Investigation of Cdc45 Function During Helicase Activation

by

Ramón Yamil Ríos-Morales
S.B. Molecular and Cellular Biology
University of Puerto Rico, Rio Piedras, 2011

Submitted to the Department of Biology
in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

at the

MASSACHUSSETTS INSTITUTE OF TECHNOLOGY

May 2017 [June 2017]

Signature of Author Signature redacted
Department of Biology
May 17, 2017

Certified by Signature redacted
Stephen P. Bell
Thesis Advisor

Accepted by Signature redacted
Amy E. Keating
Professor of Biology
Co-Chair, Biology Graduate Committee

Investigation of Cdc45 Function During Helicase Activation

by

Ramón Yamil Ríos-Morales
S.B. Molecular and Cellular Biology
University of Puerto Rico, Rio Piedras, 2011
San Juan, Puerto Rico

Submitted to the Department of Biology on May 17, 2017 in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Biology

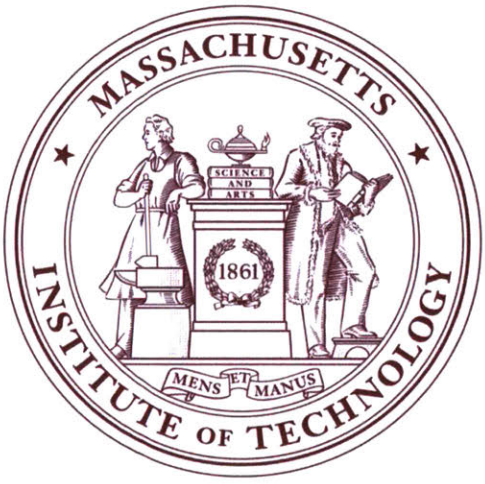
ABSTRACT

Eukaryotic DNA replication requires the stepwise loading of Mcm2-7 helicase complexes at origins of replication. These loaded helicases need to be activated during DNA replication initiation to unwind the DNA double helix and provide a ssDNA template for replicative DNA polymerases. Eukaryotic cells segregate helicase loading and activation events to ensure a single round of DNA replication per cell cycle. Upon entry into S-phase, Cdc45 and GINS associate with the Mcm2-7 complex to form the CMG complex, which is the active eukaryotic replicative helicase. Consistent with a role in stimulating helicase activity, in addition to its role during DNA replication initiation, Cdc45 is also required during DNA elongation. In this thesis, I have investigated how Cdc45 functions during DNA replication. I characterized four lethal and three temperature- sensitive point mutations in *CDC45*. Intriguingly, the temperature-

sensitive mutants were specifically defective for DNA replication initiation and not for elongation. This suggests that the requirements for Cdc45 function during DNA replication initiation are distinct from those involved in replication elongation.

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

Dedicated to my family, in particular, my late grandfather, Eligio Rafael Morales Rivera
and the people of Puerto Rico.



ACKNOWLEDGEMENTS

I was always encouraged by my family to educate myself as much as I can. My parents did not have the opportunity to go to college and my older brother was the first in the family to obtain a bachelor's degree. In 2011, I followed my brother's footsteps and graduated with B.S. in Molecular and Cellular Biology from the University of Puerto Rico, Rio Piedras. During my sophomore year I was doing research on muscular nicotinic acetylcholine receptors and it didn't take long for me to decide I wanted to seek a career in research and higher education. On September 2011, I joined the MIT Biology Ph.D. program to fulfill that goal and start a career in academic research. This chapter in my career is coming to an end and I want to acknowledge the people that have been part of it.

First, I would like to thank the Minority Access to Research Careers Program (MARC) for providing a path to develop me and other students in Puerto Rico. Through this program, I met Dr. Mandana Sassanfar, who gave me the opportunity to participate in the MIT Summer Research Program in 2010. I would like to thank Mandana for giving me the opportunity to explore new research areas in biology and the MIT scientific community. I want to thank Tania Baker for mentoring during the summer internship and support during graduate school applications.

I would like to thank Dr. Stephen P. Bell for his guidance, advice and mentoring during my thesis project. More than a mentor, Steve has been a friend to me in and outside the lab. Thanks Steve for pushing me to work hard but also thanks for the laughs, beers and Red Sox games. I would like to thank members of the Bell laboratory and Elaine Aidonidis for keeping the lab running. I would like to thank Adrian Olivares and Adam Shoemaker for their friendship and good research conversations.

I would like to thank my family for been supportive throughout these years and making themselves feel close despite the distance. To my mother, Wanda I. Morales-Fernández, thank you for all the values you taught me; they helped me to work hard and always persevere no matter what. I would like to specially thank my late grandfather, Eligio R. Morales-Rivera, for always encouraging me to focus on education and sports. You always supported me in everything and were always there for me. I will be forever grateful to God for having you in my life.

Finally, I would like to thank a group of amazing friends that made living in Boston more enjoyable. Thank you Juan R. Contreras-Aguirre, Enrique García-Rivera, Baldin Llorens-Bonilla, Carlos D. González-Huertas, Juan A. Santiago-González, Edward Betancourt-Agosto, Hector J. De Jesús-Cortés, Carlos J. Acosta-Vega and Alfredo Álvarez-Lamela.

TABLE OF CONTENTS

Abstract	2
Dedication	4
Acknowledgements	5
Chapter I: Introduction	8
Cell Cycle Review	9
Mcm2-7 Helicase Loading in *Saccharomyces cerevisiae*	15
Origins of Replication	15
The Helicase-Loading Proteins: The Origin Recognition Complex, Cdc6 and Cdt1	18
The Mcm2-7 Complex	21
Sequential Helicase Loading at Origins of Replication	28
Mcm2-7 Helicase Activation in *Saccharomyces cerevisiae*	37
Overview	37
Cdc45 and the CMG Complex	40
Chapter II: *CDC45* Mutants Are Defective in DNA Replication Initiation	49
Summary	50
Introduction	51
Results	54
In vivo Characterization of *CDC45* Site-directed Mutants	54
CDC45 Temperature-sensitive Mutants Are Selectively Defective for DNA Replication Initiation	66
Temperature-sensitive Cdc45 proteins Are Defective for CMG Formation *in vitro*	71
Discussion	79
Experimental Procedures	87
Chapter III: Discussion and Future Directions	96
References	105

CHAPTER I:

INTRODUCTION

I. CELL CYCLE OVERVIEW

Cells are the most basic unit of life. Even a unicellular organism is able to reproduce, metabolize and adapt to the environment. Still more impressive is the ability of multicellular organisms to communicate between different cell types both directly and indirectly via cell-cell interactions or secreted molecules that influence cell behavior. Proteins and non-coding RNAs encoded in the genome of each organism carry out these processes. Thus, it is of extreme and fundamental importance for cells to protect and preserve their genomes.

Throughout evolution, cells have evolved multiple mechanisms including the cell cycle, DNA repair and the DNA damage checkpoints to protect and maintain genome integrity. In particular, eukaryotic DNA replication is tightly regulated during the cell cycle to ensure the genome is duplicated exactly once per cell division. To this end, crucial and distinct steps required for DNA synthesis and chromosome segregation are carefully segregated (Figure 1). Importantly, deregulation of these events can lead to severe consequences including cancer and developmental abnormalities (Donley *et al.*, 2013; Kerzendorfer *et al.*, 2013). I will focus this overview on the current view of the cell cycle control in *Saccharomyces cerevisiae*.

FIGURE 1. *Saccharomyces cerevisiae* Cell Cycle

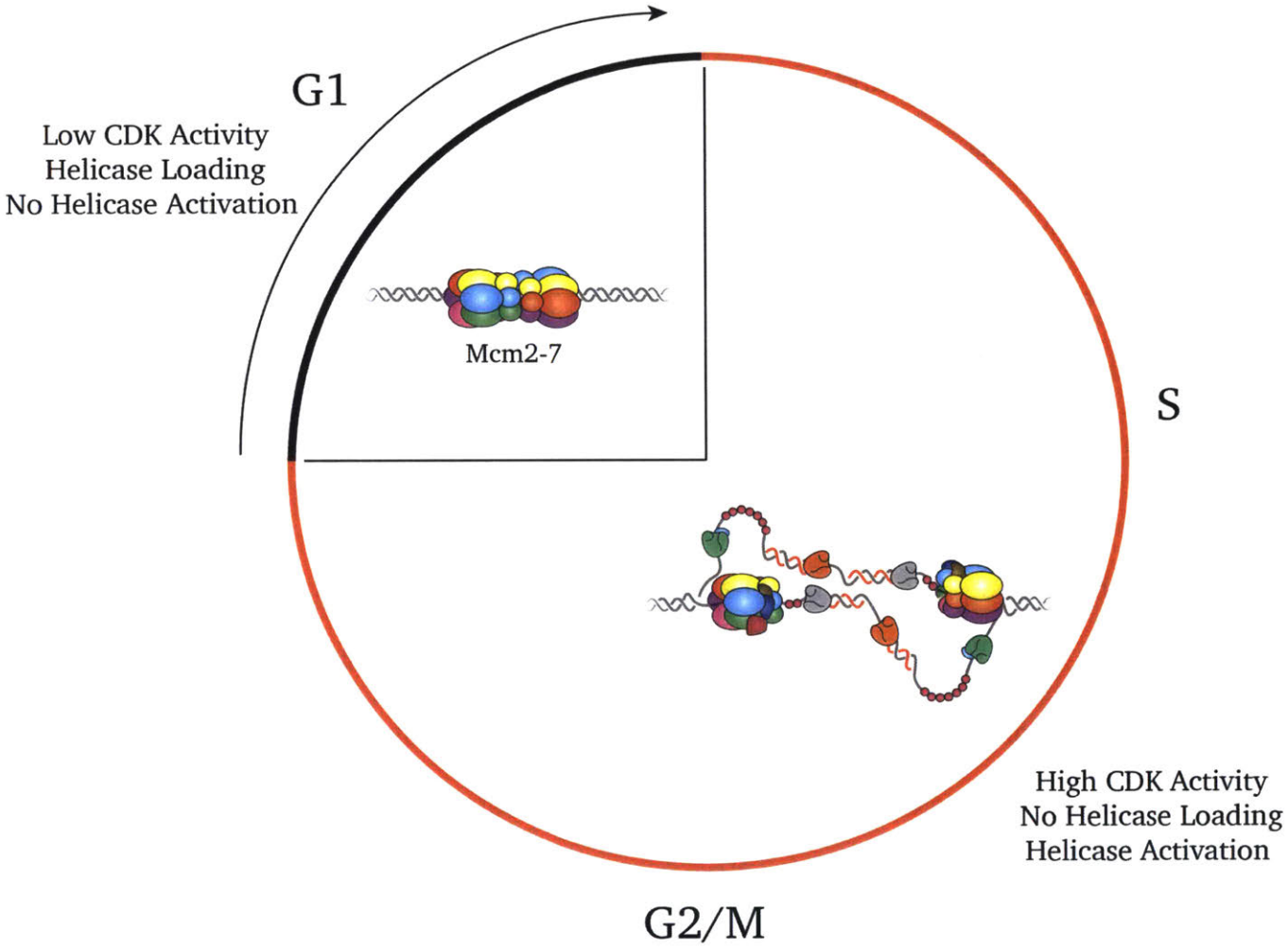


Figure 1. Cell-cycle Control of DNA Replication. Eukaryotic cells segregate Mcm2-7 helicase loading from activation to ensure a single round of chromosome duplication each cell division. During G1, low CDK activity allows helicase loading but prevents helicase activation. Once the cell enters S-phase, high CDK activity promotes helicase activation but inhibits new Mcm2-7 helicase loading events.

Cyclin-dependent kinases (CDKs) are the major effectors that control the order and timing of cell cycle progression. These kinases are composed of two subunits. One subunit (the Cdk) provides the kinase catalytic core that phosphorylates targeted proteins while the second subunit (the cyclin) activates and regulates kinase activity. Changes in cyclin concentration during the cell cycle generate the oscillations in CDK activity and it is CDK activity that establishes the cell cycle control. Indeed, it was the fluctuating levels of cyclins during cell cycle progression that led to their discovery (Evans *et al.*, 1983). The first Cdk (Cdc28) was isolated and studied almost a decade before and later shown to physically interact with cyclins (Hartwell *et al.*, 1973; Nurse *et al.*, 1980; Draetta *et al.*, 1989). Interestingly, a single Cdk protein is present in *S. cerevisiae* and *S. pombe* whereas human cells possess at least four that are involved in cell-cycle control (and many others that are not). Like other kinases, cyclin-Cdk pairs target and phosphorylate specific substrates to change their enzymatic activity and/or create sites of that are recognized by other proteins.

Cyclins are regulated by protein expression, degradation and post-translational modification level. There are three main groups of cyclins produced at different stages in the cell cycle: G1/S-, S-, and M-phase cyclins (Figure 2). Each cyclin-Cdk complex sequentially triggers the activity of the next to ensure an ordered chain of events. During early G1-phase, all three G1/S-, S-, and M-CDK complexes are inactivated by three mechanisms: inhibition of cyclin protein

expression, cyclin degradation by the Anaphase-Promoting Complex (APC) and inhibition of S-CDK activity by Subunit Inhibitor of Cdk 1 protein (Sic1). This period of low Cdk activity is permissive for loading of the **mini**chromosome **m**aintenance 2-7 (Mcm2-7) replicative helicases onto origins of replication. Importantly, helicases loaded in G1-phase are inactive and remain so until cells enter S-phase.

When the cell commits to enter a new cell cycle, G1 and S-cyclins are expressed leading to rapid degradation of Sic1 and APC inhibition (Verma *et al.*, 1997; Kramer *et al.*, 2000). The appearance of S-CDK activity is the critical step to trigger Mcm2-7 helicase activation and initiation of DNA synthesis. As cells reach the end of S-phase, M-phase cyclins are expressed and gradually accumulate. At the onset of mitosis, M-CDKs become active and trigger cell progression through G2/M transition and mitosis. M-CDKs also leads to APC activation, which targets the S- and M- cyclins for degradation. In addition, Sic1 expression increases during late mitosis and in conjunction with high APC activity, helps to rapidly lower CDK activity from the end of mitosis until G1-CDKs start to accumulate late in the following G1-phase.

FIGURE 2. Cyclin Oscillation During Cell Cycle

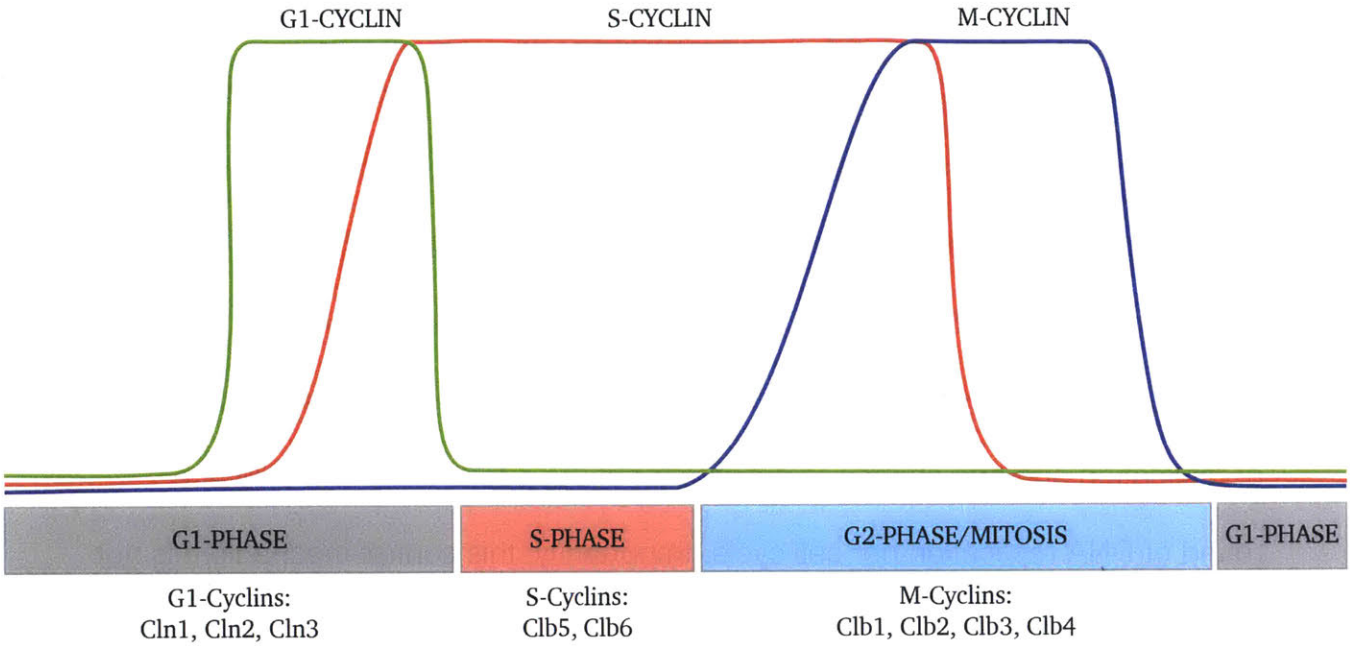


Figure 2. Cyclin Oscillation During Cell Cycle. Levels of the three cyclin types oscillate during the cell cycle. In contrast, cyclin-dependent kinase (Cdk) levels are constant. Formation of active G1-Cdk complexes commits the cell for division. G1-CDK activity promotes the expression of S-phase cyclins and inhibits CDK inhibition by Sic1. S-CDK activity triggers S-phase and chromosome duplication. M-cyclins are expressed towards the end of S-phase accumulate. At the onset of mitosis, M-CDK activity promotes the G2/M transition and mitosis. *Saccharomyces cerevisiae* cyclins are listed at the bottom of the figure.

The oscillation between low and high states of CDK activity program cells to duplicate their chromosomes only once per cell cycle. Two key steps of replication initiation are carefully segregated during the cell cycle. Helicase loading is carefully restricted to G1-phase by CDK inhibition of this event. In contrast, helicase activation requires S-CDK activity and, thus, can only occur after cells enter S-phase. By segregating Mcm2-7 helicase loading from helicase activation events, cells have a neat way to limit chromosome duplication events. During each cell division there is only one chance to load helicases onto origins of replication and one chance to activate them. As a result, there is only one round of DNA replication per cell cycle. Importantly, this control mechanism is not limited by origin quantity and works equally well for one hundred or one hundred thousand origins.

II. MCM2-7 HELICASE LOADING IN *SACCHAROMYCES CEREVISIAE*

A. ORIGINS OF REPLICATION

Mcm2-7 helicase loading onto DNA occurs at specific sites in the genome known as origins of replication. At least one origin of replication per chromosome is required for the faithfully duplication of the chromosome. A single origin of replication is commonly found in archaeal and bacterial species, whose genomes consist of smaller circular chromosomes. On the other hand, eukaryotic cells have linear chromosomes each of which has many origins of replication. For example, the budding yeast *Saccharomyces cerevisiae* that I have studied, contains around four hundred origins of replication distributed across its sixteen chromosomes (Siow *et al.*, 2012). Having multiple sites competent for replication initiation increases the rate of chromosome replication by distributing the work among many replication forks. In addition, this approach provides extra origins that can complete replication when adjacent replication forks stall.

The discovery of origins of replication in *Saccharomyces cerevisiae* stemmed from studies of DNA sequences that allowed plasmids to autonomously replicate (Stinchcom *et al.*, 1979; Bell and Stillman, 1992). For this reason, these elements were named *autonomous replication sequences* (ARSs). Subsequent studies showed that a subset of these sites acted as origins in the chromosome

(Brewer and Fangman, 1987). Each yeast origins spans 100-200 base pairs and contains two groups of DNA elements. The most important of these is the *A element*, which contains the eleven base pair ARS consensus sequence (ACS) [5'-(A/T)TTA(T/C)(A/G)TTT(A/T)-3']. In addition to the *A element*, there are several *B elements* that together are also essential for ARS function (Marahrens *et al.*, 1992). The B1 element also contributes to ORC binding (Rao and Stillman, 1995). Although its biochemical function is controversial, the B2 element facilitates helicase loading (Zou and Stillman 2000; Lipford and Bell, 2001; Wilmes and Bell, 2002). The B3 element, although not present in all ARS elements, is a binding site for ARS-binding factor 1 (Abf1). At ARS1, Abf1 helps to position adjacent nucleosomes (Lipford and Bell, 2001). Although a B3 element is not found at most origins, B1 and B2 elements are present at all origins studied.

The presence of an ACS, although required, is not sufficient to promote replication initiation. There are thousands of potential ACS matches in *Saccharomyces cerevisiae* genome but only a subset are bound by ORC and used as origins of replication. Another important determinant of origin function is the presence of an asymmetric nucleosome-free region (NFR) flanking the ACS. Interestingly, an NFR is present in both functional and non-functional ACSs. However, the NFR of ORC-bound ACSs is more extended and has well-

positioned nucleosomes flanking the NFR whereas non-functional ACSs have a shorter NFR with flanking nucleosomes randomly distributed (Eaton *et al.*, 2010).

Origins initiate replication at different times and not all origins are used in every cell cycle. There are origins that typically initiate early in S-phase (early-firing origins) and others that typically initiate late in S-phase (late-firing origins). What specifically determines early- versus late-firing origins is still being investigated. Interestingly, overexpression of limiting helicase-activating factors (e.g. Sld3, Sld7 and Cdc45) induces all origins to initiate early in S phase (Tanaka *et al.*, 2011; Mantiero *et al.*, 2011). These findings suggest that origins compete for limiting factors and that early-firing origins typically win this battle relative to late-firing origins. In support of this model, Cdc45 and Sld3 have been shown to associate with early-firing origins in late G1 and Cdc45 association with late-origins is delayed (Aparicio *et al.*, 1999; Heller *et al.*, 2011; Tanaka *et al.*, 2011). The chromatin environment influences the time of origin-firing (Ferguson and Fangman, 1992). For example, if an early-firing origin is swapped with a late-firing origin, they each assume the timing of their new location rather than keeping their replication timing. One exception of this behavior is the proximal binding of the Forkhead DNA binding protein to origins of replication. Origins containing Forkhead binding sites fire early regardless of their chromosomal position (Lõoke *et al*, 2013).

B. THE MCM2-7 HELICASE-LOADING PROTEINS: THE ORIGIN RECOGNITION COMPLEX, CDC6 AND CDT1

The Origin Recognition Complex was first isolated through biochemical fractionation of yeast extracts with the goal of identifying proteins that bind *ARS1*-containing DNA (Bell and Stillman, 1992). A six-subunit complex that protected both *A* and *B1* elements was purified from these experiment and named the Origin Recognition Complex. Importantly, there was a correspondence between the mutations in the ACS that inactivated origin function also inhibited ORC DNA binding. Interestingly, ORC DNA binding depended on the addition of adenine triphosphate (ATP) addition.

ORC is a heterohexamer comprised of six essential proteins, Orc1-6, that form a 414 kDa protein complex. ORC assembles as a two-tiered partial ring structure with the Orc1-5 subunits forming the core ring body (Bleichert *et al.*, 2015; Tocilj *et al.*, 2017; Yuan *et al.*, 2017). Orc1-5 form this ring structure through protein interactions of two domains: an AAA^+-like domain (**A**TPase **a**ssociated with various cellular **a**ctivities) and a winged-helix DNA-binding domain. Although each of the Orc1-5 subunits possess the AAA^+ module, only Orc1, Orc4 and Orc5 are capable of ATP binding and only Orc1 is an ATPase (Klemm *et al.*, 1997; Yuan *et al.*, 2017).

ATP binding and hydrolysis regulate ORC function. Orc1 ATP-binding is required for origin DNA binding. Interestingly, origin DNA association inhibits ATP hydrolysis by Orc1, possibly saving this activity for a later event in helicase loading. Orc1 ATP hydrolysis is essential as a mutant in Orc4 that prevents Orc1 ATP hydrolysis (AAA+ ATPases hydrolyze ATP at composite active sites formed at the interfaces between adjacent subunits) is lethal. Biochemical characterization suggests that ORC ATP hydrolysis is not required for helicase loading but instead is involved in the repetition of this event (Bowers *et al.*, 2004; Randell *et al.*, 2006; Coster *et al.*, 2014).

The Orc1-5 ring assembles in a defined order (Orc1-Orc4-Orc5-Orc3-Orc2) with a structural gap between Orc1 and Orc2. This gap allows ORC to encircle and engage with origin DNA. Orc2 and Orc3 interact mainly with the *A element* and the flanking region towards the *B1* element (Lee and Bell, 1997). Orc4 and Orc5 mainly interact with the ACS and *B1 element*, respectively. Orc4 also contains a yeast-specific α-helix insertion that provides additional DNA binding interactions thought to contribute to origin specificity. In the context of a helicase loading intermediate, ORC also interacts with Mcm3, Mcm4, Mcm6 and Mcm7 (Yuan *et al.*, 2017).

