process is poorly understood. My work has focused on elucidating the role of Cdt1 during the Mcm2-7 helicase-loading process.

In Chapter II, we assessed the contribution of different Cdt1 regions to Mcm2-7 helicase binding, loading, and activation. *In vivo*, all three domains of Cdt1 are essential. *In vitro*, we showed that the C-terminus of Cdt1 is essential to bind Mcm2-7, and Cdt1/Mcm2-7 complex formation is required for the association of either protein with the origin. Two central regions of Cdt1 additionally mediate its origin association, and both regions are necessary for Mcm2-7 helicase loading. We demonstrated that two Cdt1 molecules are initially recruited to the origin during the Mcm2-7 loading process. Interestingly, disrupting the formation of the multi-Cdt1 intermediate prevents helicase loading. Finally, we found that the N-terminus of Cdt1 is necessary to load Mcm2-7 helicases that are competent to initiate replication during the ensuing S

phase. Collectively, these data suggest a model in which Cdt1 initiates double-hexamer formation early in the loading process and facilitates the loading of Mcm2-7 complexes that are competent for activation in S phase (Ch. II, Fig. 11B).

Spatial orientation of origin-associated Mcm2-7 hexamers

Our study has shed light on how Mcm2-7 double-hexamer formation is facilitated by the replication initiation factor Cdt1 during the loading process. We demonstrated that two molecules of Cdt1 are recruited to the origin during the helicase-loading process (Ch. II, Fig. 7) to support the loading of the Mcm2-7 double hexamer (Evrin et al, 2009; Remus et al, 2009), but the spatial orientation of the recruited Mcm2-7 hexamers could not be determined by our assays alone. We envision that Cdt1 could recruit two Mcm2-7 hexamers to the origin and facilitate head-to-head double-hexamer formation early in the loading process. Alternatively, it is possible that the two Mcm2-7 hexamers do not form a double hexamer at this stage and that Cdt1 positions each hexamer in a proper orientation for coordinated loading.

To distinguish whether Cdt1-recruited, origin-associated Mcm2-7 complexes form head-to-head double hexamers may not be straightforward. Structural analyses of a multi-Cdt1 helicase-loading intermediate would be difficult due to the complexity and inefficiency of the fully reconstituted *in vitro* helicase-loading reaction. One way to address this question would be to use a single-molecule approach coupled with our *in vitro* helicase-loading assay. For example, the N-terminus of the Mcm2-7 complex can be labeled with different color fluorescent dyes for FRET analysis. Using donor and acceptor dyes with

small Forster radii would help to ensure that FRET is specific to head-to-head double-hexamer formation and not proximally oriented Mcm2-7 hexamers.

Notably, with the advent of multi-wavelength single-molecule total internal reflection fluorescence (TIRF) microscopy (Friedman et al, 2006), additional helicase-loading components can be labeled with a third dye and simultaneously monitored without compromising the collection of emitted fluorescence. Recently, this technique was successfully used to analyze the ordered assembly of the spliceosome, another complex macromolecular machine (Hoskins et al, 2011). Similarly, coupling TIRF microscopy with our *in vitro* helicase-loading assays could provide valuable insight into the changing architecture of the helicase-loading machinery and the temporal progression of helicase-loading events in real time.

Stoichiometry of Cdc6 in the Mcm2-7 helicase-loading reaction

Our data are consistent with a single ORC/Cdc6 complex recruiting two Cdt1/Mcm2-7 heptamers to the origin to load the Mcm2-7 double hexamer (Ch. II, Fig. 7). However, the stoichiometry of Cdc6 during the helicase-loading reaction remains unknown. Are multiple Cdc6 molecules required to support double-hexamer formation? To address this question, helicase-loading and co-immunoprecipitation experiments could be performed with tagged and untagged Cdc6 in a manner analogous to our studies with Cdt1.

If multiple Cdc6 molecules are present in the helicase-loading intermediate, how do they associate with the origin to facilitate helicase loading?

It is well established that Cdc6 directly interacts with ORC (Chen et al, 2008; Speck et al, 2005; Wang et al, 1999), but the stoichiometry of Cdc6 within the ORC/Cdc6 complex is unclear. Although single-particle reconstruction of electron micrographic images reveals that Cdc6 likely binds to one region of the ORC complex (Speck et al, 2005), the possibility of oligomerization of Cdc6 or ORC-independent origin contacts of Cdc6 during helicase loading cannot be precluded. Indeed, recombinant Cdc6 was found to exist as a homodimer in its native form and bind non-specifically to dsDNA via its N-terminus (Feng et al, 2000). A later study, however, was unable to observe Cdc6 DNA binding activity at an *ARS1* origin template *in vitro* (Speck et al, 2005).