The only ORC subunit that is not required for DNA binding is Orc6 (Lee and Bell, 1997). Consistent with this observation, Orc6 is not part of the core Orc1-5 ring and instead binds to Orc3 outside the ring (Bleichert *et al.*, 2015; Yuan *et al.*, 2017). Orc6 was initially isolated using a one-hybrid system against proteins that recognize the ACS sequence and is distantly related to the transcription factor IIB (Liu *et al.*, 2011; Li and Herskowitz, 1993). Nevertheless, Orc6 is required for Mcm2-7 loading and the C-terminal domain of Orc6 binds to Cdt1 during this process (Chen *et al.*, 2007; Yuan *et al.*, 2017).

Two other proteins are required for Mcm2-7 loading at origins of replication: Cdc6 and Cdt1. Cdc6 is an AAA^{+}-ATPase that is closely related to Orc1 and robustly interacts with ORC during helicase loading (Speck *et al.*, 2005). Cdc6 binding to ORC bridges the structural gap between Orc1-2 forming a closed ring. In the ORC-Cdc6 ring, Cdc6 contributes two additional DNA binding motifs explaining previous findings that Cdc6 association enhanced ORC specificity for origin DNA (Yuan *et al.*, 2017; Speck *et al.*, 2005 & 2007).

Cdt1 (**C**dc10-**d**ependent **t**ranscript) is another licensing factor required for Mcm2-7 helicase loading. In *Saccharomyces cerevisiae,* Cdt1 and Mcm2-7 are imported to the nucleus as a complex and this interaction is required for the recruitment of either protein to origins of replication (Tanaka and Diffley, 2002; Takara and Bell, 2011). Cdt1 interacts with the Mcm2-7 complex through a wing

helix domain located at the C-terminal of Cdt1 (Takara and Bell, 2011; Yuan *et al.*, 2017). Primarily, Cdt1 binds to Mcm6 and this interaction displaces an inhibitory domain of Mcm6 creating a binding surface for ORC-Cdc6 (Fernández-Cid *et al.*, 2013; Yuan *et al.*, 2017). In addition, recent studies suggest a role of Cdt1 to stabilize the Mcm2-7 ring in an open conformation during helicase loading (Zhai *et al.,* 2017; Ticau *et al.*, 2017).

C. THE MCM2-7 COMPLEX

After the elucidation of the DNA structure (Watson and Crick, 1953), it was clear that the DNA double helix needed to be unwound to allow chromosome duplication. In *Saccharomyces cerevisiae* and higher eukaryotes, the Mcm2-7 complex functions as the major replicative helicase during S-phase. The Mcm2-7 helicase is a heterohexamer complex composed of six homologous but distinct *MCM2-7* genes. *MCM2*, *MCM3* and *MCM5* were isolated in the initial screen for mutants defective in mini-chromosome maintenance (Maine *et al.*, 1984). *MCM4* and *MCM7* were identified as cell cycle division mutants, and *MCM6* as a chromosome segregation mutant in S*chizosaccharomyces pombe* (Henessy *et al*, 1991; Moir *et al.*, 1982; Takahashi *et al.*, 1994). Together, the Mcm subunits form either an open or closed ring arranged in a defined order: Mcm5-Mcm3-Mcm7-Mcm4-Mcm6-Mcm2 (Figure3A)(Davey *et al.*, 2003). When

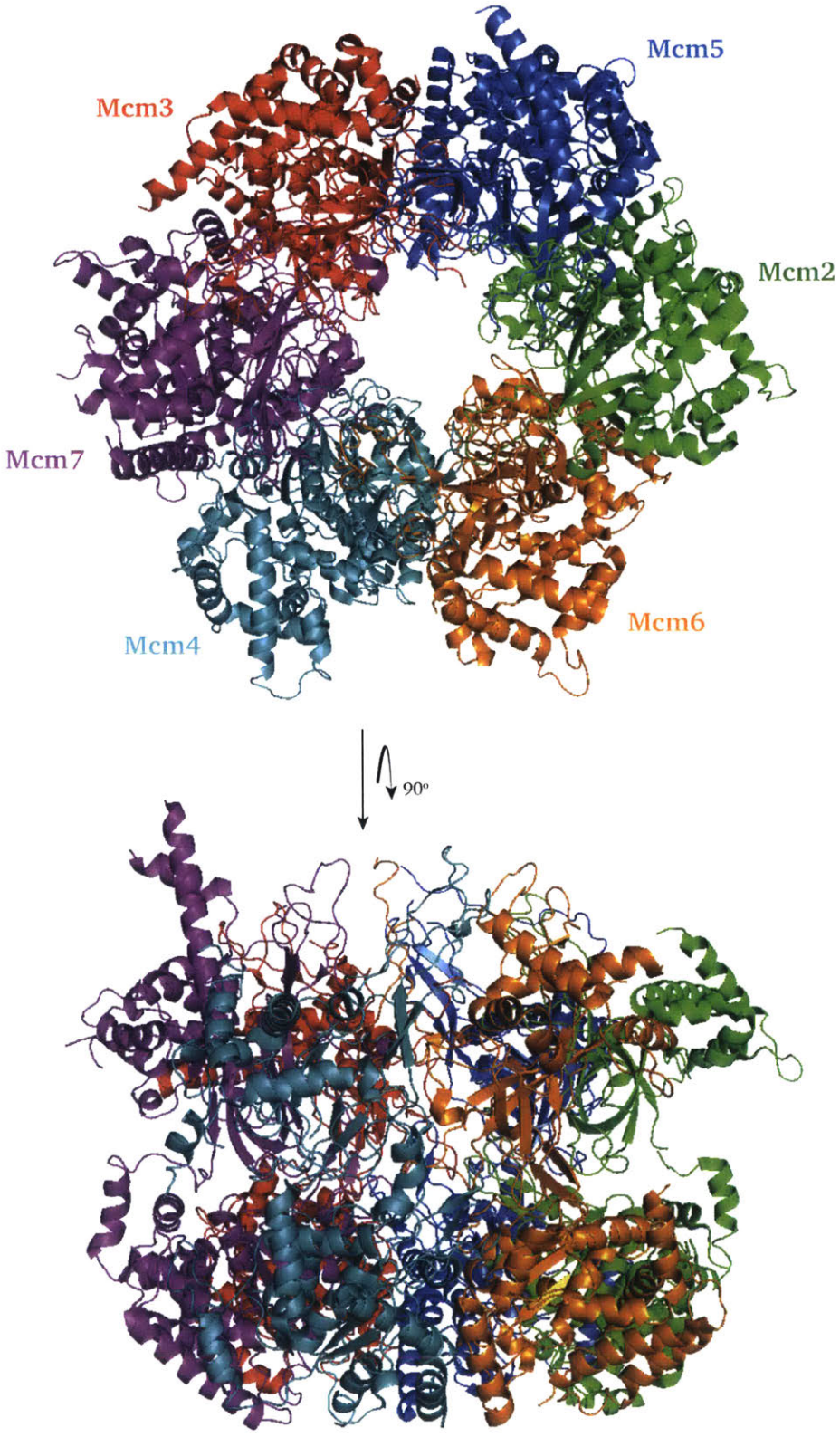
the ring opens, it occurs between the Mcm2 and Mcm5 subunits (Bochman and Schwacha, 2008; Costa *et al.*, 2011; Samel *et al.*, 2014).

The Mcm2-7 ring can be divided into an N- and C-terminal tier (Figure 3B) (Li *et al.*, 2015; Sun *et al.*, 2013). The C-terminal tier is composed of the highly conserved AAA+ ATPase domain found in each subunit. As with other AAA+ ATPases, the six ATP-binding and hydrolysis sites found in the Mcm2-7 complex are located at the interface between each pair of subunit. One subunit provides the majority of the ATPase active site whereas the other primarily provides a critical arginine residue required for ATP hydrolysis. Interestingly, mutating different ATPase sites in the Mcm2-7 ring results in different defective phenotypes ranging from initial recruitment, Cdt1 release and loading (Coster *et al.*, 2014; Kang *et al.*, 2014). The C-terminal domains of the Mcm2-7 complex also contain β-hairpin structures predicted to bind single-stranded DNA (ssDNA) during DNA unwinding (Li *et al.*, 2015).

The folded N-terminal domains of the Mcm2-7 subunits can be divided into three smaller motifs (Fletcher *et al.*, 2003; Li *et al.*, 2015). The *A subdomains* mediate intermolecular interactions with the rest of the Mcm2-7 hexamer. The *B subdomains* contain zinc finger motifs involved in interactions between Mcm2-7 complexes formed during helicase loading. The *C subdomains* are oligonucleotide/oligosaccharide binding motifs (OB-fold) that bind ssDNA. In

addition to these domains, Mcm2, Mcm4 and Mcm6 contain extended N-terminal tails that regulate helicase activation (see below).

FIGURE 3. Architecture of *Saccharomyces cerevisiae* Mcm2-7 Complex



B

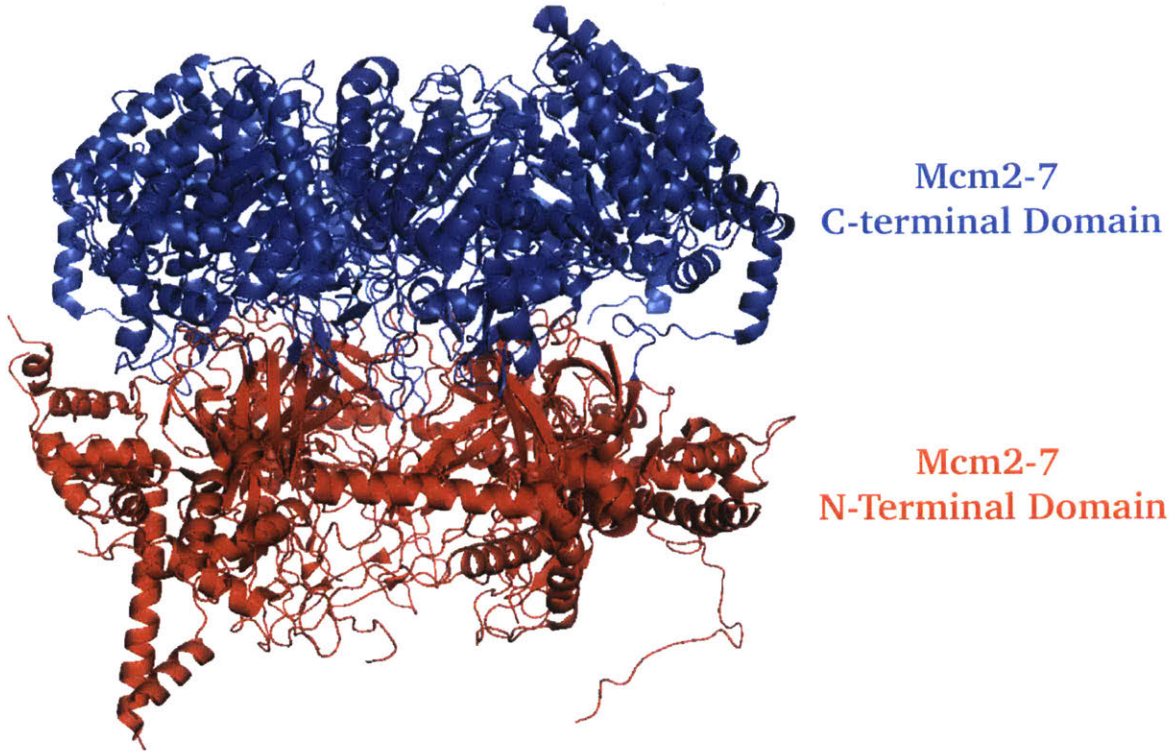


Figure 3. Architecture of the Mcm2-7 complex. *Saccharomyces cerevisiae* Mcm2-7 cryo-EM structure was adapted from Li *et al.*, 2015. (A) Top and side views of the Mcm2-7 hexamer. Mcm subunits are colored and labeled (Mcm2 = green, Mcm3 = red, Mcm4 = cyan, Mcm5 = Blue, Mcm6 = orange, Mcm7 = purple). (B) The Mcm2-7 hexamer forms a two lobed ring. The N- and C-terminal domains of Mcm subunits fold into separate rings. N- and C-terminal domains are colored red and blue, respectively.

FIGURE 4. Mcm2-7 Complex N-terminal Subdomains

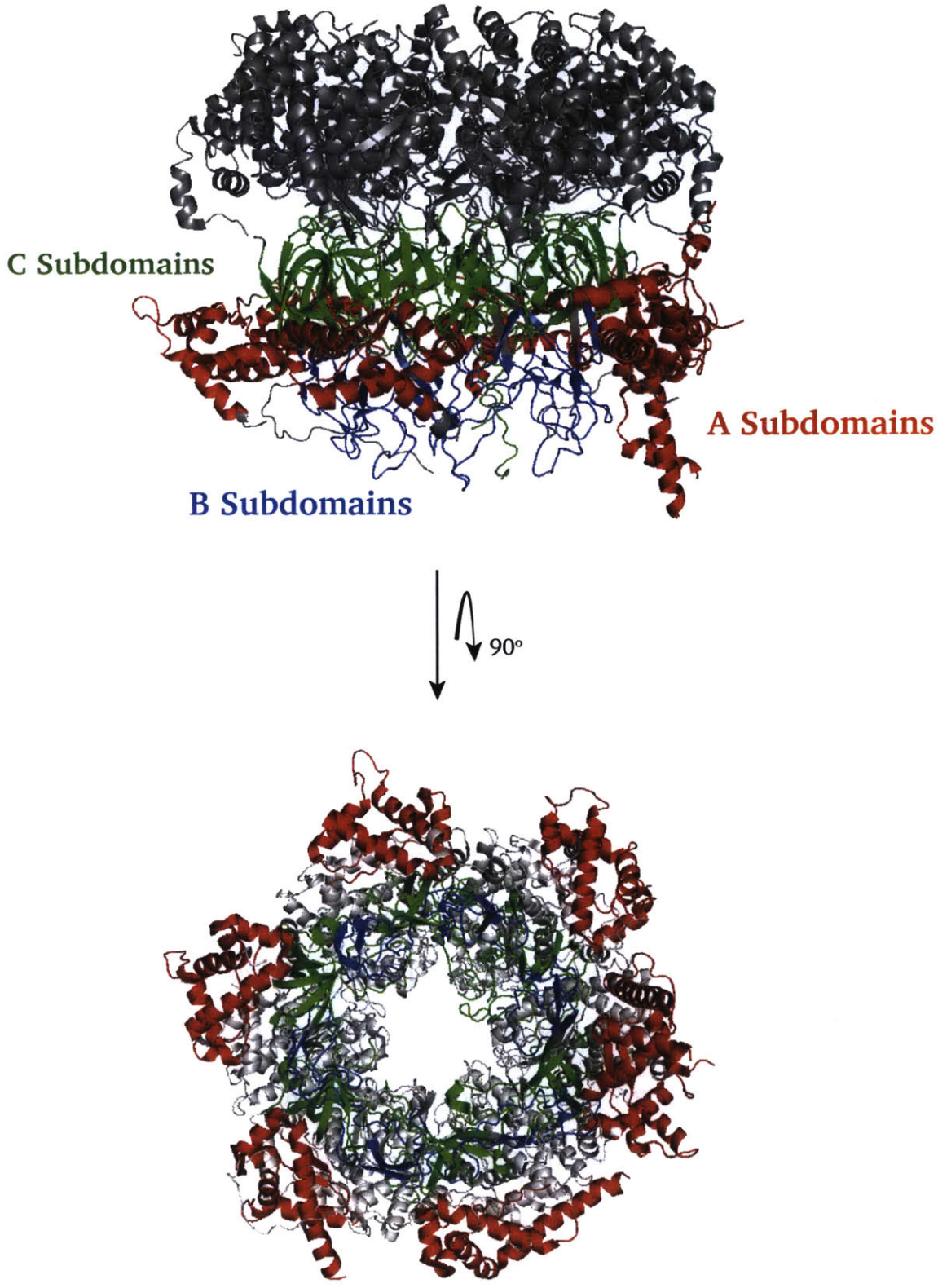


Figure 4. Mcm2-7 N-terminal Domains. *Saccharomyces cerevisiae* Mcm2-7 cryo-EM structure was adapted from Li *et al.*, 2015. *Top* Side view of the Mcm2-7 hexamer. The C-terminal domains of Mcm2-7 are colored in dark grey. The *A*, *B*, and *C subdomains* are labeled and colored red, blue and green, respectively. *Bottom* Mcm2-7 ring was rotated 90° from the top image.

D. SEQUENTIAL MCM2-7 HELICASE LOADING AT ORIGINS OF REPLICATION

Mcm2-7 replicative helicases are loaded onto origins of replication as head-to-head double hexamers enclosing dsDNA to mark all potential origins (Figure 3). Helicase loading begins with the sequential binding of ORC and Cdc6 to origins of replication during G1-phase. The resulting ORC-Cdc6 complex then recruits the Mcm2-7 helicase in complex with Cdt1 (Tanaka and Diffley, 2002; Remus *et al.*, 2009). Together, ORC-Cdc6-Cdt1-Mcm2-7 form a short lived complex (OCCM) prior to helicase loading (Figure 4). The resulting complex is stabilized by inhibition of ATP hydrolysis. A long-standing question in the field inquired if Mcm2-7 helicases were loaded sequentially one at a time or both hexamers loaded in one event.

Recent studies have provided significant evidence supporting the former model (Ticau *et al.*, 2015; Sun *et al.*, 2013). First, an electron microspcopy (EM) structure of the OCCM contained a single ORC and Mcm2-7 complex supported a one at a time loading model (Sun *et al.*, 2013; Yuan *et al*, 2017). Single-molecule experiments demonstrated that a single Mcm2-7/Cdt1 complex is recruited to DNA-bound ORC-Cdc6. Once the OCCM complex is formed, Cdc6 is rapidly released followed by Cdt1 and the first Mcm2-7 helicase loaded. Importantly, ORC remains bound to DNA after Cdt1 and Cdc6 release. This is

followed by a second Cdc6 and second Mcm2-7/Cdt1 being recruited. Finally, the second Cdc6 is released followed by the apparently simultaneous release of the second Cdt1 and ORC. Interestingly, a single ORC complex is sufficient to efficiently load both Mcm2-7 hexamers whereas two Cdc6 and Cdt1 molecules are required.

During helicase loading, the Mcm2-7 ring must open to allow DNA access to the central channel. The Mcm2-7 complex is exclusively open at the Mcm2-Mcm5 interface known as the Mcm2-5 "gate" (Bochman and Schwacha, 2008; Costa *et al.*, 2011). Importantly, artificial linking of this interface (but not any others) prevents Mcm2-7 loading (Samel *et al.*, 2014). The open ring state is maintained during helicase recruitment to origin DNA. Once loaded, the Mcm2-5 gate closure occurs concomitant with Cdt1 release suggesting a connection between these two steps (Ticau *et al.*, 2017). This mechanism is repeated to load a second Mcm2-7 hexamer. However, the second Cdt1 release and gate closing are delayed compared to the first Mcm2-7 loading event. This observation suggests that despite two hexamers being loaded separately, they are part of a single concerted event.

After the two hexamers are loaded, they encircle dsDNA (Evrin *et al.*, 2009). However, these loaded Mcm2-7 double hexamers are not fixed at origins of replication and can slide on the DNA away from their initial loading site

(Remus *et al.*, 2009). Interestingly, this includes being moved along the DNA by other processes such as transcription (Gros *et al.*, 2015). Nonetheless, displaced Mcm2-7 complexes are competent for DNA replication initiation.

Helicase loading is restricted to the G1 phase of the cell cycle. Outside of G1, CDK activity inhibits helicase loading by multiple mechanisms. First, CDK phosphorylates Cdc6 leading to its degradation (Honey and Futcher, 2007; Drury *et al.*, 2010). Second, CDK promotes Cdt1-Mcm2-7 nuclear export by targeting a nuclear export localization signals in Mcm3 (Labib *et al.*, 1999; Nguyen *et al.*, 2000; Tanaka and Diffley, 2002; Liku *et al.*, 2005). In addition, CDK phosphorylates both Orc2 and Orc6 blocking the Cdt1-binding sites on Orc6 (Nguyen *et al.*, 2001; Wilmes *et al.*, 2004; Chen and Bell, 2011). Lastly, a CDK-binding motif on Orc6 recruits Clb5 (the primary S-phase cyclin) to ORC and sterically blocks Mcm2-7 helicase loading.

FIGURE 5. Sequential Mcm2-7 Helicase Loading at Origins of Replication

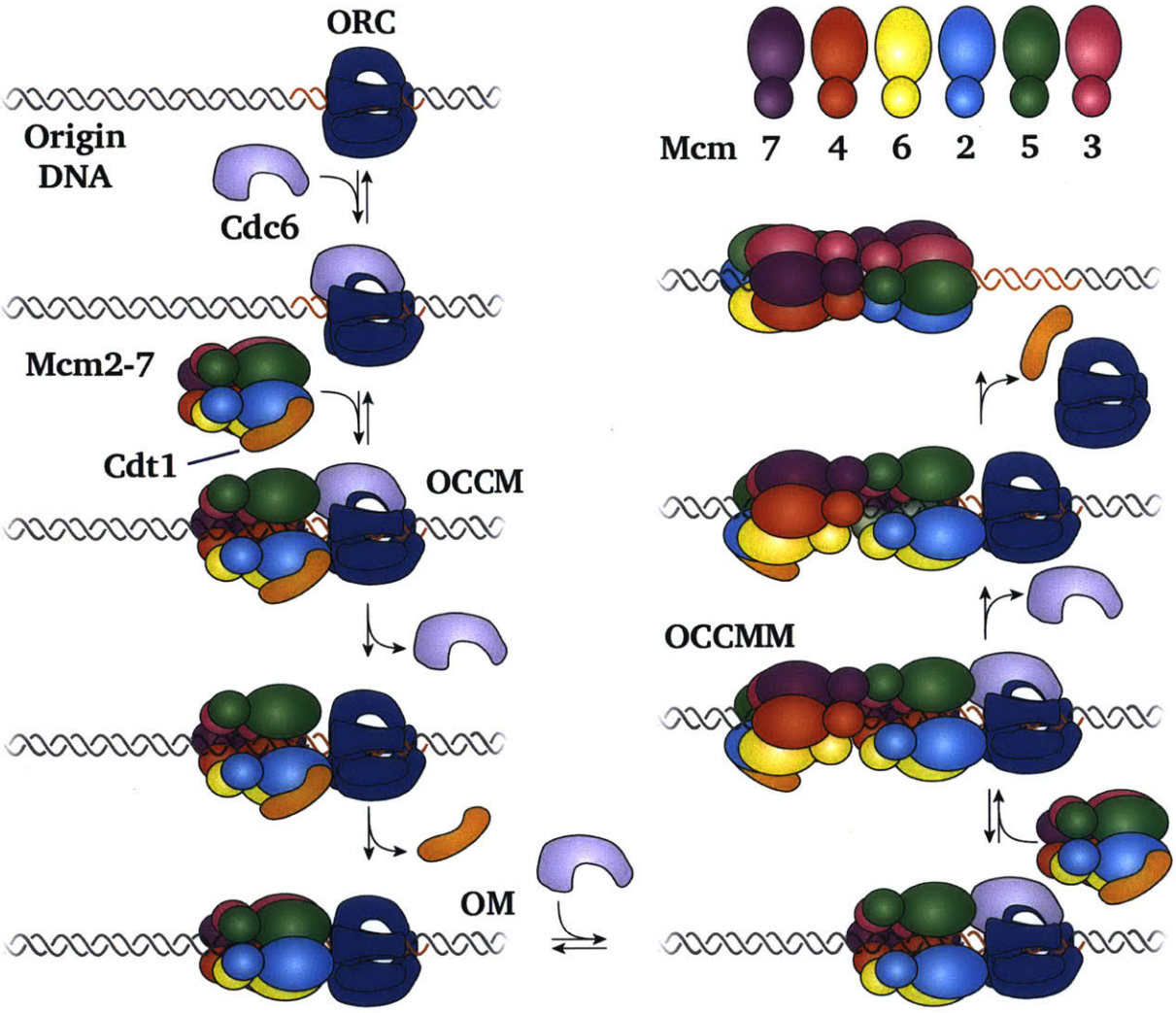
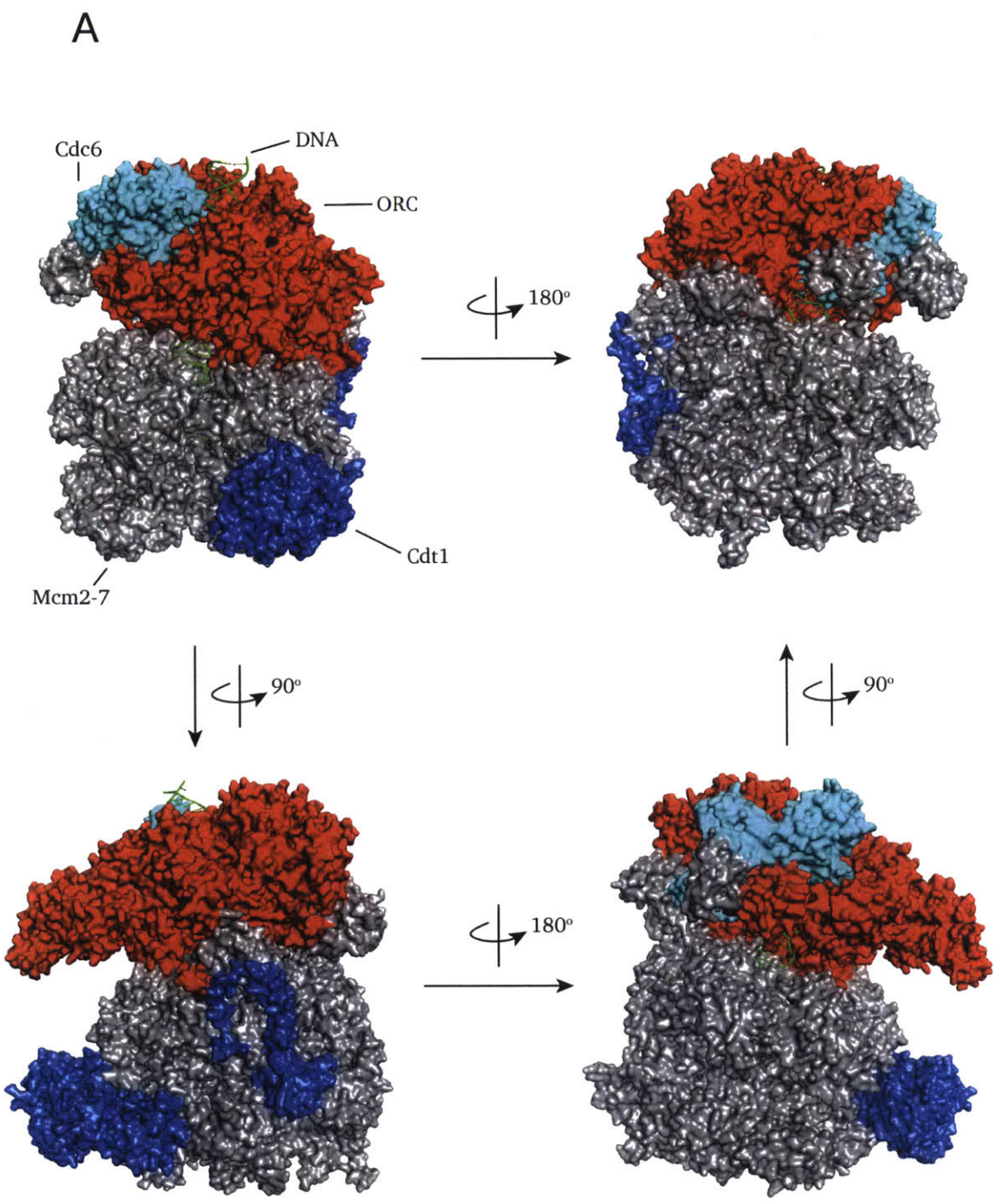
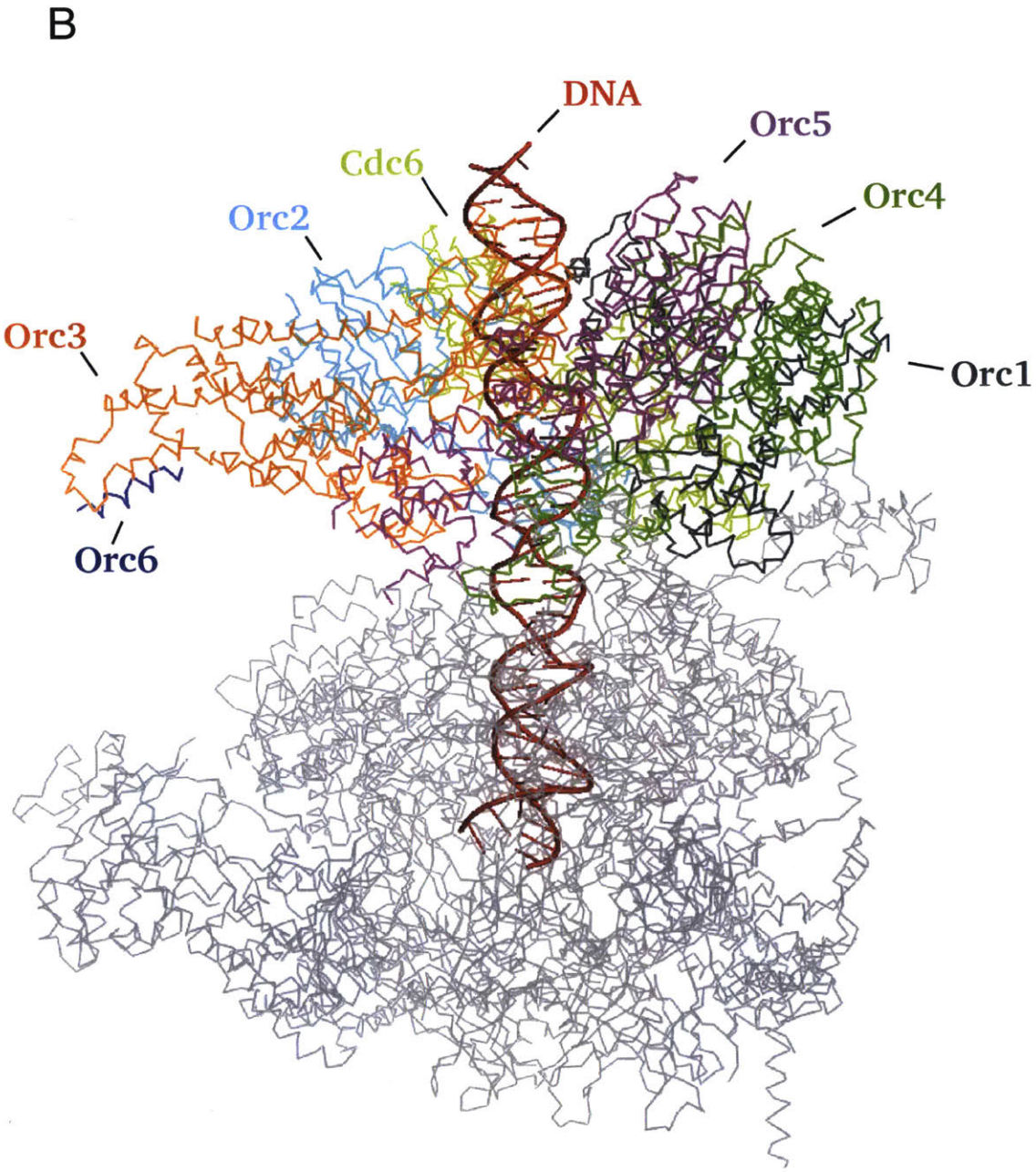


Figure 5. Mcm2-7 Helicase Loading at Origins of Replication. Helicase loading begins with ORC binding to origin DNA followed by Cdc6 binding to ORC. Mcm2-7 helicases are recruited to ORC in complex with Cdt1 to form the OCCM complex. Loading of the first Mcm2-7 helicase triggers Cdc6 and Cdt1 release from DNA. A second Cdc6 molecule binds to ORC and recruits a second Mcm2-7/Cdt1 complex to form the OCCMM complex. The second Mcm2-7 is then loaded followed by Cdc6, Cdt1 and ORC release. This last step results in Mcm2-7 double hexamers formation encircling dsDNA.

FIGURE 6. *Saccharomyces cerevisiae* OCCM Structure




B
DNA
Orc5
Cdc6
Orc4
Orc2
Orc3
Orc1
Orc6

C

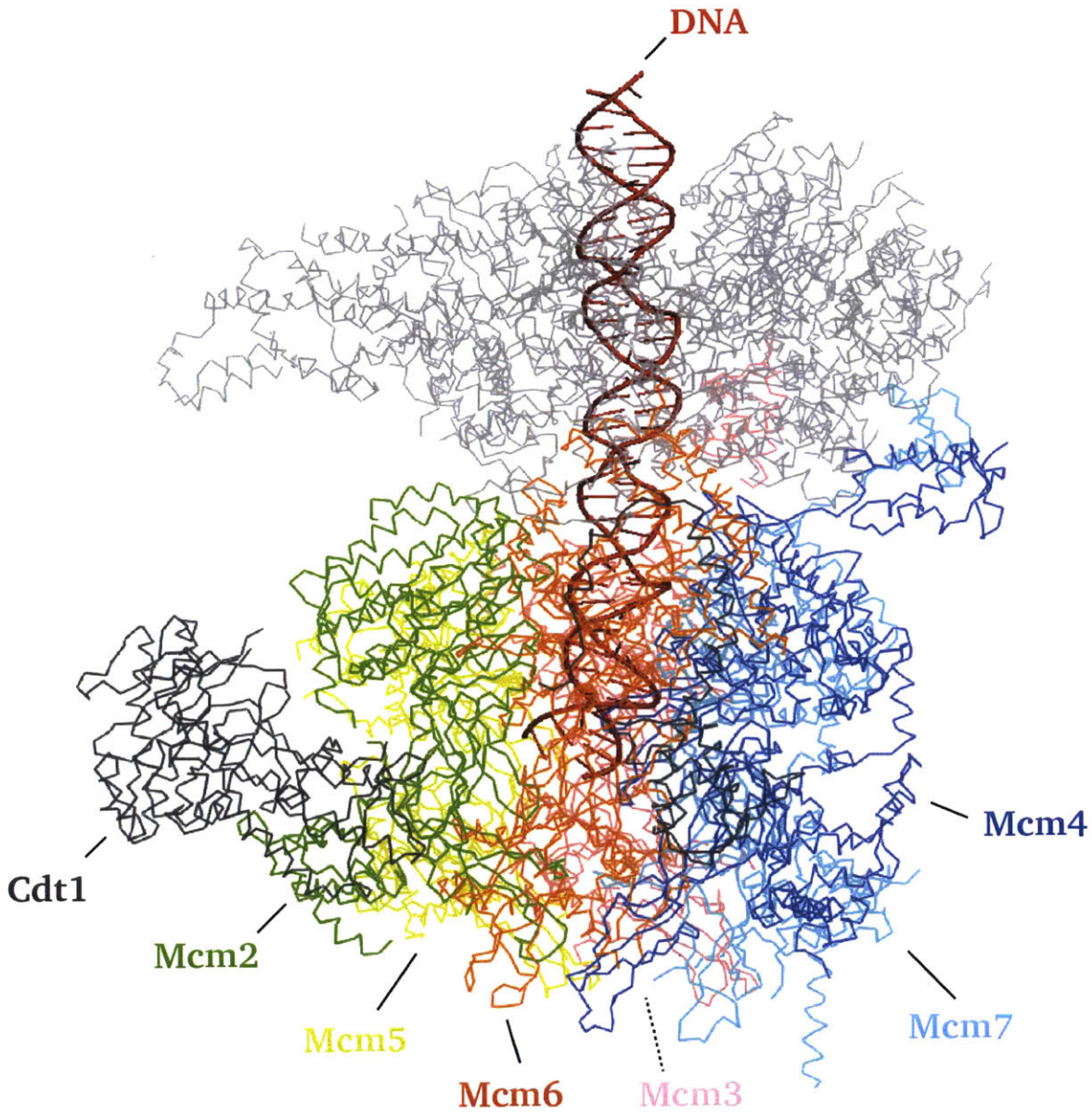


Figure 6. Architecture of the OCCM Complex. *Saccharomyces cerevisiae* OCCM cryo-EM structure was adapted from Yuan *et al.*, 2017. (A) 360° view of the OCCM complex bound to DNA. Space filling model of the OCCM complex is colored (ORC = red, Cdc6 = cyan, Cdt1 = blue, Mcm2-7 = gray, DNA = green). (B and C) Arrangement of ORC and Mcm2-7 subunits in the OCCM Complex. Each single subunit and DNA is colored and labeled accordingly.

III. MCM2-7 HELICASE ACTIVATION IN *SACCHAROMYCES CEREVISIAE*

A. OVERVIEW

Although the Mcm2-7 complex is the core motor of the eukaryotic replicative helicase, the initially loaded Mcm2-7 helicases are inactive and require substantial remodeling to activate. Activation of the Mcm2-7 helicase is associated with dramatic changes in the Mcm2-7 complex and the DNA it is bound to. In its active form, the Mcm2-7 complex functions as a single hexamer that encircles ssDNA. This is in stark contrast to the loaded Mcm2-7 double hexamer that encircles dsDNA (Fu *et al.*, 2011; Remus *et al.*, 2009; Evrin *et al.*, 2009). Thus, during Mcm2-7 activation the double-hexamer interface must be disrupted, the DNA within Mcm2-7 must be unwound and one DNA strand ejected. The order of these events and their mechanism remains unknown.

Mcm2-7 activation is triggered in S phase by the association of two key activators, Cdc45 and the GINS complex (Psf1, Psf2, Psf3 and Sld5). Together, these proteins form the active replicative helicase, the **C**dc45/**M**cm2-7/**G**INS (CMG) complex. Formation of this complex requires several other proteins and kinases. In the next section, I will address the current view in the field regarding the helicase activation process and the key players involved (Figure 4).

FIGURE 7. MCM2-7 Helicase Activation

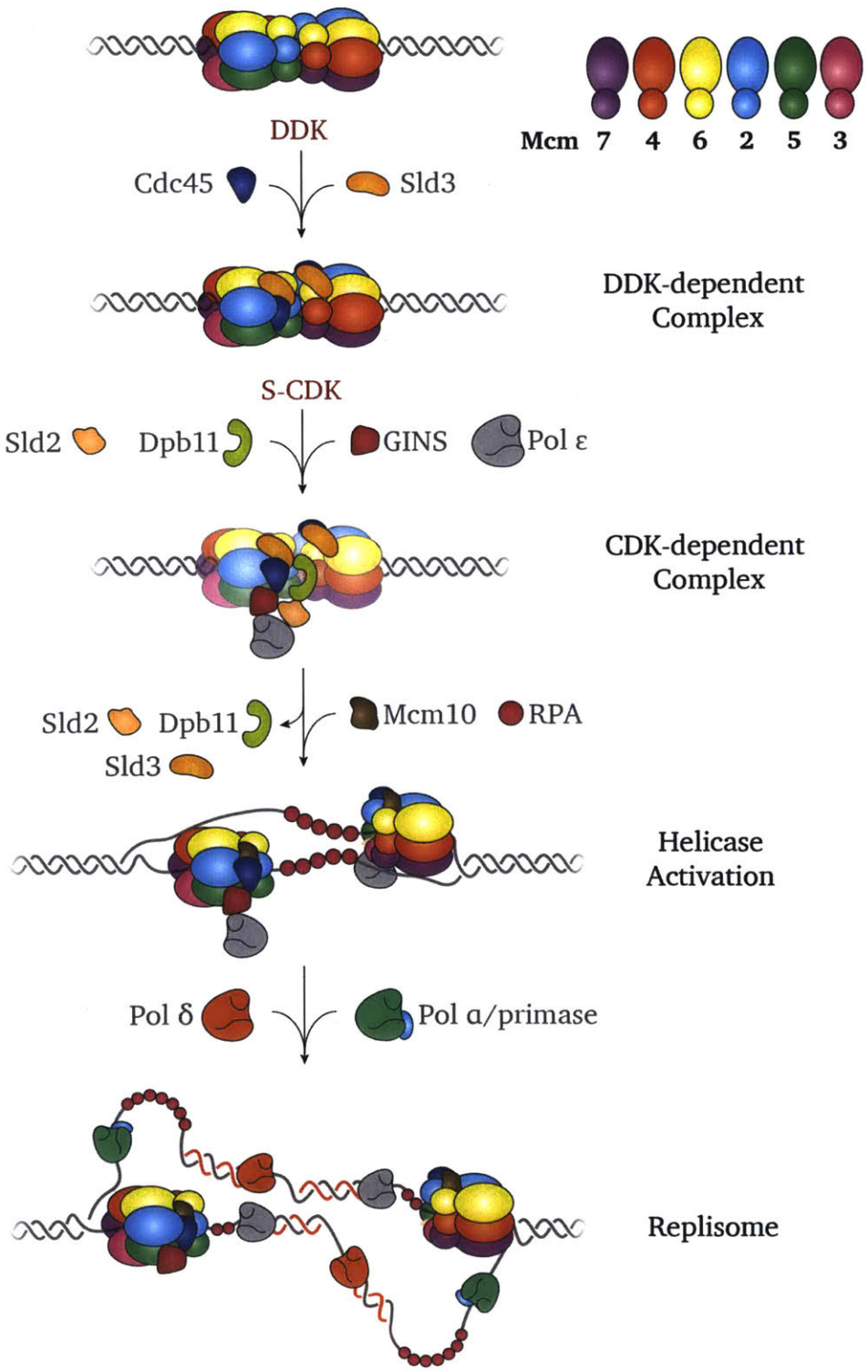


Figure 7. Mcm2-7 Helicase Activation Model. Loaded helicases are phosphorylated by DDK leading to the recruitment of Cdc45 and Sld3. S-CDK phosphorylates Sld2 and Sld3 and promotes the recruitment of GINS, Sld2, Dpb11 and Pol ε. Mcm10 recruitment activates the helicase leading to initial DNA unwinding and RPA recruitment. CMG helicases unwind DNA on opposite directions providing a ssDNA template for DNA Pol α/primase, DNA Pol δ and DNA Pol ε.

B. CDC45 AND THE CMG COMPLEX

Cdc45 is a 74 kDa essential protein that is conserved across all eukaryotes and one of the two key Mcm2-7 helicase activators. Cdc45 was first isolated in a cold-sensitive screen of cell-cycle mutants (Moir *et al.*, 1982). Interestingly, this study also made the first connection between Cdc45 and the Mcm2-7 complex. After isolating Cdc45, Moir found two genes that suppress the cold-sensitive phenotype: *CDC46* and *CDC47*, which subsequently were renamed *MCM5* and *MCM7*. Subsequent studies found Cdc45 is essential for DNA replication, interacts with the Mcm2-7 complex and moves with the replication fork (Hopwood *et al.*, 1996; Zou *et al.*, 1997; Aparicio *et al.*, 1997; Masuda *et al.*, 2003).

Cdc45 function during DNA replication initiation was initially studied in *Xenopus laevis* extracts. These studies revealed that Cdc45 was required to promote the recruitment of Pol α/primase and Replication Protein A (RPA). Moreover, inhibition of DNA unwinding did not impair Cdc45 association suggesting that Cdc45 functions prior to initial DNA unwinding replication initiation (Mimura and Takisawa, 1998; Walters and Newport, 2000).

Consistent with its role as part of the replicative helicase, Cdc45 is required during DNA elongation as part of the replisome (Tercero *et al.*, 2000;

Pacek *et al.*, 2006). Cdc45 degradation during S-phase halts DNA synthesis and renders cells unable to finish DNA replication. Importantly, this effect is reversible after inducing new Cdc45 protein synthesis suggesting that Cdc45 actively functions in the context of the CMG at replication forks and not solely during initial helicase activation.

All the data regarding Cdc45 function during DNA replication were bound together in 2006 with the isolation of the CMG complex (Moyer *et al.*, 2006). This complex was biochemically purified from *Drosophila melanogaster* embryos and showed robust helicase activity *in vitro* suggesting that the CMG complex was the eukaryotic replicative helicase. The CMG helicase was also shown to translocate on DNA in a 3'→5' direction (Moyer *et al.*, 2006), which means that it would move on the leading strand template (as opposed to the 5'→3' polarity of the bacterial replicative DNA helicase, DnaB). In collaboration with GINS, Cdc45 promotes helicase activation by directly stimulating Mcm2-7 ATP hydrolysis, helicase activity and DNA binding (Ilves *et al.*, 2010). How Cdc45 and GINS stimulate helicase activity remains unclear.

Cdc45 differentially associates at origins of replication (Aparicio *et al.*, 1999). Interestingly, Cdc45 associates with early-firing origins but not with late-firing origins in late G1. This observation suggests that the early presence of Cdc45 at an origin contributes to the temporal programming of origin firing in S-

phase. Although Cdc45 associates with early-firing origins in G1 it also late-firing origins but only at the time of their initiation of replication (Aparicio *et al.*, 1999). In addition, Cdc45 recruitment to late-firing origins is suppressed by activation of the DNA damage checkpoint (Zegerman *et al.*, 2010).

Cdc45 association with the Mcm2-7 complex requires the activity of the Dbf4-dependent kinase (DDK) (Owens *et al.*, 1997; Heller *et al.*, 2011). DDK is a two-protein complex composed of the Cdc7 catalytic and Dbf4 regulatory subunits and is essential for DNA replication initiation. DDK is a Serine/Threonine (S/T) kinase and preferentially phosphorylates loaded Mcm2-7 complexes at multiple sites in the N-terminal regions of Mcm4 and Mcm6 (Francis *et al.*, 2009; Sheu and Stillman, 2010; Randell *et al.*, 2010). There are two classes of motifs that are recognized and phosphorylated by DDK (Randell *et al.*, 2010). Intrinsic DDK sites (S/T-D/E) are characterized by the presence of an acidic residue at the +1 position (i.e. D/E). The second group of DDK target sites (S-S-P/Q) is not intrinsically phosphorylated by DDK. Instead, they require a priming phosphorylation event at position +1 to be targeted by DDK (Randell *et al.*, 2010). Mcm4 and Mcm6 have a mixture of both types of sites. Mutating all DDK sites either in Mcm4 or Mcm6 does not cause lethality suggesting some level of redundancy between sites. However, mutating both Mcm4 and Mcm6 DDK sites causes cell death.

DDK-dependent recruitment of Cdc45 requires the initiation factor Sld3. (Labib, 2010; Heller *et al.*, 2011). *In vivo*, Cdc45 and Sld3 recruitment are interdependent (Tanaka and Diffley, 2002). However, Sld3 can be recruited in the absence of Cdc45 *in vitro* suggesting that Sld3 recruits Cdc45 to origins of replication (Deegan *et al.*, 2016). Sld3 binds to DDK-phosphorylated peptides on Mcm4 and Mcm6. It is important to note that not all Sld3 binding sites are DDK sites suggesting that DDK phosphorylation might cause a conformational change on the Mcm2-7 complex that exposes the Sld3 (and potentially other protein) binding sites. This is supported by studies revealing that deletion of Mcm4 N-terminal extension bypasses DDK function (Sheu and Stillman, 2010). It is possible that DDK phosphorylation of the Mcm4 N-terminal extension reveals additional binding sites for Sld3 and by deleting this region they are constitutively available.

S-CDK activity is also required for CMG formation. The essential functions of S-CDK during this process is the phosphorylation of Sld2 and Sld3 (**S**ynthetically **L**ethal with **D**pb11-1). Sld3 and Sld2 phosphorylation promotes both proteins to associate and bind Dpb11 through two distinct pairs of BRCT (BRCA1 C-terminus) domains localized at the N- and C-terminal ends of Dpb11, respectively. (Tanaka *et al.*, 2007). The Sld2-Dpb11 interaction induces the association of GINS and Pol ε to form a weakly associated set of proteins called the pre-loading complex (pre-LC) (Muramatsu *et al.*, 2010). GINS binds directly

to a region between the BRCT domains of Dpb11 (Tanaka *et al.*, 2013). Similarly, the Dpb2 subunit of Pol ε interacts with GINS to recruit this polymerase to the pre-LC. Importantly, the Dpb2-GINS interaction is required for CMG formation making Pol ε important for replication initiation even before any DNA synthesis (Sengupta *et al.*, 2013). Sld3 association with Dpb11 recruits the pre-LC to origins of replication via interactions with DDK-phosphorylated Mcm2-7 complexes (Fang *et al.*, 2016). These interactions bring GINS to loaded Mcm2-7-Cdc45 complexes to form the CMG. It remains to be elucidated how and when Sld2, Sld3 and Dpb11 are released after the CMG is formed and whether their release triggers any step during this process.