Which Cdt1 binding site of Orc6 is disrupted by CDK activity?

In this thesis, we demonstrated that loading of the Mcm2-7 helicase onto origin DNA proceeds via formation of a transient multi-Cdt1 helicase-loading intermediate. The origin recruitment of Cdt1 depended on two distinct ORC/Cdc6 binding sites (Ch. II, Fig. 7D and 11A) that we suspect interact, at least in part, with the two distinct Cdt1 binding sites on the N- and C-terminal domains of Orc6 (Chen et al, 2007). Notably, recent work has proposed that CDK primarily inhibits ORC function outside of G1 phase by inhibiting the N-terminal Cdt1 binding site of Orc6, which results in a two-fold reduction in Cdt1 origin recruitment and nearly complete inhibition of Mcm2-7 loading (Chen & Bell, 2011). These data are consistent with our findings that indicate disrupting formation of the multi-Cdt1 intermediate impairs Mcm2-7 loading (Ch. II, Fig. 7 and 8).

We hypothesize that each ORC/Cdc6 binding site of Cdt1 has a cognate binding site on Orc6, but it is presently unclear which of the two ORC/Cdc6 binding sites of Cdt1 binds the CDK-regulated Cdt1 binding site at the N-terminus of Cdc6. This question can be addressed by different means *in vitro*. For example, we could use ΔN292 Cdt1, a IDR1 deletion mutant that possesses only one intact ORC/Cdc6 binding site (Ch. II, Fig. 7D), and CDK-treated ORC in our *in vitro* helicase-loading assays. CDK-dependent inhibition of ΔN292 Cdt1 origin recruitment would suggest that the intact central ORC/Cdc6 binding site of ΔN292 Cdt1 binds the CDK-regulated N-terminal Cdt1 binding site of Orc6. If the N-terminal Cdt1 binding site of Orc6 were the cognate binding site of the disrupted ORC/Cdc6 binding site of Cdt1, CDK treatment would not be expected to alter the origin recruitment of ΔN292 Cdt1.

The influence of Cdt1 on the replication-competence of loaded Mcm2-7

Cdt1 function is essential for helicase loading in G1 phase, but uniformly down-regulated before the initiation of DNA replication in S phase by various means in different organisms (Arias & Walter, 2007), suggesting that the role of Cdt1 is specific to G1 phase. Interestingly, we demonstrated that the N-terminus of Cdt1 is dispensable for Mcm2-7 binding, origin recruitment, and topologically linked origin-DNA loading *in vitro* (Ch. II, Fig. 11A), but a N-terminal Cdt1 deletion mutant is unable to complement a *CDT1* deletion *in vivo* (Ch. II, Fig. 1). These data were reconciled when we showed that the N-terminal domain of Cdt1 is important for influencing the competence of loaded Mcm2-7 complexes to participate in helicase activation and replication initiation *in vitro* (Ch. II, Fig. 9).

We propose that Mcm2-7 complexes loaded by a ΔN271 (N-terminal domain) Cdt1 deletion mutant are either single hexamers that cannot become activated when not part of a double-hexameric unit or double hexamers that are loaded in a conformation that is refractory to activation. Coupling a structural approach like electron microscopy with our *in vitro* helicase-loading assay could directly demonstrate whether single or double hexamers are loaded at origin DNA. If only single hexamers are loaded, we envision that the hexamers might uniformly be loaded in one orientation such that head-to-head double hexamer formation is not possible. In this possibility, the N-terminal domain of Cdt1 could be specifically required to load Mcm2-7 hexamers in one orientation onto DNA. Alternatively, if a ΔN271 Cdt1 deletion mutant loads Mcm2-7 double hexamers, the N-terminal domain of Cdt1 might play a role in positioning the individual hexamers of the double hexamer in the proper orientation relative to one another to support subsequent helicase activation. By an analogous method to the proposed analysis of double-hexamer formation above, FRET could also help determine the inter-hexamer interactions that take place at the N-termini of two Mcm2-7 hexamers within a double-hexameric unit. For example, does the N-terminus of Mcm2 of one hexamer interact with the N-terminus of Mcm2 (or a different Mcm subunit) of the second hexamer? Elucidation of inter-hexamer interactions could provide insight into the process by which the Mcm2-7 double hexamer is normally activated by Cdc45 and GINS association and the potential ring-opening and –closing events that might take place during helicase activation.

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