A cryo-EM structure of the *Drosophila melanogaster* CMG revealed where Cdc45 and GINS are localized in relationship to the Mcm2-7 complex (Costa *et al.*, 2011). Cdc45 showed interactions with both Mcm2-7 rings and GINS supporting previous evidence proposing their interaction (Masuda *et al.*, 2003; Gambus *et al.*, 2006). Cdc45 bridged the gap between the Mcm2-Mcm5 gate suggesting that Cdc45 binding closes and locks the Mcm2-7 ring. This structure provided evidence on the physical morphology of the CMG and provided the first model for helicase activation. However, this structure lacked enough resolution to pinpoint the exact amino acids mediating protein interactions between Cdc45 and the rest of the CMG.

A higher resolution Cryo-EM of *Saccharomyces cerevisiae* CMG revealed that Cdc45 interacts only with Mcm2 and Mcm5 N-terminal domains and not with the C-terminal domains (Figure 8B)(Yuan *et al.*, 2016). GINS also interacts Mcm5 and Mcm3 N-terminal domains through interactions with Psf3. Psf2 also localizes to the Mcm5-3 interface whereas Psf1 and Sld5 extend outward from the Mcm2-7 ring. In addition, Psf1 and Psf2 interact with Cdc45. These studies identified two conformations of the CMG complex. Interestingly, Mcm2-7 C-terminal domains are tilted ~10° between the two conformations whereas the Mcm2-7 N-terminal ring remained rigid. The C-terminal domains of the Mcm2-7 complex contain the ATPase motifs suggesting that ATPase activity controls the transition between conformations. Notably, the C-terminal Mcm2-5 interface is open in one conformation whereas Cdc45 and GINS hold the N-terminal domain closed. This suggests that one function of Cdc45 and GINS is to stabilize the N-terminal ring of the Mcm2-7 complex to prevent the CMG helicase from falling off DNA.

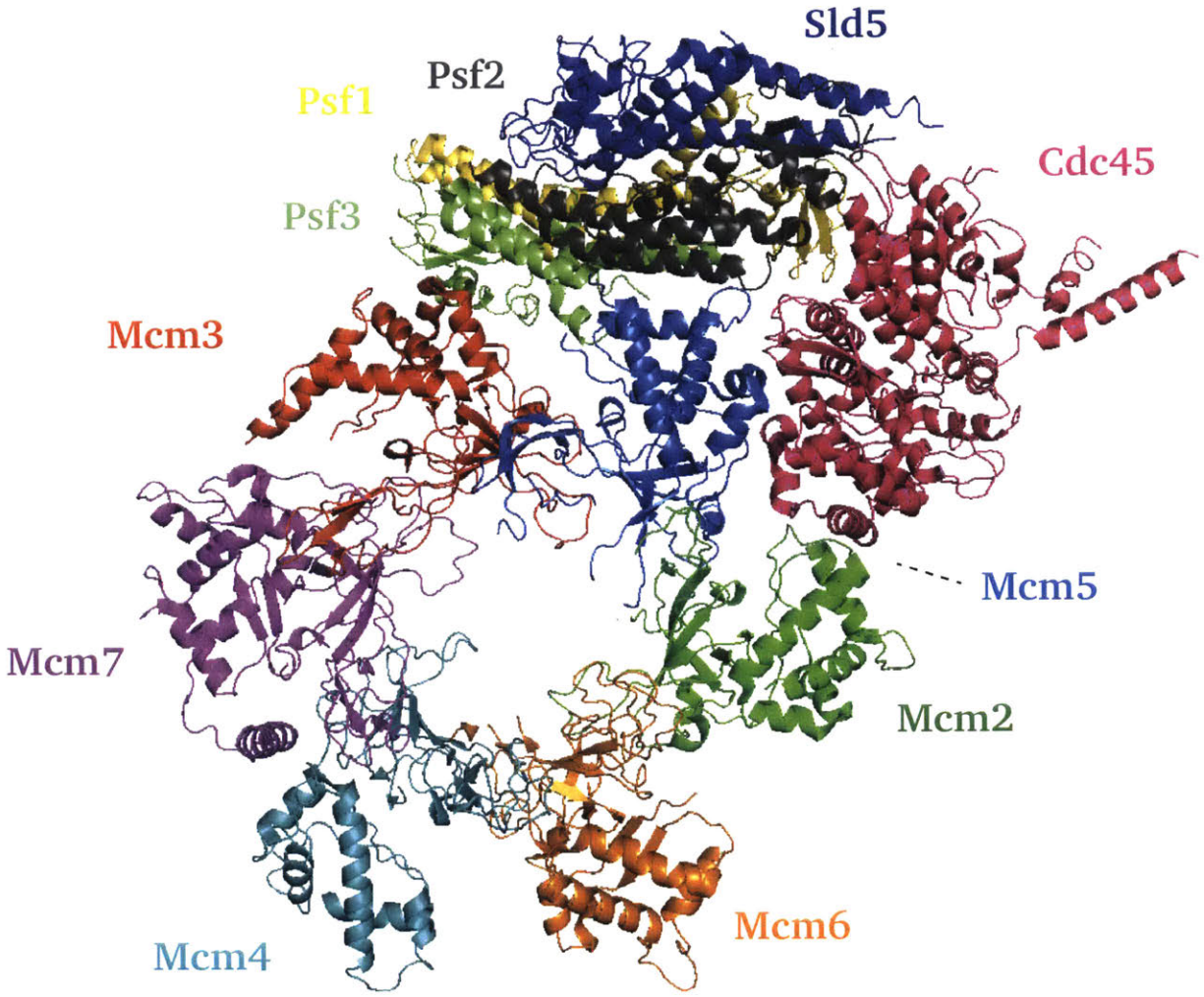
CMG formation is not sufficient to promote helicase activation and origin firing. Recent studies have shown that initial DNA unwinding is dependent on Mcm10 activity (Kanke *et al.,* 2012). Removing Mcm10 prevents the recruitment of the ssDNA-binding protein RPA suggesting that Mcm10 activates the CMG helicase. However, it is possible that the CMG prior to Mcm10 function is active but not engaged with the DNA, similar to a running engine in neutral. Several

functions have been proposed for Mcm10. It is possible that Mcm10 functions by separating the double CMG complexes (Quan *et al.*, 2015), initial DNA melting or ssDNA strand exclusion from the CMG central channel. In addition to its role in helicase activation, Mcm10 also functions during DNA elongation *in vivo* and *in vitro* (Lõoke *et al.*, 2017). How Mcm10 mediates this process remains unclear, although interactions with the N-terminus of Mcm2 (near the Cdc45 binding site) are clearly involved.

Although the effects of Cdc45 removal during DNA replication have been studied, little is know about how Cdc45 functions during helicase activation and elongation. The goal of this thesis is to investigate how Cdc45 functions during DNA replication. In chapter II, I describe our genetic and biochemical approach to address these questions. I have found several *CDC45* mutants that impair Cdc45 function. Interestingly, we found mutants defective for DNA replication initiation but not elongation. This suggests that Cdc45 has at least have two functions, one during Mcm2-7 activation and another in the context of the CMG during DNA elongation.

FIGURE 8. Cryo-EM Structure of *Saccharomyces cerevisiae* CMG

A



B

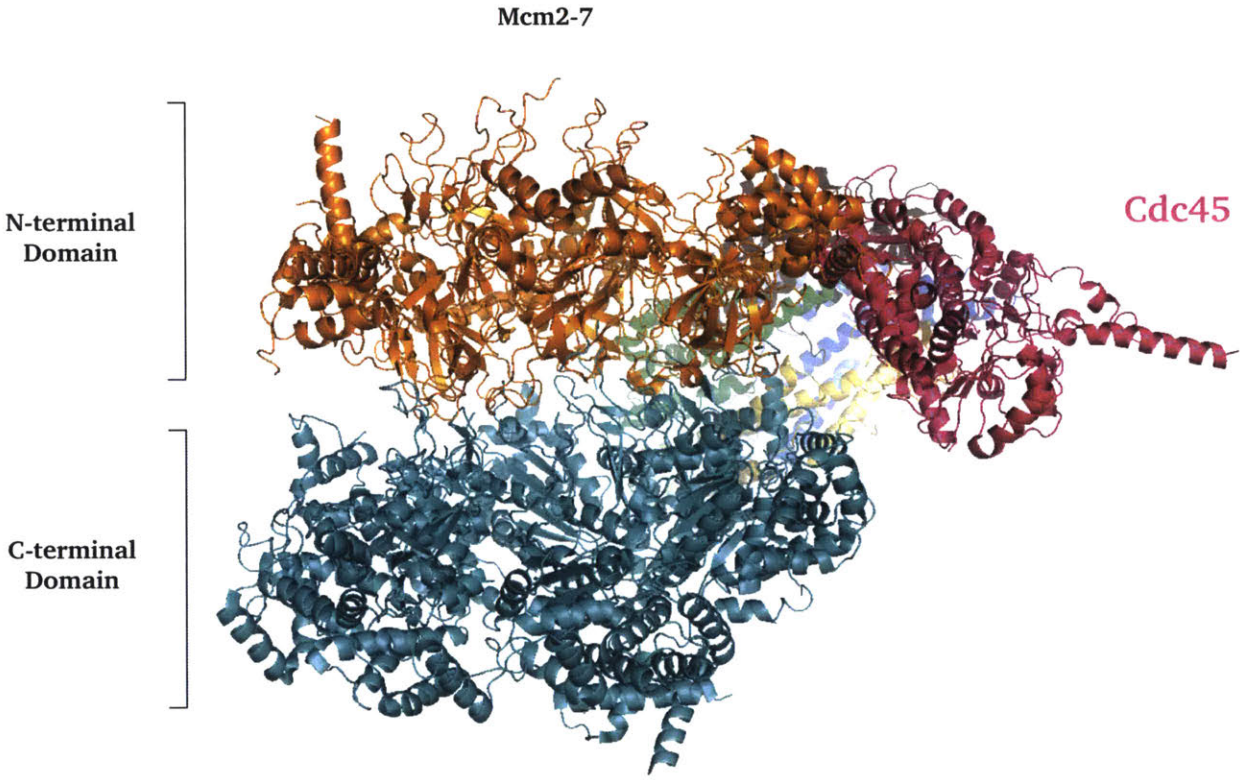


Figure 8. Cryo-EM Structure of *Saccharomyces cerevisiae* CMG. CMG structure was adapted from Yuan *et al.*, 2016. (A) Single subunits of the CMG are color coated and labeled. Cdc45 is located at the interface between Mcm2 and Mcm5. GINS is adjacent to Cdc45 and localizes to the Mcm3 and Mcm5 interface. C-terminal domains of each Mcm2-7 subunit are omitted for clarity. (B) Cdc45 interacts with the N-terminal domain of Mcm2 and Mcm5. N- and C-terminals domains of the CMG are labeled orange and teal, respectively.

CHAPTER II:

CDC45 Mutants are Defective in DNA Replication Initiation

I would like to acknowledge Bena Chan for the initial design of *CDC45* mutants and plasmid constructions.

SUMMARY

Cdc45 is required for DNA replication initiation and elongation. During DNA replication initiation, Cdc45 is essential to activate loaded Mcm2-7 helicases at origins of replication. In addition, Cdc45 function is required for CMG helicase activity during DNA replication elongation at replication forks. How Cdc45 mediates helicase activation and subsequent function during DNA elongation remains unclear. We designed mutations in *CDC45* with the goal of identifying protein domains or residues critical for Cdc45 function. These mutations could result in Cdc45 defects during DNA replication initiation, elongation or both. We demonstrate that three Cdc45 mutants are temperature-sensitive and these mutants are specifically defective for DNA replication initiation but not elongation. These data suggest that Cdc45 functions differently during DNA replication initiation and elongation.

INTRODUCTION

Eukaryotic DNA replication begins with the loading of Mcm2-7 complexes at origins of replication during G1-phase. Two Mcm2-7 hexamers are loaded onto dsDNA as a head-to-head double hexamer in an inactive state (Evrin *et al.*, 2009; Remus *et al.*, 2009; Ticau *et al.*, 2015 and 2017). During S phase, Mcm2-7 helicases are activated by the association of Cdc45 and GINS to form the active Cdc45/Mcm2-7/GINS helicase (CMG)(Moyer *et al.*, 2006; Ilves *et al.*, 2010). Formation of the CMG requires two kinases: the Dbf4-dependent Cdc7 kinase (DDK) and the S-phase cyclin-dependent kinase (S-CDK). DDK phosphorylation of the Mcm2-7 complex promotes the association of Cdc45 and Sld3 (Heller *et al.*, 2011; Deegan *et al.*, 2016). Subsequently, S-CDK promotes the recruitment of a complex between Dpb11, Sld2, GINS and Pol ε (Tanaka *et al.*, 2007; Zegerman and Diffley, 2007; Muramatsu *et al.*, 2010; Yeeles *et al.*, 2015). Although Cdc45 and GINS association leads to CMG formation, this initial CMG complex is not active. The initially formed CMG requires Mcm10 for initial DNA unwinding and the recruitment of the rest of the DNA replication machinery (Heller *et al.*, 2011; Kanke *et al.*, 2012; Watase *et al.*, 2012).

Helicase activation requires dramatic remodeling of the loaded Mcm2-7 double hexamer. The active CMG functions as a single hexamer encircling ssDNA whereas the Mcm2-7 helicases are initially loaded around dsDNA as

head-to-head, double hexamers. During Mcm2-7 activation the double-hexamer interface must be disrupted, the DNA within the Mcm2-7 must be unwound and one DNA strand ejected. How this transition is achieved remains elusive.

Cdc45 is one of two helicase activators and is required for both DNA replication initiation and elongation (Mimura and Takisawa, 1998; Walters and Newport, 2000; Tercero *et al.*, 2000; Heller *et al.*, 2011). Structural studies have identified interactions between Cdc45 and the Mcm2-7 complex (Costa *et al.*, 2011; Yuan *et al.*, 2016). Cdc45 is located at the Mcm2-5 interface and interacts only with the N-terminal domains of Mcm2 and Mcm5. Together with GINS, Cdc45 is thought to stabilize a closed-ring state of the N-terminal domains of Mcm2-7 while the C-terminal ATPase ring switches between a closed and cracked-ring conformation (Yuan *et al.*, 2016). In addition, Cdc45 interacts with Pol ε in the context of the CMG, suggesting that Cdc45 has a distinguish feature of elongation by connecting the active helicase with DNA polymerases.

Although the effects of Cdc45 loss during DNA replication have been studied, how Cdc45 functions during helicase activation and elongation is poorly understood. *In vitro* studies demonstrate that Cdc45 and GINS association stimulates Mcm2-7 ATPase and helicase activity suggesting one model for helicase activation (Ilves *et al.*, 2010). In contrast, recent studies have shown that initial DNA unwinding is dependent on Mcm10 activity (Kanke *et al.*, 2012). It is

possible that prior to Mcm10 function, Cdc45 still stimulate CMG ATPase activity but the CMG is maintained in a conformation not competent for DNA unwinding. Although Mcm10 triggers initial DNA unwinding, Cdc45 function is still required for this process (Kanke *et al.*, 2012). Importantly, Cdc45 function is not limited to DNA replication initiation but also is essential for DNA elongation. Degrading Cdc45 at replication forks halts DNA replication and renders cells unable to finish S-phase (Tercero *et al.*, 2000).

In this thesis, we designed and tested Cdc45 mutations to investigate the underlying mechanism of Cdc45 function. Using both genetics and biochemical approaches, we demonstrate that Cdc45 function during DNA replication initiation is separate from its role during DNA elongation. In particular, we have focused on the function of three temperature-sensitive *CDC45* alleles defective for DNA replication initiation but not elongation. Our findings suggest for the first time that Cdc45 function during DNA replication initiation is different from its function from DNA elongation.

RESULTS

In vivo Characterization of *CDC45* Site-directed Mutants

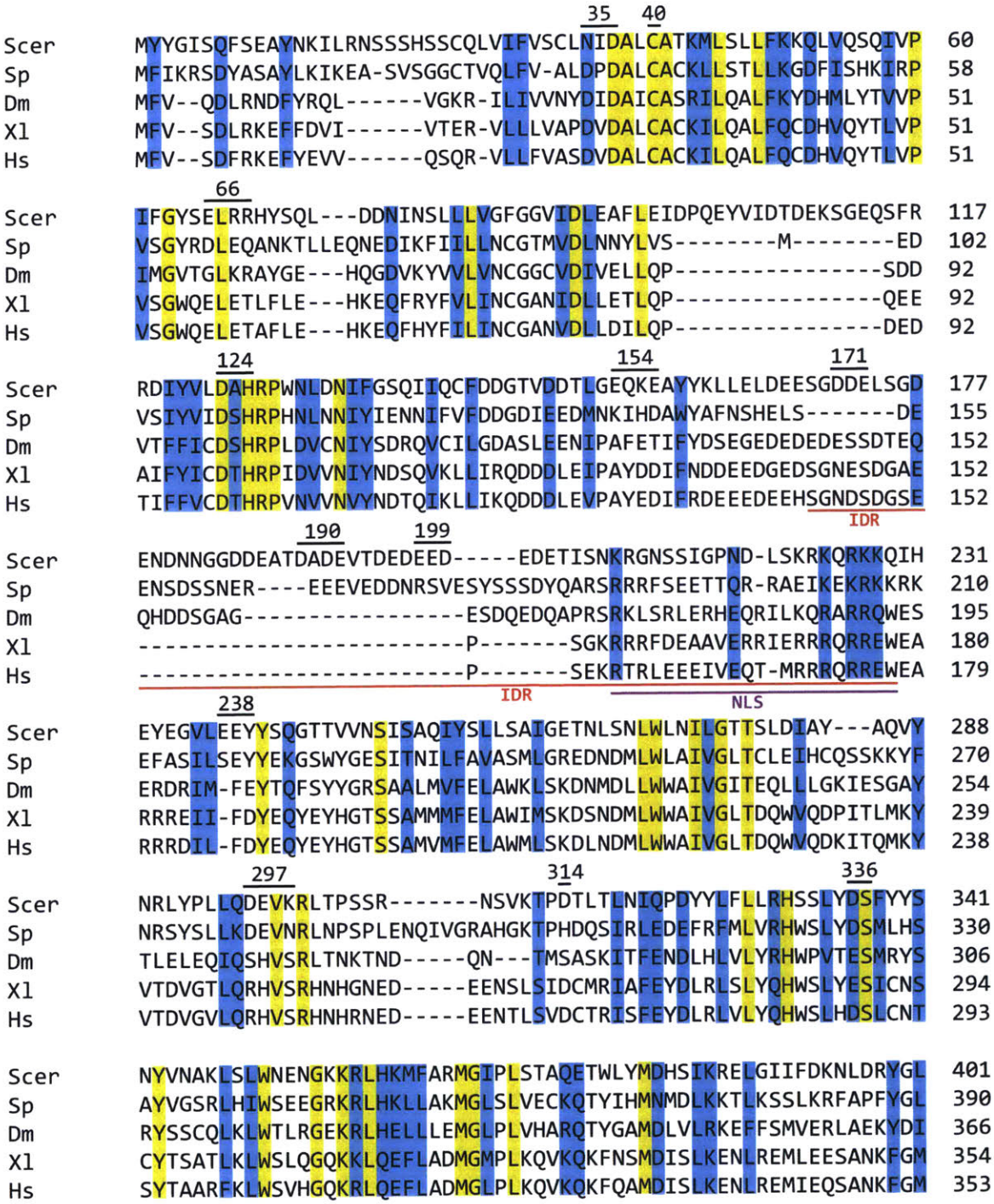
To better understand the role of CDC45 during DNA replication, we designed nineteen *CDC45* mutants based on sequence conservation and segments of charged residues (Figure 1 and 2). We targeted regions of four or more charged residues in addition to residues conserved among eukaryotes. Targeted residues were mutagenized to alanine and their viability tested using a Cdc45 'swapper' strain.

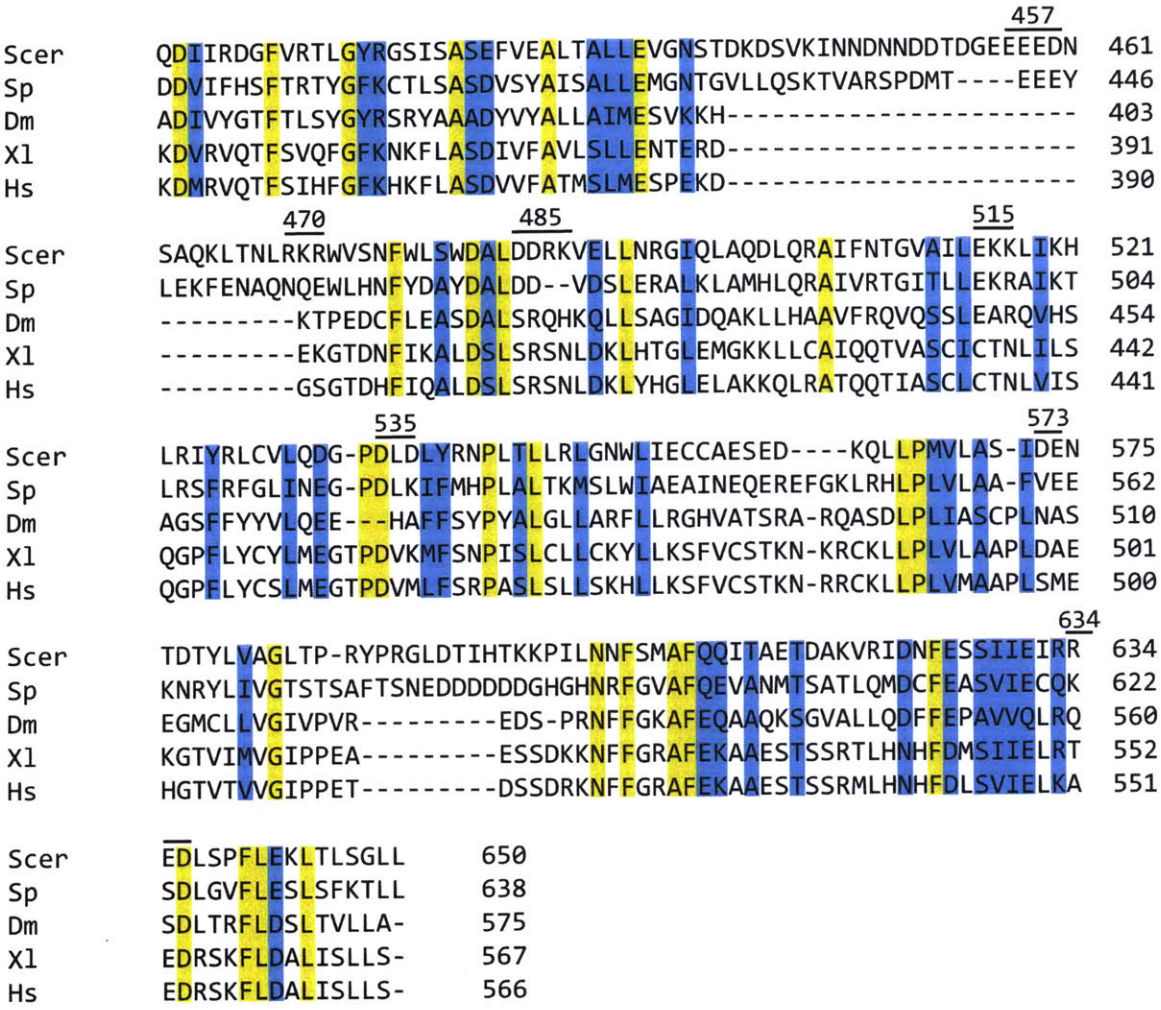
Four of nineteen mutants resulted in a lethal phenotype (Figure 3). Two of these mutants, *cdc45-35* and *cdc45-66*, reside in a region previously found to be homologous to the RecJ N-terminal DDH domain (Sanchez-Pulido *et al.*, 2011). The other two lethal mutants, *cdc45-171* and *cdc45-199*, are located in a region of Cdc45 predicted to be a disordered region (Romero *et al.*, 1997; Li *et al.*, 1999). In *S.* cerevisiae, this region spans from amino acids 169-229 and contains a nuclear localization signal (210-229). Importantly, computational models predict an intrinsic disordered region (IDR) to be a conserved feature of Cdc45 from all eukaryotes despite poor primary sequence conservation (Figure 1 and 4). To test if this region is dispensable for *CDC45* function, we deleted amino acids 169-209 (*cdc45ΔIDR*, we retained 210-229 as it contained the Cdc45 NLS) and tested for

complementation. Although point mutations in the IDR were lethal, *cdc45ΔIDR* was viable (Figure 5). None of the mutants analyzed were sensitive to hydroxyurea (HU) and methyl methanesulfonate (MMS)(Figure 6). However, three mutants were viable at 25°C but lethal when grown at 37°C: *cdc45-124*, *cdc45-238* and *cdc45-485* (Figure 7).

FIGURE 1. *CDC45* Sequence Alignment

A




457
Scer QDIIRDGFVRTLGYRGSISASEFVEALTALLEVGNSTDKDSVKINNDNNDDTDGEEEEDN 461
Sp DDVIFHSFTRTYGFKCTLSASDVSYAISALLEMGNTGVLLQSKTVARSPDMT----EEEY 446
Dm ADIVYGTFTLSYGYRSRYAAADYVYALLAIMESVKKH---------- 403
Xl KDVRVQTFSVQFGFKNKFLASDIVFAVLSLLENTERD---------- 391
Hs KDMRVQTFSIHFGFKHKFLASDVVFATMSLMESPEKD---------- 390
470
485
515
Scer SAQKLTNLRKRWVSNFWLSWDALDDRKVELLNRGIQLAQDLQRAIFNTGVAILEKKLIKH 521
Sp LEKFENAQNQEWLHNFYDAYDALDD--VDSLERALKLAMHLQRAIVRTGITLLEKRAIKT 504
Dm ---------KTPEDCFLEASDALSRQHKQLLSAGIDQAKLLHAAVFRQVQSSLEARQVHS 454
Xl ---------EKGTDNFIKALDSLSRSNLDKLHTGLEMGKKLLCAIQQTVASCICTNLILS 442
Hs ---------GSGTDHFIQALDSLSRSNLDKLYHGLELAKKQLRATQQTIASCLCTNLVIS 441
535
573
Scer LRIYRLCVLQDG-PDLDLYRNPLTLLRLGNWLIECCAESED----KQLLPMVLAS-IDEN 575
Sp LRSFRFGLINEG-PDLKIFMHPLALTKMSLWIAEAINEQEREFGKLRHLPLVLAA-FVEE 562
Dm AGSFFYYVLQEE---HAFFSYPYALGLLARFLLRGHVATSRA-RQASDLPLIASCPLNAS 510
Xl QGPFLYCYLMEGTPDVKMFSNPISLCLLCKYLLKSFVCSTKN-KRCKLLPLVLAAPLDAE 501
Hs QGPFLYCSLMEGTPDVMLFSRPASLSLLSKHLLKSFVCSTKN-RRCKLLPLVMAAPLSME 500
634
Scer TDTYLVAGLTP-RYPRGLDTIHTKKPILNNFSMAFQQITAETDAKVRIDNFESSIIEIRR 634
Sp KNRYLIVGTSTSAFTSNEDDDDDDGHGHNRFGVAFQEVANMTSATLQMDCFEASVIECQK 622
Dm EGMCLLVGIVPVR---------EDS-PRNFFGKAFEQAAQKSGVALLQDFFEPAVVQLRQ 560
Xl KGTVIMVGIPPEA---------ESSDKKNFFGRAFEKAAESTSSRTLHNHFDMSIIELRT 552
Hs HGTVTVVGIPPET---------DSSDRKNFFGRAFEKAAESTSSRMLHNHFDLSVIELKA 551
Scer EDLSPFLEKLTLSGLL 650
Sp SDLGVFLESLSFKTLL 638
Dm SDLTRFLDSLTVLLA- 575
Xl EDRSKFLDALISLLS- 567
Hs EDRSKFLDALISLLS- 566

B

Cdc45 Site-directed Mutations
35-37 (NID -> AAA)
40 (C -> A)
66-69 (ELRR -> ALAA)
124-126 (DAH -> AAA)
154-157 (EQKE -> AQAA)
171-173 (DDE -> AAA)
190-193 (DADE -> AAAA)
199-201 (EED -> AAA)
238-240 (EEY -> AAA)
297-300 (DEVK -> AAVA)
314 (D -> A)
336-337 (DS -> AA)
457-460 (EEED -> AEAA)
470-472 (RKR -> AAA)
485-488 (DDRK -> AAAA)
515-517 (EEK -> AKA)
535-537 (DLD -> ALA)
573-574 (DE -> AA)
634 (RED -> AAA)

Figure 1. *CDC45* Sequence Alignment. (A) *CDC45* sequence alignment of indicated species was obtained using EMBL-EBI Clustal Omega. The Intrinsic Disordered Region (IDR) and nuclear localization signal (NLS) of *S. cerevisiae* are underlined in red and purple, respectively. Site directed mutants are underlined in black and labeled by the position of the first mutated amino acid. Blue and yellow bars indicated very similar and identical residues, respectively. (B) Summary table of *CDC45* mutants.

FIGURE 2. Localization of *CDC45* Mutants

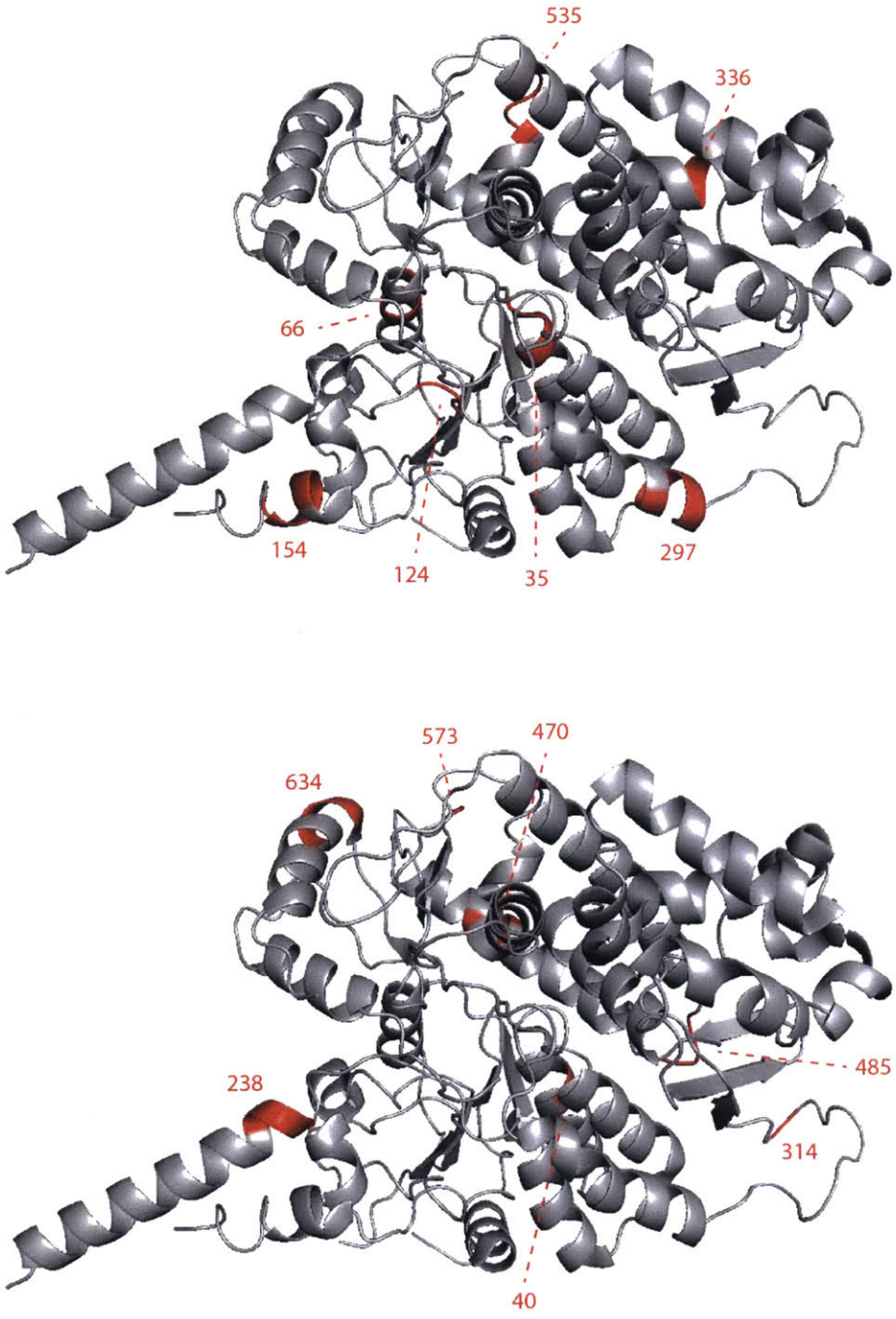


Figure 2. Localization of *CDC45* mutants within the Cdc45 structure (adapted from Yuan *et al.*, 2016). *cdc45-171*, *cdc45-190*, *cdc45-199*, *cdc45-457*, *cdc45-515* were not visible in the structure.

FIGURE 3. *In vivo* Analysis of *CDC45* Mutants.

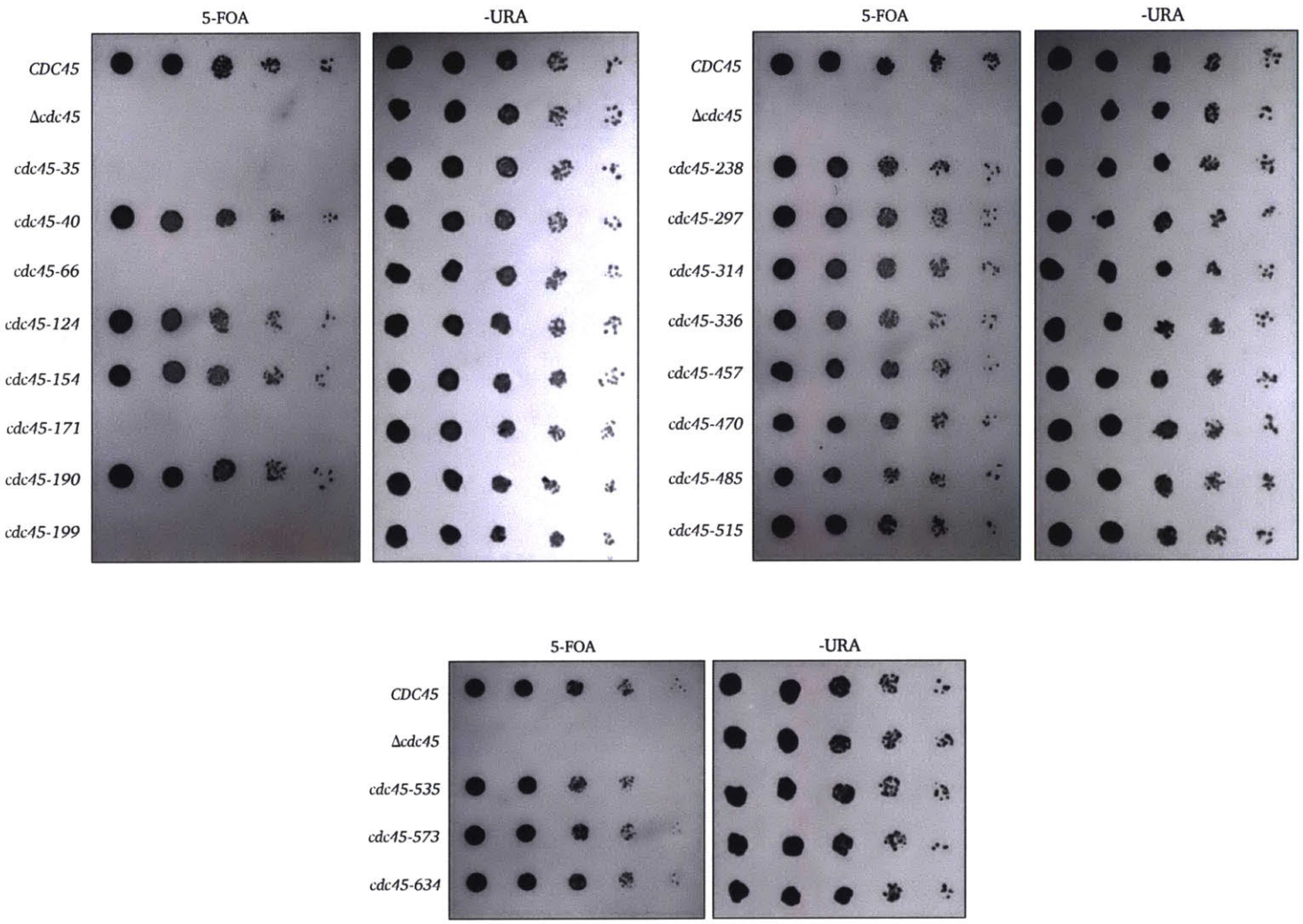


Figure 3. *In vivo* Analysis of *CDC45* Mutants. A plasmid shuffle assay was used to analyze nineteen *CDC45* mutants.The tester yeast strain was constructed with the endogenous *CDC45* gene deleted while carrying a copy of *CDC45* on a plasmid with the *URA3* gene that can be counter-selected by plating cells on 5-FOA. Five-fold serial dilutions of cells were grown on indicated plates for 3d at 25°C. Four out of nineteen *CDC45* mutants resulted to be lethal.

FIGURE 4. The Presence of an Intrinsic Disordered Region in Cdc45 is Conserved.

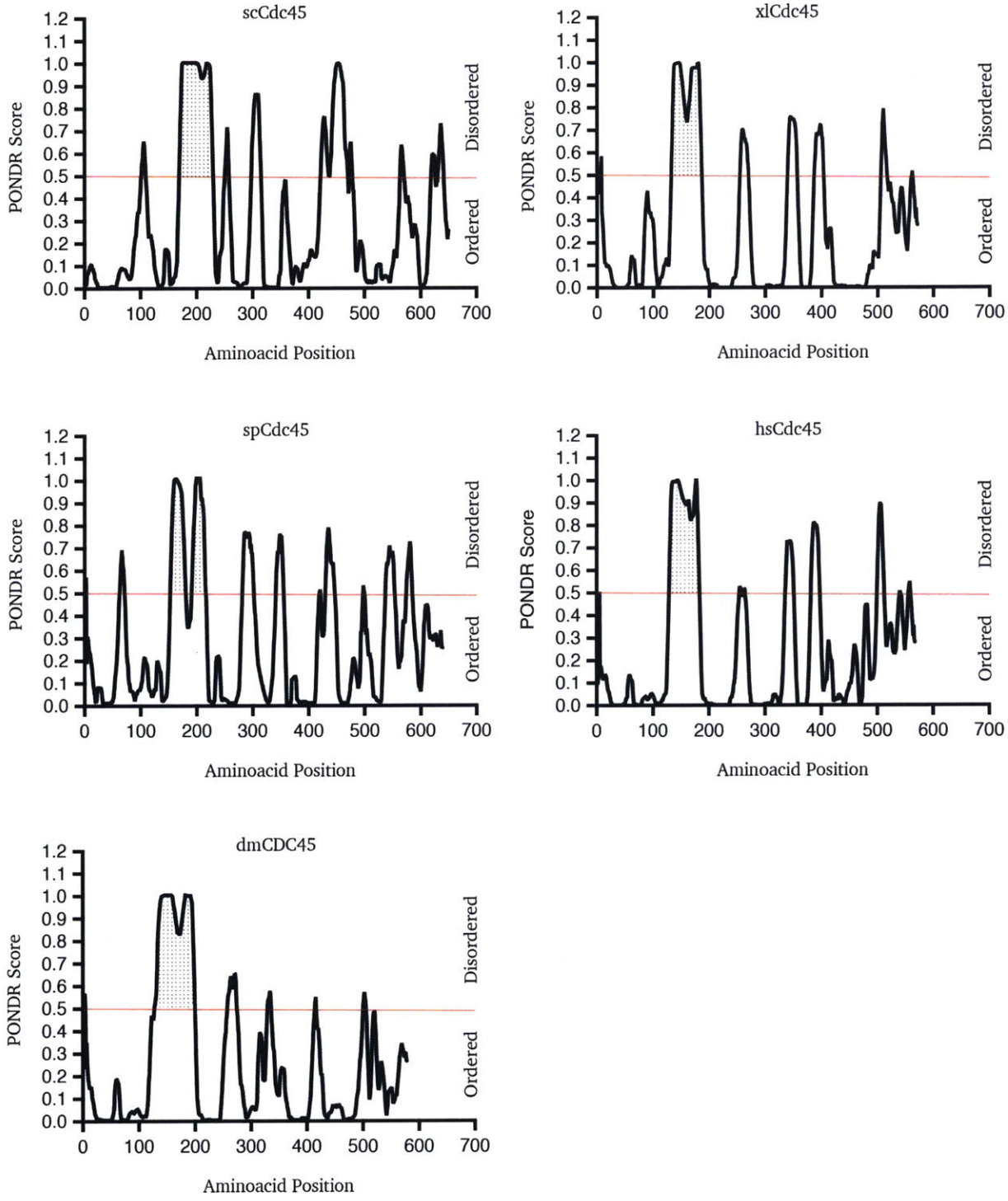


FIGURE 4. Intrinsic Disordered Region in Cdc45 is Conserved Among Eukaryotes. PONDR® protein disorder prediction was used to analyze indicated Cdc45 protein (Sc = *S. cerevisiae*, Sp = *S. pombe*, Dm = *D. melanogaster*, Xl = *X. laevis*, Hs = *H. sapiens*). Highest confidence protein regions predicted to be disordered are shown as dotted lines (Sc = 169-229, Sp = 153-215, Dm = 125-196, Xl = 129-183, Hs = 128-182). In each case, this region is found after the RecJ homology region of Cdc45.

FIGURE 5. The Cdc45 IDR Is Dispensable for Cell Viability

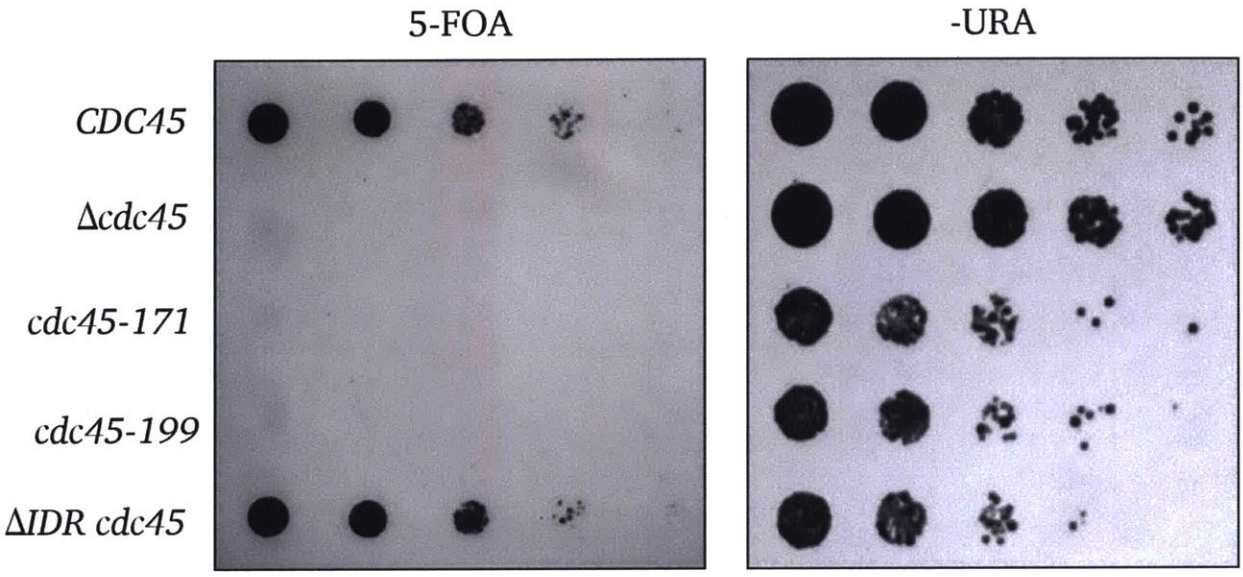


Figure 5. The *CDC45* IDR Is Dispensable for Cell Viability. Mutants in the Cdc45 IDR, *cdc45-171* and *cdc45-199*, are lethal. Five-fold serial dilutions of cells were grown on indicated plates for 3d at 25°C.

FIGURE 6. *CDC45* Mutants Are Not Sensitive to DNA Replication Stress Agents

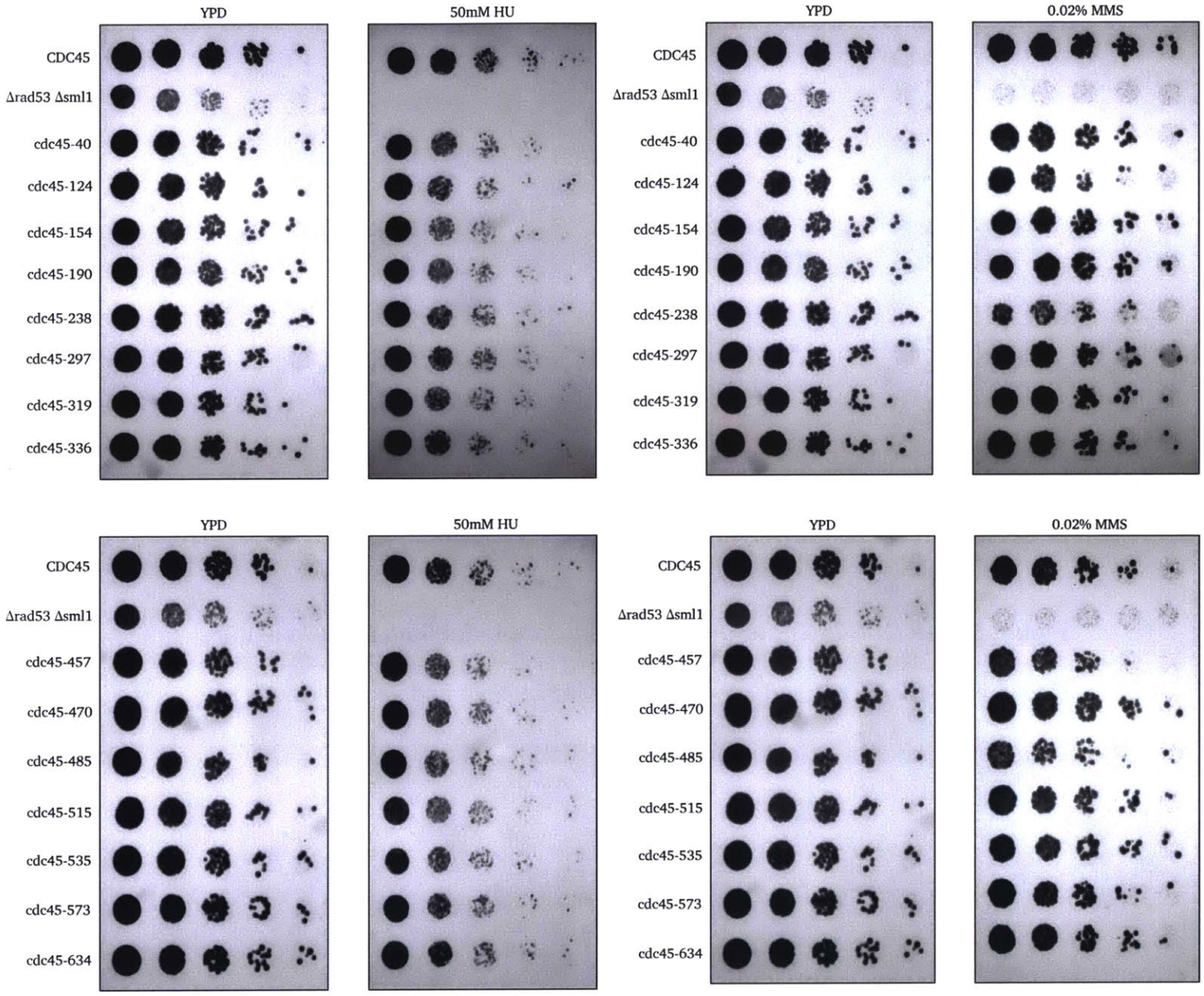


Figure 6. *CDC45* Mutants Are Not Sensitive to Hydroxyurea (HU) and Methyl Methanesulfonate (MMS). Five-fold serial dilutions of viable *cdc45* mutants were grown on indicated plates for 4 days at 25°C.

FIGURE 7. Three *CDC45* Mutants Are Lethal When Grown at 37°C.

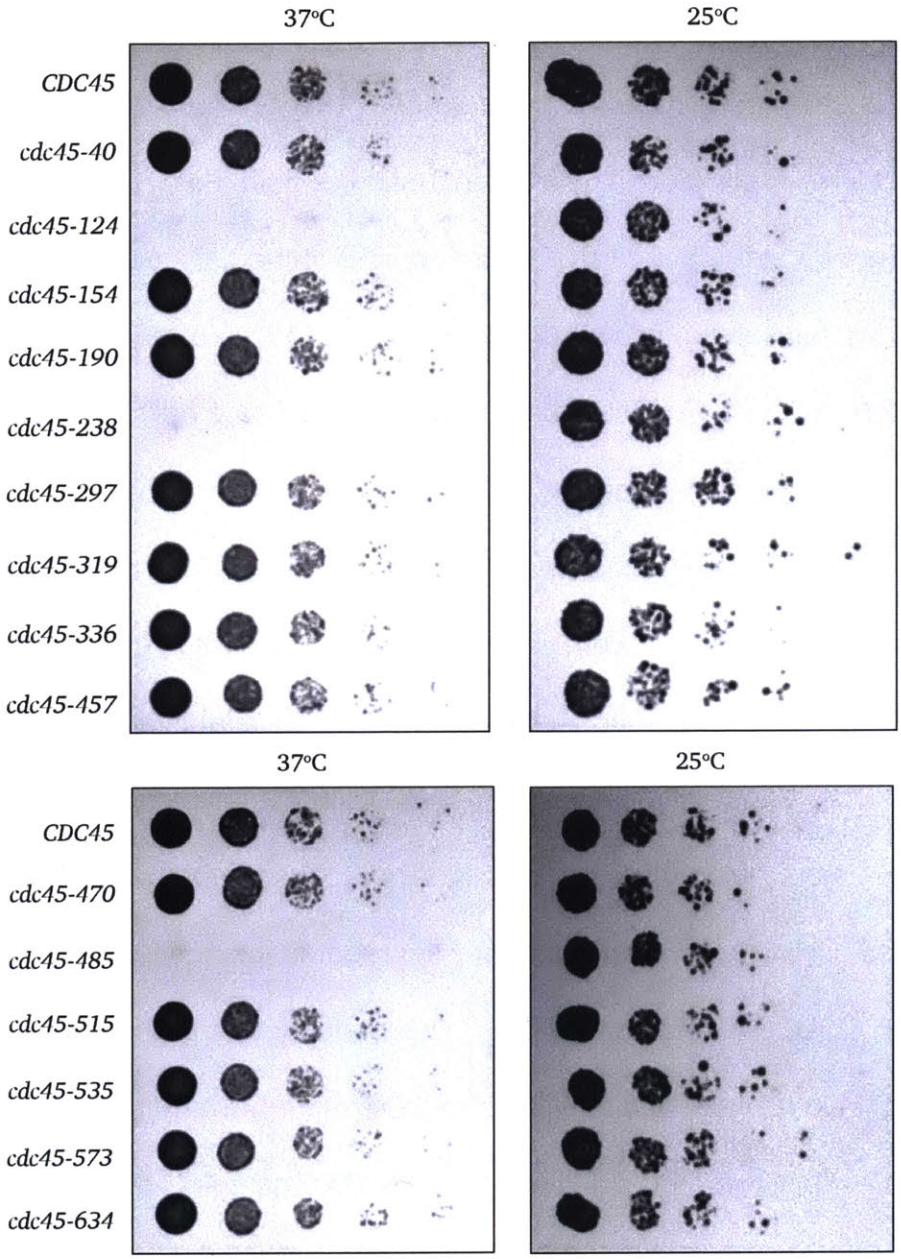


Figure 7. Three *CDC45* Mutants Are Lethal When Grown at 37°C. Five-fold serial dilutions of cell containing the indicated *CDC45* mutations as the only copy of *CDC45* were grown on YPD plates for 2d at 37°C and 3d at 25°C.

Cdc45 Temperature-sensitive Mutants Are Selectively Defective for DNA Replication Initiation

We next asked if these temperature-sensitive (ts) mutations were defective in DNA replication initiation, elongation or both. First, asynchronous cells were grown at the permissive temperature and then shifted to restrictive conditions. In this setting, *cdc45-124*, *cdc45-238* and *cdc45-485* mutants accumulated cells with G1 DNA content whereas wild-type cells remained asynchronous (Figure 8). Because defects in replication elongation would be expected to arrest throughout S-phase, an arrest with G1 DNA content suggested that these *cdc45* were defective in DNA replication initiation but competent for elongation. In addition, all three *cdc45* mutants accumulated cells with sub-G1 DNA content at later time points. This phenotype suggests that these mutants are going through reductional anaphase, a process during which unreplicated chromosomes are randomly segregated into the daughter cells (Piatti *et al.*, 1995; Tercero *et al.*, 2000). This phenotype is proposed to occur when cells have no replication initiation and therefore have no replication intermediates to signal incomplete replication. That the *cdc45* mutants exhibit reductional anaphase suggests that these mutants completely prevent entry into S phase.

To test for an initiation defect directly, we tested the ability of the *cdc45-ts* mutants to enter S-phase after inactivation during a G1 arrest. Each of the mutants showed strong defects in S-phase entry after the inactivation during G1-phase, maintaining G1 DNA content 1.5 hours after G1 release (Figure 9A). In contrast, wild-type cells completed S-phase in the same time frame.

Next, we asked if *cdc45-124*, *cdc45-238* and *cdc45-485* were competent for replication elongation at the non-permissive temperature. To this end, we arrested cells in early S-phase and raised them to the non-permissive temperature. We then monitored progress through S-phase after release from the S phase arrest at the non-permissive temperature. Unlike inactivation during G1, inactivation of the mutants during S-phase resulted in little or no defects in completion of S-phase. FACS analysis revealed that *cdc45-124*, *cdc45-238* and *cdc45-485* mutants resumed DNA elongation and finished S-phase. Consistent with the known role of Cdc45 during replication elongation, a strain containing a temperature-sensitive degradation allele (*cdc45-td*) was unable to complete S-phase (Figure 9B). Taken together, these findings indicate that *cdc45-124*, *cdc45-238* and *cdc45-485* mutants are defective in DNA replication initiation but not elongation at the non-permissive temperature.

FIGURE 8. Temperature-sensitive Alleles of *cdc45* Accumulate Cells with G1 DNA Content at the Restrictive Temperature

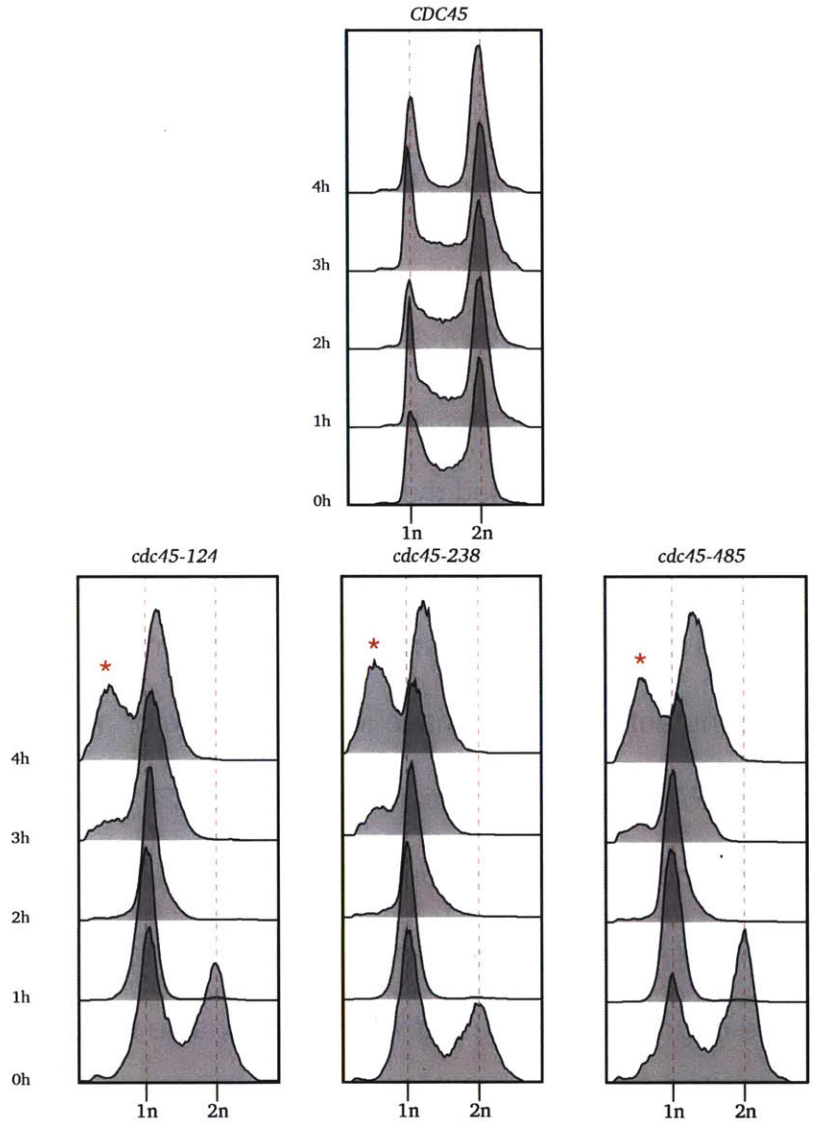


Figure 8. Temperature-sensitive alleles of *cdc45* Accumulate Cells with G1 DNA Content at the Restrictive Temperature. Asynchronous cultures of the indicated mutants were grown at 25°C in YPD and shifted to 37°C for 4h. Samples were collected at indicated time points and analyzed using FACS. Red asterisk denotes cells with less than G1 DNA content as a result of reductional anaphase.

FIGURE 9. Temperature-sensitive alleles of *cdc45* Are Defective for DNA Replication Initiation

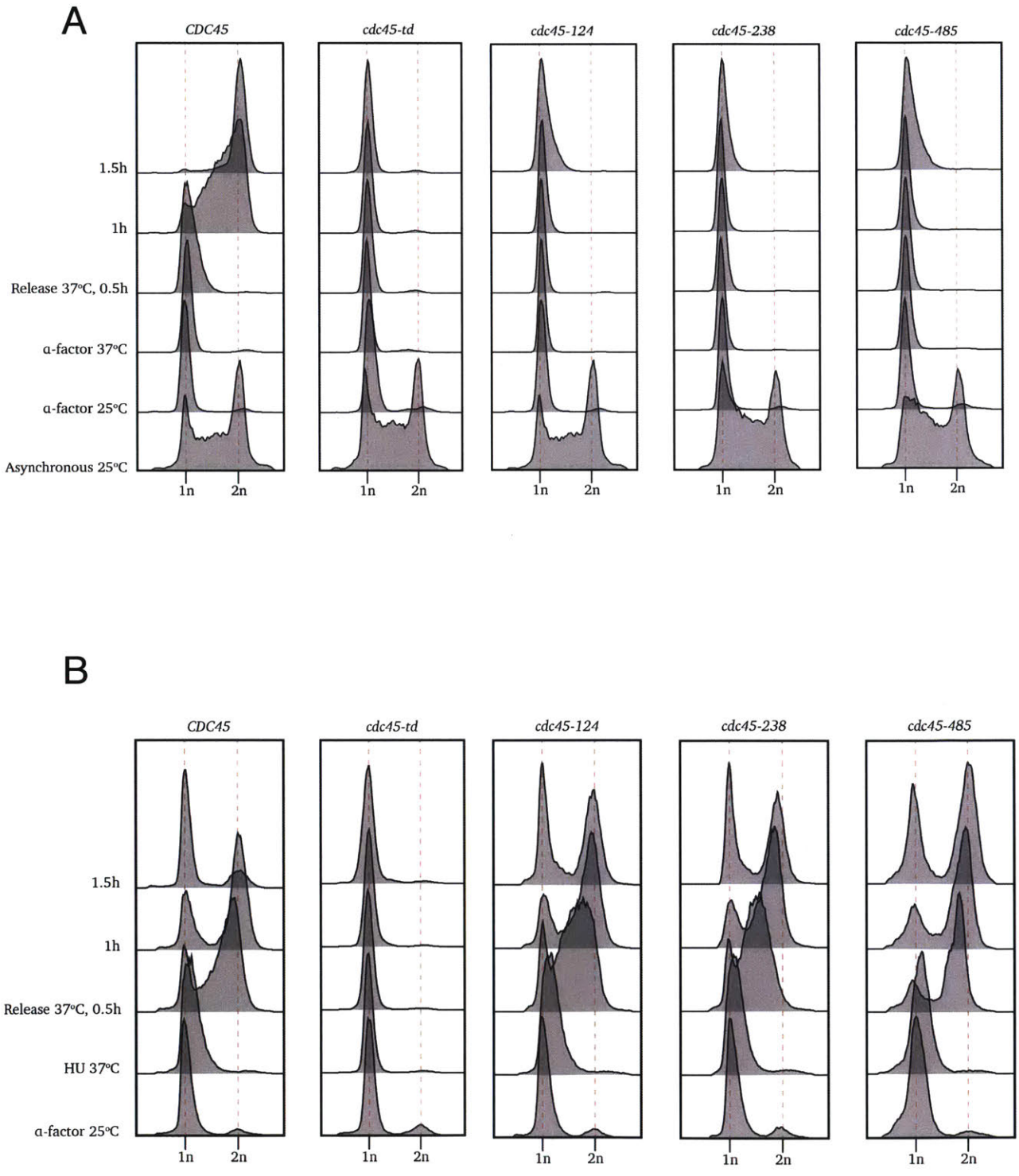


Figure 9. Temperature-sensitive Alleles of *cdc45* Are Defective for DNA Replication Initiation. (A) Asynchronous cells were arrested in G1 using α-factor at 25°C followed by incubation at 37°C and release. Samples were taken at the indicated time points after G1-phase release and analyzed for DNA content using FACS. (B) Cells were arrested in G1 at 25°C for 3 hours and released into YPD medium containing 200mM Hydroxyurea (HU) for 30min. After 30min of HU treatment, cells were shifted to 37°C for an additional 1h followed by release into YPD medium lacking HU. Cells were harvested at indicated times and analyzed for DNA content using FACS.

Temperature-sensitive Cdc45 proteins Are Defective for CMG Formation *in vitro*

To assess the underlying defects of the Cdc45-124, Cdc45-238 and Cdc45-485 mutant proteins, we purified and tested these proteins using a reconstituted DNA replication assay (Figure 10). Briefly, purified helicase-loading and helicase-activation and replication-elongation factors were sequentially incubated with origin-containing DNA attached to beads (Yeeles *et al.*, 2015: Lõoke *et al.*, 2017). Protein complex formation was monitored by assessing the proteins retained on the origin DNA and DNA replication was monitored through incorporation of radiolabeled dCTP.

All three Cdc45 mutants were defective for DNA replication, but the extent of defect varied between the mutants (Figure 11A). The strongest defects were observed for Cdc45-238 and Cdc45-485. In contrast, Cdc45-124 showed an intermediate defect. Cdc45-124 supported 50% of wild-type Cdc45 nucleotide incorporation; Cdc45-238 and Cdc45-485 mutants showed 8% and 20%, respectively (Figure 11B). Importantly, the lengths of the DNA replication products for all of the mutants were similar to those observed for wild-type Cdc45, consistent with these mutants being defective in DNA replication initiation but not elongation.

To further investigate the molecular defects of these mutants, we analyzed the replication proteins that remained associated with the DNA at the end of the replication assay (Figure 12A). It has been previously shown that Cdc45 association with the Mcm2-7 complex is required for stable GINS recruitment (Heller *et al.*, 2011) and helicase activity (Ilves *et al.*, 2010). Thus, it is possible that mutant Cdc45-ts proteins are defective during initial Mcm2-7 association, GINS association or helicase activation.

All three mutant Cdc45-ts proteins were defective for CMG formation. Similar to the extent of replication defects observed, the amount of Cdc45-124 associated with origin-DNA was approximately half of wild-type levels. In contrast, Cdc45-238 and Cdc45-485 showed stronger defects with 20% and 27% of wild-type Cdc45 association, respectively (Figure 12B). We also observed that GINS and Pol ε recruitment were affected by the Cdc45-ts mutants. Similar to the Cdc45 association defects, Cdc45-238 and Cdc45-485 showed more significant defects relative to Cdc45-124.

FIGURE 10. Outline of Reconstituted DNA Replication Assay

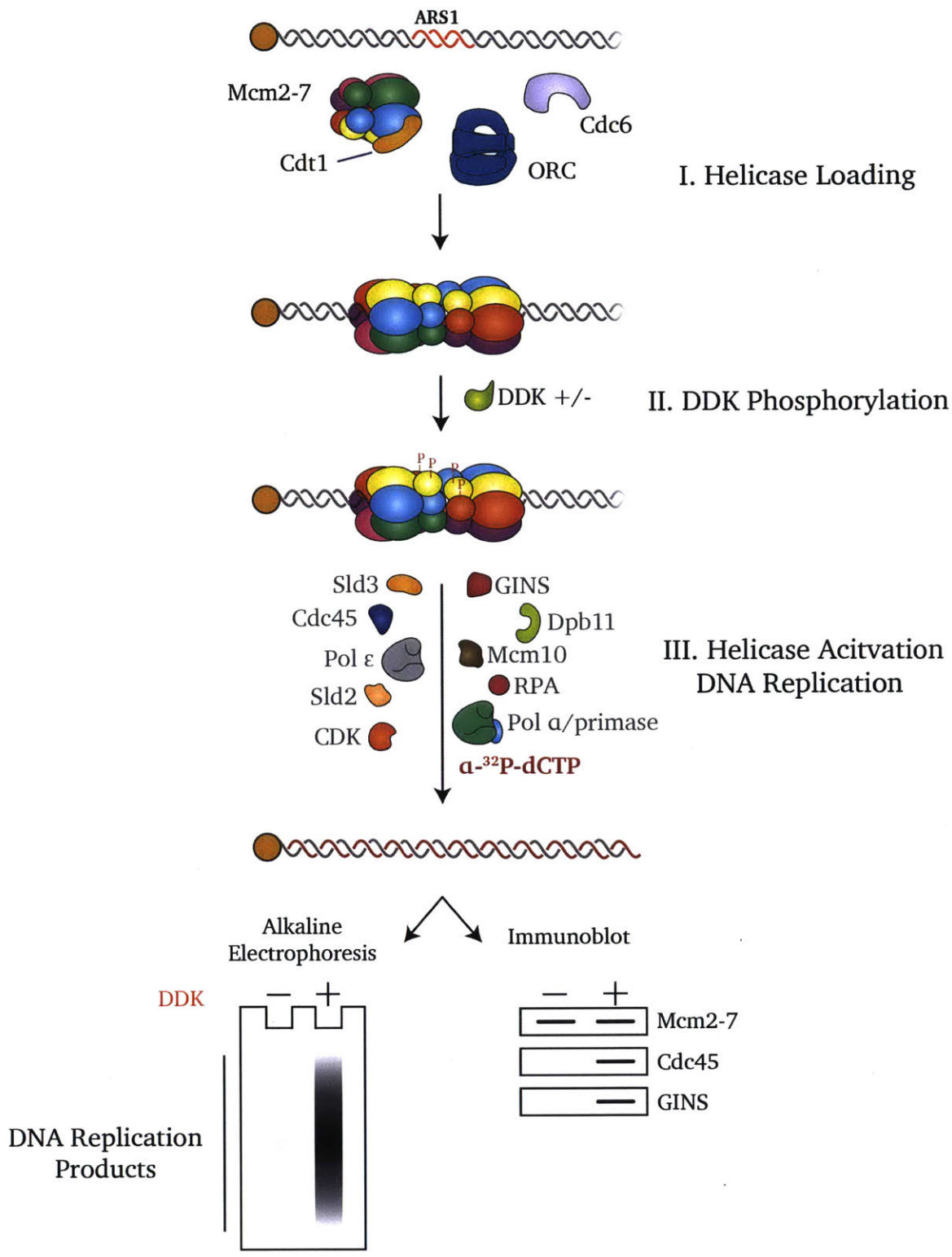
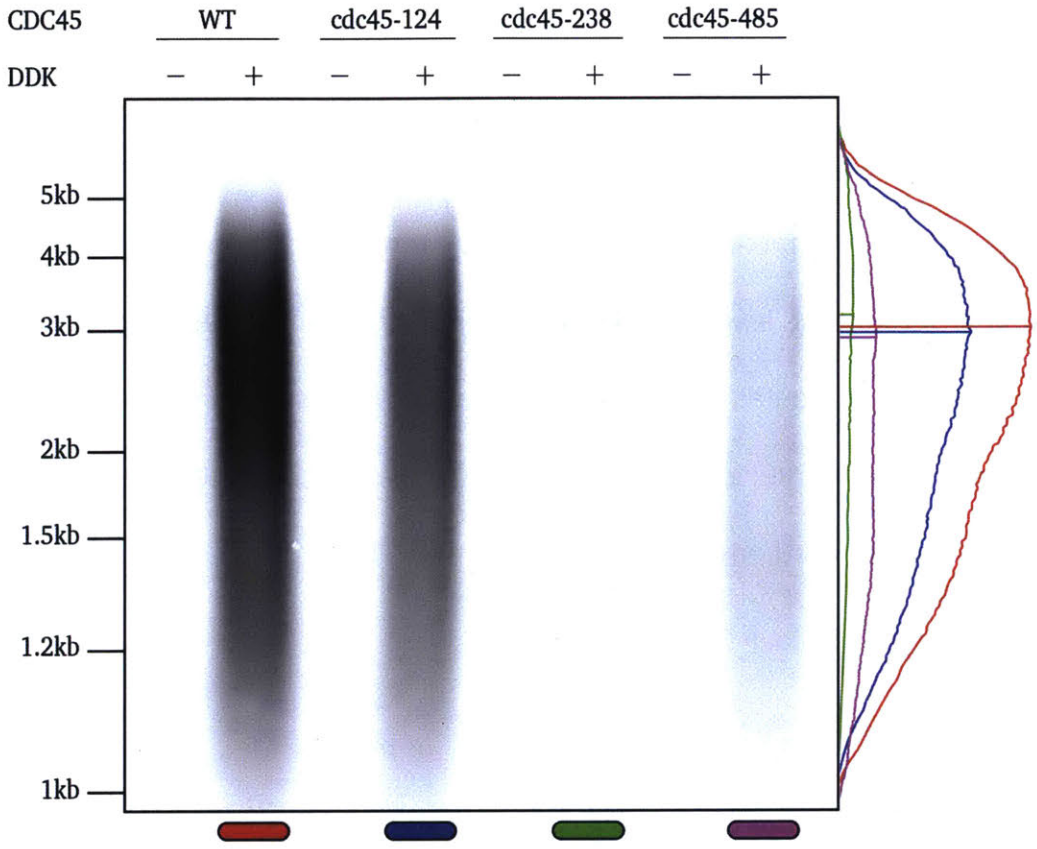


Figure 10. Outline of Reconstituted DNA Replication Assay. (I) Magnetic beads attached to *ARS1*-containing linear DNA are incubated with ORC, Cdc6 and Mcm2-7/Cdt1 to promote helicase loading. (II) Unbound proteins are removed and DNA beads are incubated with Dbf4-Cdc7 kinase to phosphorylate loaded Mcm2-7 helicases. (III) Replication factors are introduced in the presence of nucleotides to promote DNA replication initiation and elongation. Radiolabeled dCTP is used to monitor DNA replication products separated on an alkaline agarose gel. Origin-DNA-bound proteins were detected by immunoblot.

FIGURE 11. Cdc45-ts Mutants Are Defective for DNA Replication

A



DNA Replication

B

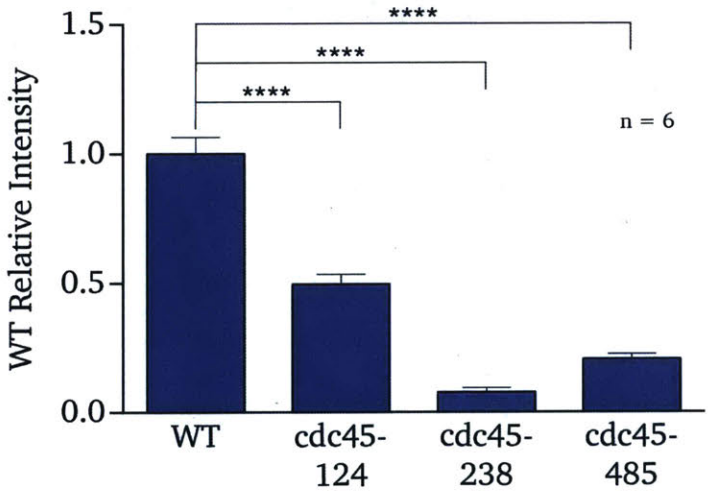
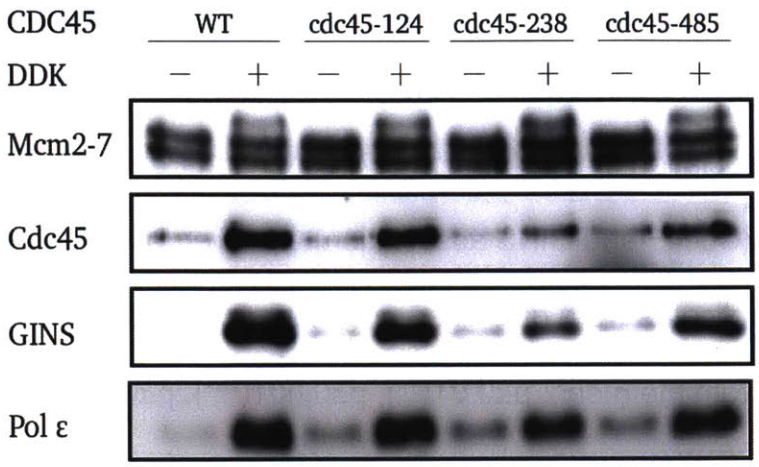


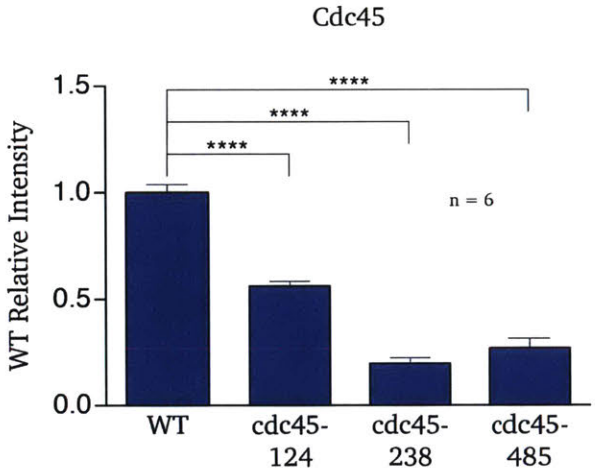
Figure 11. Cdc45-ts Mutants Are Defective for DNA Replication. (A) DNA replication products produced with the indicated Cdc45 proteins were separated on a 0.8% alkaline agarose gel and imaged using a phosphoimager. Relative intensities of +DDK lanes were quantified and plotted using ImageJ (Cdc45 = red, Cdc45-124 = blue, Cdc45-238 = green, Cdc45-485 = purple). Horizontal lines indicate the most highly represented product length for each Cdc45 protein tested (B) Relative levels of DNA replication for the indicated Cdc45 proteins from six experimental replicates of replication assays performed with the indicated Cdc45 mutant proteins were quantified and plotted. Error bars represent standard error from the mean. $p \leq 0.01$(**), $p \leq 0.001$(***), $p \leq 0.0001$(****), not significant (n.s., $p \geq 0.05$).

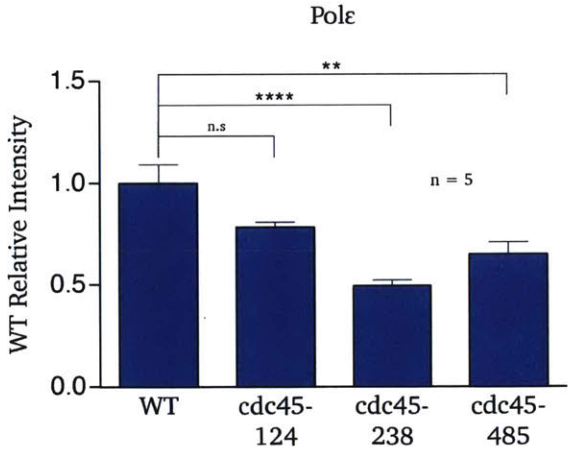
FIGURE 12. Cdc45 Mutants Show Defects in DNA Association

A



B





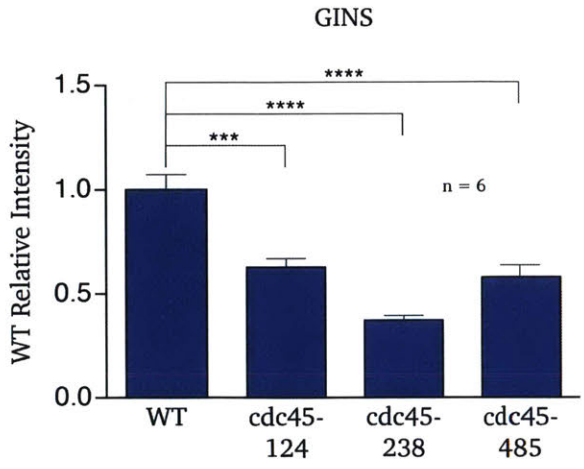


Figure 12. Cdc45 Mutants Show Defects in DNA Association. (A) Proteins associated with the DNA at the end of the replication reaction. Bead-associated proteins were washed with Buffer H+0.3M K-Glut and detected by immunoblot. (B) Relative association of Cdc45, Mcm3-7, GINS and Pol ε with origin DNA after replication. Six (Cdc45 and GINS association) and five (Pol ε association) experimental replicates were quantified and plotted. Error bars represent standard error from the mean. $p \leq 0.01$(**), $p \leq 0.001$(***), $p \leq 0.0001$(****), not significant (n.s., $p \geq 0.05$).

DISCUSSION

Our findings provide insights into the function of Cdc45 during DNA replication. We identified Cdc45 mutants selectively defective for DNA replication initiation but competent for DNA elongation *in vivo*. In addition, we demonstrate that these mutants are defective for DNA replication and CMG formation *in vitro*. Together, these data supports a model in which Cdc45 has different roles during DNA replication initiation and elongation. Another possibility is that Cdc45 has one function with different requirements during DNA replication initiation and elongation. We also found two lethal *cdc45* mutants in the IDR of Cdc45 despite this region been dispensable for Cdc45 function.

We designed and tested nineteen Cdc45 mutants based on sequence conservation and stretches of charged residues. Four of these mutants, *cdc45-35*, *cdc45-66*, *cdc45-171*, *cdc45-199* are lethal. The Cdc45-35 mutant is located at the core of Cdc45 structure. It is likely that this mutation causes protein misfolding leading to a nonfunctional copy of Cdc45 or its degradation. The residues mutated in Cdc45-66 are located at the surface of Cdc45 and do not interact with GINS or the Mcm2-7 ring. It is possible that this region has unknown interactions with other replication factors. It is also possible that structural changes in this region can cause a distant conformational change critical for Cdc45 function. Finally, Cdc45-66 mutations might also lead to protein

misfolding. We were unable to purify these two mutants suggesting they are compromised for protein folding.

Two other lethal mutants, *cdc45-171* and *cdc45-199* are located in an intrinsic disordered region (IDR) within Cdc45. A similarly sized and located IDR is a found in Cdc45 from many other organisms, suggesting that the presence of such a disordered region is a conserved feature of this protein but these regions are not obviously related in sequence. Strikingly, deletion of the IDR is dispensable for viability whereas these two mutations within the IDR are lethal. Although this region is not visible in CMG structures, *in vitro* crosslinking studies suggest that it interacts with the catalytic and largest subunit of DNA Pol ε (called Pol2, Sun *et al.*, 2015; Yuan *et al.*, 2016). Since Pol2 also interacts with Mcm2 and Mcm6, we propose that the IDR-Pol2 interactions are dispensable for Polε association. Why then are mutations in the IDR lethal? One interesting possibility is that *cdc45-171* and *cdc45-199* mutations cause a conformational changes in the IDR that block the association of Pol2 with the CMG leading to lethality. I have purified these two mutant proteins and I am currently testing the underlying defects of all these mutants *in vitro* using the DNA replication assay.

We found three temperature-sensitive mutants, *cdc45-124*, *cdc45-238* and *cdc45-485,* that are lethal when grow at 37°C. We find that *cdc45-124*, *cdc45-238* and *cdc45-485* are defective for DNA replication initiation both *in vivo* and *in*

vitro. All three mutants arrest in G1-phase when shifted to the restrictive temperature and subsequently undergo reductional anaphase. When cells pass through the G1/S transition without any replication initiation they undergo reductional anaphase, a process during which unreplicated chromosomes are randomly segregated into the daughter cells. This event results in non-viable cells with sub-G1 DNA content (Piatti *et al.*, 1995; Tercero *et al.*, 2000). This phenotype is also seen when Cdc45 is degraded in G1-arrested cells and allowed to enter S phase (Tercero *et al.*, 2000). In contrast, arresting *cdc45-124, cdc45-238* and *cdc45-485* cells in G1-phase followed by incubation at the restrictive temperature and release retained cells with G1 DNA content without undergoing reductional anaphase. Because Cdc45 is recruited to early-firing origins in late G1-phase (Aparicio *et al.*, 1999), it is possible that Cdc45-124, Cdc45-238 and Cdc45-485 molecules associated with early origins in during a G1 arrest are (partially or fully) protected from inactivation at the restrictive temperature. Once released at the restrictive temperature, these Cdc45 molecules could stimulate a low level of replication initiation that prevents reductional anaphase. Just a single helicase activation event is thought to be sufficient to activate DNA replication checkpoints that inhibit mitosis and prevent reductional anaphase. We could test this hypothesis by analyzing if the DNA damage checkpoint is active and by measuring newly incorporated DNA.

The Cdc45-ts mutants are located within distinct Cdc45 domains. The Cdc45-124 mutation (DAH->AAA) is located in the RecJ-like domain found in the N-terminal region of Cdc45. Cdc45-124 alters the Cdc45 residues that are located in the position of the DHH catalytic triad observed in RecJ. Although Cdc45 has no nuclease or ssDNA-binding activity like RecJ, the integrity of this domain might be structurally relevant for Cdc45 function during DNA replication initiation. The residues altered in Cdc45-238 (EEY->AAA) are located at the end of an α-helix connecting to the IDR domain in Cdc45. The IDR domain is not visible in the structure and it is thought to interact with Pol ε. Since Pol ε association is required for CMG formation and activation (Sengupta *et al.*, 2013), it is possible that Cdc45-238 initiation defects are due to defective Pol ε association with the IDR. Although we did not observe such a defect in our *in vitro* origin association assays (Fig. 11) *In vitro,* it is possible that there is an additional interaction between Cdc45 and Pol ε that occurs after initial Pol ε association that is required for CMG activation. Finally, the residues altered in Cdc45-485 (DDRK->AAAA) are located at the surface of Cdc45 facing GINS. In the available CMG structures, this region does not interact with GINS, Mcm2-7 or other regions in Cdc45. However, it is possible that this region is required during CMG formation or activation intermediates.

Each of the Cdc45-ts mutants are competent for DNA elongation. Unlike a mutant that leads to complete degradation of Cdc45 (Tercero *et al.*, 2000),

replication forks containing *cdc45-124, cdc45-238* and *cdc45-485* mutants each resume DNA replication after incubation at the restrictive temperature. Consistent with these mutants being specifically defective in replication initiation, the length of DNA replication products produced by reactions containing Cdc45-124, Cdc45-238 and Cdc45-485 were similar to wild type Cdc45. Taken together, these results suggest that these mutants are functional in the context of the active GMG helicase at replication forks but are defective during DNA replication initiation.

Multiple lines of evidence indicate that the Cdc45-ts mutants are defective in replication initiation. Each of the mutant proteins are defective for origin DNA association *in vitro*, an event that is required for both the formation and initial DNA unwinding by the CMG complex. We also found that downstream recruitment of GINS and Pol ε was impaired. There are at least three possible steps that could be defective in Cdc45-124, Cdc45-238 and Cdc45-485 mutants. One possibility is that they are defective for initial association with loaded Mcm2-7 helicases. Second, they are competent for association but are defective for CMG formation and this leads to reduced stability of Cdc45 association. Third, it is possible that they can form a CMG but the resulting complex becomes unstable upon helicase activation. This third possibility is less likely since this effect would most likely affect elongation.

Our findings suggest that Cdc45 function during helicase activation is different than its role during DNA elongation. There are two possible explanations for this apparent separation of function. One possibility is that Cdc45 performs at least one distinct function during replication initiation that is not required during replication elongation. In this case, the Cdc45-ts mutants would each have targeted a region of Cdc45 involved in this function. Alternatively, it is possible that the Cdc45-ts can only be inactivated if they are free in solution. This type of defect could explain the lack of elongation defects since the mutant Cdc45 proteins would have already been incorporated into CMG complexes at replication forks in the experiments testing for elongation. Finally, it is also possible that the Cdc45-ts mutants are temperature sensitive for synthesis. Again, the proteins already incorporated into the CMG in the elongation experiments would be resistant to inactivation in this case. We could test these hypotheses by arresting cells in HU and degrading cdc45-ts proteins using a temperature-sensitive degron to halt DNA replication. We could then induce cdc45-ts (without degron) and analyze if DNA replication is restarted.

These are the first mutants shown to separate Cdc45 function in DNA replication but we have yet to identify any mutants defective in DNA replication elongation. However, we cannot rule out the possibility of finding other mutants selectively defective for DNA replication elongation. Degradation of Cdc45 during DNA elongation halts DNA replication and stops fork movement. Since Cdc45

and GINS stimulate Mcm2-7 ATPase activity, it is possible that Cdc45 mutants defective in Mcm2-7 ATPase stimulation but not CMG formation would be defective for DNA replication elongation.

Figure 13. Structural Position of Cdc45 Mutants

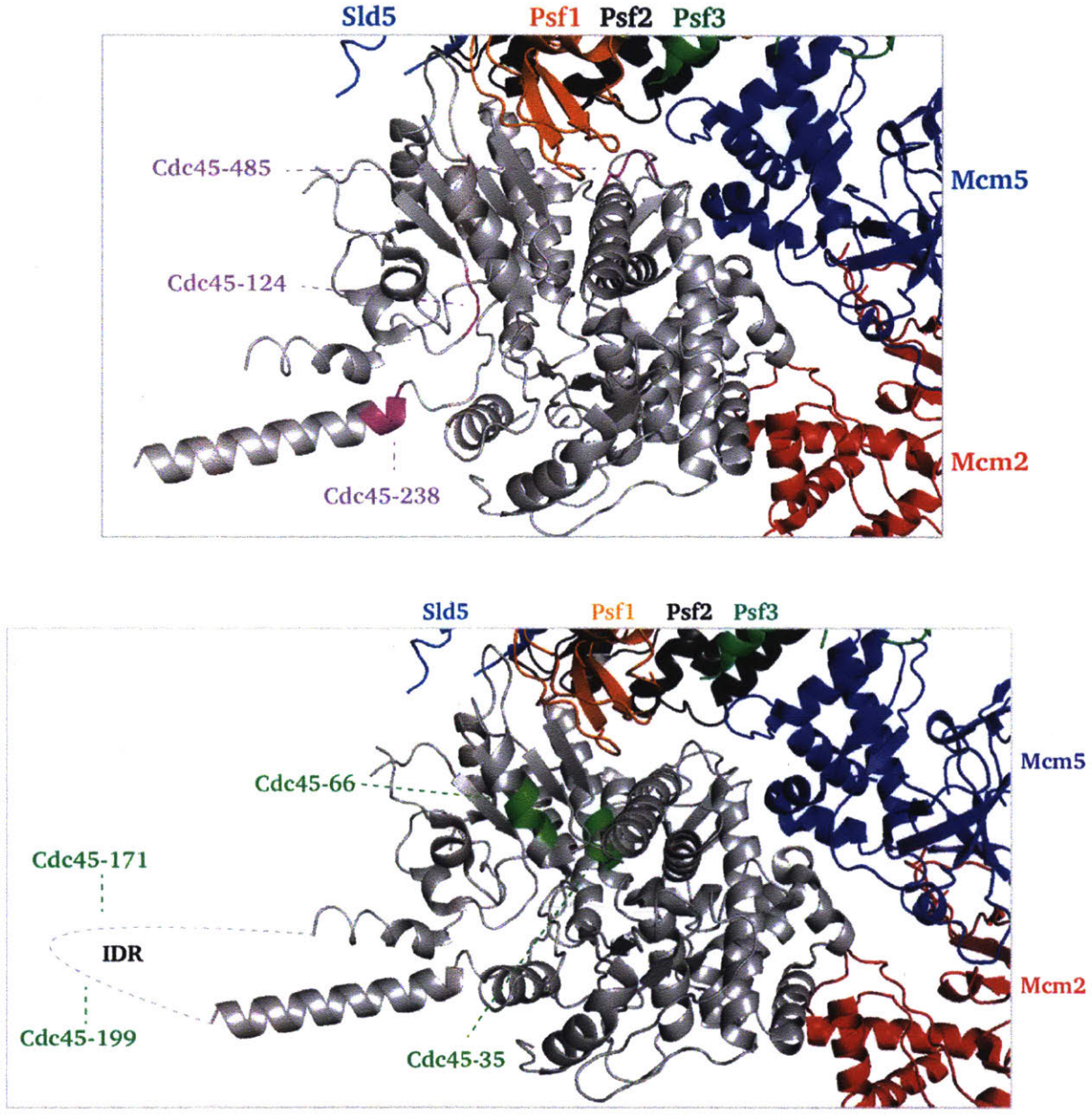


Figure 13. Structural Position of Cdc45 Mutants. *Top* Cdc45 temperature-sensitive mutants location relative to the CMG. Cdc45-ts mutants are labeled in purple. *Bottom* Cdc45 lethal mutations are shown in green. The IDR region is not visible in the CMG structure and is shown as a dashed line. This CMG structure was adapted from Yuan *et al.*, 2016.

EXPERIMENTAL PROCEDURES

Yeast Strains and Mutant Construction

All nineteen mutants were constructed by site directed PCR mutagenesis. All mutagenized sequences were sequenced to confirm that only the desired mutation was made. Mutagenesis was performed using pYR01 as template (for yeast integration). Plasmid vectors for Cdc45 protein purification were also constructed by site directed PCR mutagenesis of pMM33. All *Saccharomyces cerevisiae* strains were congenic with W303 (*ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-1 can1-100*) and are summarized in Table 1. We tested the viability of these mutants using a Cdc45 'swapper' strain in which the chromosomal copy of *CDC45* was deleted and a wild-type copy maintained in vector harboring a *URA3* marker. Each mutant gene was integrated into the swapper strain and tested for viability on media that selected against the presence of the plasmid containing the wild-type gene (using 5-Fluorootic acid, 5-FOA).

Flow Cytometry

Cell cultures were inoculated in YPD medium and grown overnight at 25°C. Cultures were then diluted to OD_{600} 0.05 and grown for 3h at 25°C. Cells were arrested with α-factor (1μg/mL) for 3h at 25°C. Cells were release from the α-factor arrest by washing twice with fresh YPD+50μg/mL pronase(EMD Millipore) medium. For experiments in Figure 8A, cells were shifted to 37°C for 1h

after the initial α-factor arrest at 25°C. Cells were then released into pre-warmed YPD+50µg/mL pronase medium and time points taken. For experiments in Figure 8B, cells were released from the α-factor arrest into YPD+50µg/mL pronase+200mM hydroxyurea(Acros Organics) medium for 30min at 25°C. Cells were shifted to 37°C for 1h in YPD+200mM hydroxyurea medium. Cells were released from hydroxyurea by washing three times with fresh pre-warmed YPD medium. Cells were then inoculated in fresh pre-warmed YPD medium and time points taken. Samples (1mL) were centrifuged, supernatant removed and resuspended 1mL of 70% ethanol. Cells were centrifuged a second time and resuspended in 500µL of 50mM NaCitrate+RNAseA (0.02mg/mL final concentration) and incubate for 2h at 50°C. Proteinase K was added to 0.8mg/mL final concentration and incubate for 2h at 50°C. Samples were sonicated (20% amplitude, 2s pulses [5x]), DNA labeled with Syntox green and analyzed using FACS.

Protein Purification

ORC, Cdc6, Mcm2-7/Cdt1 were purified as previously described (Kang *et al.*, 2014). DDK, Dpb11, Pol ε, S-CDK, Mcm10 and Pol α-primase were purified as previously described (Lõoke *et al.*, 2017). RPA was purified from yeast as described in Yeeles *et al.*, 2015.

Yeast Cell Growth and Lysis

All yeast strains were grown in selective medium before being inoculated into 8L (12L for Sld2) of YEP+2% glycerol at 30°C. Cells were grown to an OD_{600}~1 before induction with galactose (2% final concentration). After 4-6 h, the cells were harvested and washed with 250 mL of chilled water+1mM PMSF. The cells were then resuspended in approximately half-packed cell volume of the indicated lysis buffer containing a Roche® protease inhibitor cocktail and frozen drop-wise into liquid nitrogen. The frozen cells were lysed using a SPEX SamplePrep freezer/mill. Lysed cell powder was transferred to ultracentrifugation tubes and thawed on ice. The lysate was cleared by centrifugation in a Beckman ultracentrifuge at 160,000g for 1h. All purification steps were done at 4°C.

Flag Affinity Purification

Cleared cell lysates were incubated with the indicated amount of packed anti-Flag M2 affinity gel (Sigma) for 2h at 4°C. Bound proteins were washed with the indicated buffers and eluted with the indicated buffer+0.2mg/mL 3xFlag peptide (MDYKDHDGDYKDHDIDYKDDDDK; Koch Institute Swanson Biotechnology Center). The first eluate was collected by flowing 1CV (column volume) of elution buffer over resin. Four additional eluates were collected after a 30min incubation with the elution buffer.

Buffers

The following buffers were used for protein purification: buffer H (50 mM HEPES-KOH at pH 7.6, 1 mM EDTA, 1 mM EGTA, 5 mM MgOAc, 10% glycerol), buffer S (buffer H, 0.3 M KCl, 0.02% NP-40), buffer D (buffer H, 0.02% NP-40, 0.3 M KOAc), and buffer R (25 mM TrisCl at pH 7.2, 10% glycerol, 1 mM DTT).

Cdc45

Cdc45-3xFlag was overexpressed from yMM33. Purification of Cdc45 was based on a previously published protocol (Yeeles et al. 2015) with the following modifications. Cells were resuspended in buffer H+500mM KGlut, 1M sorbitol, 3 mM ATP, Roche® protease inhibitor cocktail and 1mM PMSF. After lysis, the lysate was incubated with 1.5 mL of anti-Flag M2 affinity gel equilibrated with buffer H+500 mM KGlut, 2 mM ATP. The resin was washed with 20 CV of buffer H, 500 mM KGlut, 2 mM ATP, 1mM PMSF followed by 10 CV of 20 mM potassium phosphate buffer (pH 7.4), 150 mM KOAc, and 10% glycerol (Buffer C). Cdc45 was eluted in buffer C+ 3xFlag peptide. Fractions were pooled and incubated with 1.5mL hydroxyapatite column previously equilibrated with buffer C. Protein was washed with 80 mM potassium phosphate buffer (pH 7.4), 150 mM KOAc, and 10% glycerol and eluted with 300 mM potassium phosphate buffer (pH 7.4), 150 mM KOAc, and 10% glycerol. Cdc45 fractions were pooled and dialyzed against buffer H + 0.3 M KGlut.

GINS

Sld5, Psf1, Psf3, and Psf2-3C-6xHis-Flag were overexpressed from ySK136. Cells were resuspended in buffer H+500mM KCl, 1M sorbitol, 0.02% NP-40, 2 mM ATP, Roche® protease inhibitor cocktail and 1mM PMSF. After lysis, the cleared lysate was diluted to 0.3 M KCl with buffer H+protease inhibitors. The lysate was then incubated with 1.5 mL of anti-Flag M2 affinity gel previously equilibrated with buffer S. The resin was washed with 20 CV of buffer S followed by 10 CV of buffer H, 0.02% NP-40, and 0.1 M KCl. GINS was eluted in the previous buffer + 3xFlag peptide. GINS- containing fractions were pooled and the flag tag on Psf2 was removed with an overnight incubation (16 h) with HRV 3C protease. GINS was flowed over 1mL Complete His-tag resin to remove uncut GINS and HRV 3C protease before applying the flow-through to a 1mL HiTrap Q HP column (GE healthcare). GINS was eluted with a 20 CV gradient of 0.1-1M KCl in buffer H + 0.02% NP-40. The peak fractions were dialyzed against buffer H + 300mM potassium KOAc, 0.02% NP-40.

Sld3/7

Sld3-3xFlag and Sld7-VSV-G were overexpressed from ySK123. Sld3 was expressed with a deletion of residues 1–104 to remove a putative destruction box. Cells were resuspended in buffer H+800mM KCl, 1M sorbitol, 0.02% NP-40, 2mM ATP, Roche® protease inhibitor cocktail and 1mM PMSF. After cell lysis, the cleared lysate was diluted to 0.3 M KCl with buffer H+protease inhibitors. The

diluted lysate was incubated with 1.5 mL of anti-Flag M2 affinity gel equilibrated with buffer H+300mM KCl. The resin was washed with 30 CV of buffer H+300mM KCl and eluted in buffer H+300mM KCl+3xFlag peptide. Sld3/Sld7-containing fractions were diluted to 0.2 M KCl with buffer H and applied to a 1mL HiTrap SP HP column (GE Healthcare). The column was washed with buffer H+300mM KCl, 0.02% NP-40 and eluted with buffer H+660mM KCl, 0.02% NP-40. Sld3/7 fractions were pooled and dialysed against H+300mM KCl, 0.02% NP-40.

Sld2

3xFlag-3C-6xGly-Sld2 was overexpressed from ySK127. Cells were resuspended in buffer H+800mM KCl, 1M sorbitol, 0.02% NP-40, 2 mM ATP, Roche® protease inhibitor cocktail and 1mM PMSF. After cell lysis, the cleared lysate was dialyzed overnight (16 h) in buffer H+300mM KCl, 3 mM ATP and 1mM PMSF. The lysate was cleared by spinning at 11,000rpm for 20 min. The lysate was incubated with 1.5 mL of anti-Flag M2 affinity gel equilibrated with buffer H+300mM KCl, 1mM ATP. The resin was washed with 30 CV of the same and eluted in buffer H+300mM KCl+3xFlag peptide. Sld2-containing fractions were diluted to 0.2M KCl with buffer H and applied to a 1mL HiTrap SP HP column. Sld2 was eluted with a 15 CV gradient of 0.2-1M KCl in buffer H, 0.02% NP-40, and 1mM ATP.

Reconstituted DNA Replication Assay

The DNA plasmid template p*ARS1*-Nco (7.6kB) was linearized, biotinylated and coupled to streptavidin-coated magnetic beads as described previously (Heller *et al.*, 2011). Each incubation step was performed in a thermomixer (Eppendorf) with shaking at 1250rpm at 25°C. A DynaMag-2 magnet (ThermoFisher Scientific) was used to magnetize DNA beads and remove the reaction mix after each step. Mcm2-7 loading was performed by incubating 2.0pmol of ORC, 4.0pmol of Cdc6 and 6.0pmol of Mcm2-7/Cdt1 with 0.12pmol of p*ARS1*-Nco in 25mM HEPES (pH7.6), 10mM MgOAc, 1mM DTT, 300mM KGlut, 20mM creatine phosphate, 5mM ATP, 10%glycerol, 0.02% NP-40 and 0.2µg of creatine kinase. Loading was performed in a 10µL reaction for 25min. The reaction mix was removed and DDK phosphorylation was performed in a 10µL reaction containing 50mM HEPES (pH7.6), 3.5mM MgOAc, 1mM DTT, 225mM KGlut, 1mM ATP, 10%glycerol, 0.02% NP-40 and 1.0pmol of DDK for 25min and the DDK reaction mix was removed.

DNA replication was initiated by adding the following amount of proteins: 0.7pmol CDK, 1.9pmol Sld2, 1.0pmol Dpb11, 1.25pmol Pol α-primase, 1.6pmol Pol ε, 2.0pmol RPA, 2.5pmol GINS, 0.15pmol Mcm10, 1.0pmol Sld3/7, 2.6pmol Cdc45, 0.2mM rNTP, 0.04mM dNTP and 10µCi [α-^{32}P]dCTP. This proteins were added to a 30µL reaction containing 25mM HEPES (pH7.6), 12mM MgOAc, 1mM DTT, 300mM KGlut, 20mM creatine phosphate, 6mM ATP, 10%glycerol, 0.02% NP-40 and 0.3µg of creatine kinase. DNA replication reactions were

performed for 1h. Reactions were washed with buffer H+300mM KGlut, 0.5%NP-40. DNA was released from beads by boiling in SDS-PAGE sample buffer and products were separated in a 0.8% alkaline agarose gel and imaged using a phosphor screen. For immunoblot analyses, proteins were released from the DNA by incubating with 5U of DNase (Worthington) in 15 µL of buffer H+150 mM KGlut and0.01% NP-40 for 30 min at 25°C before immunoblotting. The following antibodies were used for immunoblotting: α-Mcm2-7 (UM174), α-Cdc45 (HM135), α-GINS (HM128), Pol ε (HM7602).

Table I. Strains Used in This Study

Strain Name	Genotype
yBC093	MATα *cdc45*::KanMX [pRS416-*CDC45*::*URA3*
yRR01	MATa *cdc45*::KanMX *LEU2*::pRR01-*CDC45*::*URA3*
yRR02	MATα *cdc45*::KanMX *LEU2*::pRR01-*cdc45-35*::*URA3*
yRR03	MATα *cdc45*::KanMX *LEU2*::pRR01-*cdc45-40*::*URA3*
yRR04	MATα *cdc45*::KanMX *LEU2*::pRR01-*cdc45-66*::*URA3*
yRR05	MATα *cdc45*::KanMX *LEU2*::pRR01-*cdc45-124*::*URA3*
yRR06	MATα *cdc45*::KanMX *LEU2*::pRR01-*cdc45-154*::*URA3*
yRR07	MATα *cdc45*::KanMX *LEU2*::pRR01-*cdc45-171*::*URA3*
yRR08	MATα *cdc45*::KanMX *LEU2*::pRR01-*cdc45-190*::*URA3*
yRR09	MATα *cdc45*::KanMX *LEU2*::pRR01-*cdc45-199*::*URA3*
yRR10	MATα *cdc45*::KanMX *LEU2*::pRR01-*cdc45-238*::*URA3*
yRR11	MATα *cdc45*::KanMX *LEU2*::pRR01-*cdc45-297*::*URA3*
yRR12	MATα *cdc45*::KanMX *LEU2*::pRR01-*cdc45-314*::*URA3*
yRR13	MATα *cdc45*::KanMX *LEU2*::pRR01-*cdc45-336*::*URA3*
yRR14	MATα *cdc45*::KanMX *LEU2*::pRR01-*cdc45-457*::*URA3*
yRR15	MATα *cdc45*::KanMX *LEU2*::pRR01-*cd45-470*::*URA3*
yRR16	MATα *cdc45*::KanMX *LEU2*::pRR01-*cdc45-485*::*URA3*
yRR17	MATα *cdc45*::KanMX *LEU2*::pRR01-*cdc45-515*::*URA3*
yRR18	MATα *cdc45*::KanMX *LEU2*::pRR01-*cdc45-535*::*URA3*
yJT18	MATa *cdc45*::*cdc45-td*::*TRP1 ubr1*::*GAL-UBR1*::*HIS3*
yRR19	MATa *cdc45*::*cdc45-124 bar1*::*TRP1*
yRR20	MATa *cdc45*::*cdc45-238 bar1*::*TRP1*
yRR21	MATa *cdc45*::*cdc45-485 bar1*::*TRP1*
yRR22	MATα *cdc45*::KanMX *LEU2*::pRR01-*cdc45Δ169-209*::*URA3*
yRR23	MATa *pep4*::unmarked *LEU2*::*GAL-cdc45-124-3xFlag*
yRR24	MATa *pep4*::unmarked *LEU2*::*GAL-cdc45-238-3xFlag*
yRR25	MATa *pep4*::unmarked *LEU2*::*GAL-cdc45-485-3xFlag*
yMM016	MATa *pep4*::unmarked *LEU2*::*GAL-CDC45-3xFlag*
ySK136	MATa *pep4*::unmarked *bar1*::*hisG URA3*::*pGAL-SLD5 LEU2*::*pGAL-PSF2-3C-His-FLAG LYS2*::*pGAL-PSF1+PSF3*
ySK123	MATa *pep4*::unmarked *bar1*::*hisG LEU2*::*pGAL1-Δ1-104-SLD3-3xFLAG HIS3*::*pGAL-SLD7-VSV-G*
ySK127	MATa *pep4*::unmarked *bar1*::*hisG LEU2*::*pGAL-3xFLAG-3C-SLD2*

CHAPTER III:

Discussion and Future Directions

Key Conclusions

Our studies suggest that Cdc45 has two separate functions during DNA replication or one function with different requirements for initiation and elongation. We showed that *cdc45-ts* mutants are defective for DNA replication initiation but are competent for DNA elongation. When grown at the restrictive temperature, *cdc45-ts* mutants accumulate cells with G1 DNA and subsequently undergo reductional anaphase indicating that these mutants are defective in DNA replication initiation. Consistent with this result, *cdc45-ts* mutants are defective for CMG formation *in vitro*. Inactivation of Cdc45-ts mutants during S-phase did not compromise its function in the context of the CMG. Consistent with this finding, the length of DNA replication products made with Cdc45-ts mutants were similar to that of wild-type Cdc45. We also found two lethal mutants in the IDR of Cdc45. These mutations are particularly interesting since we also observe that the IDR region is dispensable for cell viability.

Cdc45 Function During DNA Replication

Cdc45 is essential for both DNA replication initiation and elongation (Tercero *et al.*, 2000). Together with GINS, Cdc45 associate with the Mcm2-7 hexamer to form the active CMG helicase (Ilves *et al.*, 2010). It is not clear whether Cdc45 function during helicase activation is the same as its function in the context of the CMG at replication forks. In this study, we designed and tested nineteen mutations in *CDC45*. We found four lethal and three temperature-sensitive mutations. *In vitro* and *in vivo* analyses revealed that temperature-sensitive mutants *cdc45-124*, *cdc45-238* and *cdc45-485* are defective in DNA replication initiation but not elongation. This result supports the hypothesis that Cdc45 function during DNA replication initiation is different that its role during DNA elongation. Another possibility is that Cdc45 has one function that is required at different levels during DNA replication initiation and elongation. In support of this model, we did not find any mutants only defective for DNA elongation and the three Cdc45-ts alleles are located at very different regions of the protein. However, our mutational analysis was not so comprehensive that we could eliminate the possibility that elongation mutants might exist.

It is clear that Cdc45, together with GINS, activates the Mcm2-7 helicase (Ilves *et al.*, 2010). Conditional degradation of Cdc45 during DNA elongation halts DNA replication suggesting that Cdc45 is required for Mcm2-7 helicase

activaty (Tercero *et al.*, 2000). However, these studies did not address if Cdc45 degradation also removes GINS from the CMG leading to helicase inactivation. Interestingly, DNA replication can be resumed after *de novo* synthesis of Cdc45 but is not know whether replication forks that were inactivated by Cdc45 degradation can successfully restart upon Cdc45 expression or DNA synthesis was carried out by new origin-firing events. DNA sequencing could be used to analyze DNA content and determine if new origins are fired after Cdc45 degradation and new synthesis. Future experiments should also address whether conditional degradation of GINS, in the context of active CMG at replication forks, halts DNA replication similar to the effects observed by Cdc45 degradation (Tercero *et al.*, 2000).

The current model in the field suggests that Cdc45 and GINS activate the Mcm2-7 helicase by remodeling the Mcm2-7 ring to optimize ATPase and helicase activity (Costa *et al.*, 2011; Yuan *et al.*, 2016). In the context of the active CMG, two ATP-binding sites (located at the C-terminal domains) formed by Mcm2-5 and Mcm5-3 are particularly important for CMG helicase activity (Ilves *et al.*, 2010). Mutating the adjacent ATP-binding sites (Mcm6-2 and Mcm3-7 interfaces) showed weaker defects in helicase activity. Interestingly, the two critical ATP-binding sites are located at the hinge point between two CMG conformations (Yuan *et al.*, 2016). Similarly, Cdc45 and GINS bind to the N-terminal interfaces of Mcm2-5 and Mcm5-3, respectively. This architecture

positions Cdc45 and GINS close but not in direct contact with the Mcm2-5 and Mcm5-3 ATP-binding sites. Together these observations suggest that Cdc45 and GINS association with the Mcm2-7 ring remodel the Mcm2-5 and Mcm5-3 interfaces to stimulate ATPase and helicase activity. This leads to one important question, do both Cdc45 and GINS cooperate to activate the Mcm-7 helicase or is only one factor is sufficient?

Cdc45 is required for GINS recruitment to the Mcm2-7 complex (Heller *et al.*, 2011). One possibility is that Cdc45 interactions with GINS facilitate GINS association with the Mcm2-7 ring. Consistent with this model, Cdc45 directly interacts with all four GINS subunits (Yuan *et al.*, 2016). However, GINS also directly interacts with Mcm5 and Mcm3. This suggests that another possible function of Cdc45 is to expose GINS binding sites in the Mcm2-7 complex and/or to stabilize GINS interactions with the Mcm2-7. ATP-binding mutants in the Mcm2-6 interface prevents GINS, but not Cdc45 binding to the Mcm2-7 complex (Kang *et al.*, 2014). This observation suggests that state of ATP binding and hydrolysis of the Mcm2-7 ring can influence GINS binding. I propose that Cdc45 binding to the Mcm2-7 induces a conformational change in the Mcm2-7 ring that modifies the ATPase activity of the Mcm2-6. This change in ATPase activity creates or stabilizes GINS binding motifs on Mcm2-7. Testing this hypothesis would require Mcm2-7 ATPase or other mutants that selectively bind GINS but not Cdc45. It would be challenging to test this hypothesis since ATPase mutants

are generally defective for helicase loading and GINS does not form a complex the Mcm2-7 in the absence of Cdc45. One alternative would be to compare the current CMG structure with a structure of the Cdc45-Mcm2-7 intermediate. I would be also interesting to test if the Cdc45-ts mutants described in this thesis are defective in modulating the Mcm2-7 ring during GINS recruitment.

Cdc45 function during DNA replication elongation is less clear. It is possible that the only role of Cdc45 during this process is to maintain, together with GINS, the active conformation of the Mcm2-7 helicase. Other studies suggest that Cdc45 also helps to connect Pol ε with the helicase (Sun *et al.*, 2015; Yuan *et al.*, 2016). An intrinsic disordered region (IDR) in Cdc45 mediates this interaction. My studies have shown that this interaction is dispensable for cell viability most likely due to redundant interactions between Pol ε and the Mcm2-7 ring. However, mutating two regions of this IDR region in *CDC45* results in cell lethality. I hypothesize that these Cdc45 mutants block Pol ε association with the replisome. We could test this by testing Cdc45 lethal mutants in the reconstituted replication assay and analyze Pol ε association to the DNA.

Who Is the True Helicase Activator?

Cdc45 and GINS are sufficient to enhance Mcm2-7 ATPase and helicase activity in vitro (Ilves *et al.*, 2010). However, these experiments are in the context

of already formed single CMG complexes. We extrapolated these results and assumed for many years that Mcm2-7 helicase activation was concomitant with CMG formation during DNA replication initiation in the context of double hexamers encircling dsDNA. Recent evidence suggests that this model is not correct. Mcm10, another protein essential for DNA replication initiation, is required for initial DNA unwinding and possibly involved in double hexamer splitting (Kanke *et al.*, 2012; Quan *et al.*, 2015; Lõoke *et al.*, 2017). This leads to one question: Is Mcm10 the final helicase activator?

It is clear that CMG formation does not translate to active DNA unwinding CMG complexes. Mcm10 is required to trigger this process. However, it is possible that CMG formation does promote Mcm2-7 ATPase activity but are not competent for DNA unwinding because some other event required for this activity has yet to happen. For example, either separation of the Mcm2-7 helicases in the initially loaded double hexamer or extrusion of one of two strands such that the CMG encircles ssDNA could be required for helicase activation. It would be interesting to measuring the ATP hydrolysis rate of loaded Mcm2-7 complexes before and after CMG formation in the absence of Mcm10. It is possible that CMG formation is sufficient to activate the ATPase activity of the Mcm2-7 ring but they are not in a conformation suitable for DNA unwinding similar to an idling engine on neutral. Similarly, it would be interesting to measuring the ATP hydrolysis rate of loaded CMG complexes in the absence or presence of Mcm10.

One possible outcome is to observe an increase in ATPase activity in the presence of Mcm10 suggesting that Mcm10 stimulates the ATPase activity of the CMG triggering DNA unwinding. Another interpretation for this result would be that Mcm10 triggers initial DNA unwinding and ssDNA-binding to the CMG stimulates its ATPase activity.

Mcm10 is required for DNA unwinding but how it mediates this process remains unclear. Mcm10 binds to the A subdomain in Mcm2 and induces a conformational change that triggers helicase activation (Lõoke *et al.*, 2017). However, it remains inconclusive how this conformational affects the overall structure of the CMG or if it is sufficient to trigger initial DNA unwinding, DNA strand exclusion and double hexamer separation.

What's next?

Although we understand the requirements and key players in CMG formation and activation, we do not fully understand the order of these events. It would be interesting to understand when the accessory proteins required for CMG formation (i.e. Sld2, Dpb11 and Sld3) arrive and release from the DNA. Are they released as a complex or separately? Are any of these release events influencing CMG formation or activation? In addition to its role in CMG activation, the Sld5 subunit of GINS also acts as a scaffold protein for the Ctf4 (adaptor for

replication factors). Similar to Sld5, Psf2 does not contact the Mcm2-7 ring and may serve as a scaffold protein for unknown factors. Finally, the *CDC45* IDR domain projects outside of the Mcm2-7 central channel and could potentially serve as a scaffold protein for the recruitment of other factors (in addition to Pol ε).

REFERENCES

Aparicio, O.M., Stout, A.M., & Bell, S.P. (1999). Differential assembly of Cdc45p and DNA polymerases at early and late origins of DNA replication. *Proc. Natl. Acad. Sci. USA 96*, 9130-9135.

Aparicio, O.M., Weinstein, D.M., & Bell, S.P. (1997). Components and dynamics of DNA replication complexes in S. cerevisiae: Redistribution of MCM proteins and Cdc45p during S-phase. *Cell 91*, 59-69.

Bell, S.P., & Stillman, B. (1992). ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. *Nature 357*, 128-134.

Bleichert, F., Botchan, M.R., & Berger, J.M. (2015). Crystal structure of the eukaryotic origin recognition complex. *Nature 519*, 321-326.

Bochman, M. L., & Schwacha, A. (2008). The Mcm2-7 complex has in vitro helicase activity. *Molecular Cell 31*, 287-293.

Bowers, J.L., Randell, J.C.W., Chen, S., & Bell, S.P. (2004). ATP hydrolysis by ORC catalyzes reiterative Mcm2–7 assembly at a defined origin of replication. *Molecular Cell 16*, 967-978.

Brewer, B. J., & Fangman, W. L. (1987). The localization of replication origins on ARS plasmids in S. cerevisiae. *Cell 51(3)*, 463-471.

Chen, S., and Bell, S.P. (2011) CDK prevents Mcm2–7 helicase loading by inhibiting Cdt1 interaction with Orc6. *Genes and Development 25*, 363-372.

Chen, S., de Vries, M.A., & Bell, S.P. (2007). Orc6 is required for dynamic recruitment of Cdt1 during repeated Mcm2-7 loading. *Genes & Development 21*, 2897-2907.

Costa, A., Ilves, I., Tamberg, N., Petojevic, T., Nogales, E., Botchan, M.R., & Berger, J.M. (2011). The structural basis for MCM2-7 helicase activation by GINS and Cdc45. *Nature Structural & Molecular Biology 18 (4)*, 471-477.

Coster, G., Frigola, J., Beuron, F., Morris, E. P., & Diffley, J. F. X. (2014). Origin licensing requires ATP binding and hydrolysis by the MCM replicative helicase. Mol. Cell 55, 666–677.

Davey, M.J. Indiani, C., O'Donnell, M. (2003). Reconstitution of the Mcm2-7p heterohexamer, subunit arrangement, and ATP site architecture. *Journal of Biological Chemistry 278*, 4491-4499.

Deegan, T. D., Yeeles, J. T., Diffley, J. F. X. (2016). Phosphopeptide binding by Sld3 links Dbf4-dependent kinase to MCM replicative helicase activation. *EMBO Journal 35*, 961-973.

Donley, N., & Thayer, M. J. (2013). DNA replication timing, genome stability and cancer: Late and/or delayed DNA replication timing is associated with increased genomic instability. *Seminars in Cancer Biology 23,* 80-89.

Donovan, S., Harwood, J., Drury, L.S., & Diffley, J.F.X. (1997). Cdc6p-dependent loading of Mcm proteins onto pre-replicative chromatin in budding yeast. *Proc. Natl. Acad. Sci. USA 94*, 5611-5616.

Draetta, G., Luca, F., Westendorf, J., Brizuela, L., Ruderman, J., & Beach, D. (1989). Cdc2 protein kinase is complexed with both cyclin A and B: evidence for proteolytic inactivation of MPF. *Cell 56*, 829-838.

Drury, L.S., Perkins, G., & Diffley, J.F.X. (2000). The cyclin-dependent kinase Cdc28p regulates distinct modes of Cdc6p proteolysis during the budding yeast cell cycle. *Current Biology 10*, 231-240.

Eaton, M.L., Galani, K., Kang, S., Bell, S.P., & MacAlpine, D.M. (2010). Conserved nucleosome positioning defines replication origins. *Genes & Development 24*, 748-753.

Evans, T., Rosenthal, E.T., Youngbloom, J., Distel, D., & Hunt, T. (1983). Cyclin: A protein specified by maternal mRNA is sea urchin eggs that is destroyed at each cleavage division. *Cell 33*, 389-396.

Evrin, C., Clarke, P., Zech, J., Lurz, R., Sun, J., Uhle, S., ... Speck, C. (2009). A double-hexameric MCM2-7 complex is loaded onto origin DNA during licensing of eukaryotic DNA replication. *Proc. Natl. Acad. Sci. USA 106(48)*, 20240-20245.

Evrin, C., Fernandez-Cid, A., Zech, J., Herrera, M.C., Riera, A., Clarke, P., Brill, S., Lurz, R., & Speck, C. (2013). In the absence of ATPase activity, pre-RC formation is blocked prior to MCM2-7 hexamer dimerization. *Nucleic Acids Research 41*, 3162-3172.

Fang, D., Cao, Q., & Lou, H. (2016). Sld3-MCM interaction facilitated by Dbf4-dependent kinase defines an essential step in eukaryotic DNA replication initiation. *Frontiers in Microbiology 7*, 85.

Fernández-Cid, A., Riera, A., Tognetti, S., Herrera, M.C., Samel, S., Evrin, C., ... & Speck, C. (2013). An ORC/Cdc6/MCM2-7 complex is formed in a multistep reaction to serve as a platform for MCM double-hexamer assembly. *Molecular Cell 50*, 577-588.

Ferreira, M.G., Santocale, C., Drury, L.S., & Diffley, J.F.X. (2000). Dbf4p, an essential S phase-promoting factor is targeted for degradation by the Anaphase-Promoting Complex. *Molecular and Cellular Biology,* 242-248.

Ferguson, B.M., & Fangman, W.L. (1992). A position effect on the time of replication origin activation in yeast. *Cell 68*, 333-339.

Fletcher, R.J., Bishop, B.E., Leon, R.P., Sclafani, R.A., Ogata, C.M., & Chen, X.S. (2003). The structure and function of MCM from archaeal M. thermoautotrophicum. *Nature Structural Biology 10(3)*, 160-167.

Francis, L. I., Randell, J. C., Takara, T. J., Uchima, L., & Bell, S. P. (2009). Incorporation into the prereplicative complex activates the Mcm2-7 helicase for Cdc7-Dbf4 phosphorylation. *Genes and Development 23*, 643-654.

Fu, Y. V., Yardimci, H., Long, D.T., Ho, T.V., Guainazzi, A., Bermudez, V.P., ... Walter, J. (2011). Selective bypass of a lagging strand roadblock by the eukaryotic replicative DNA helicase. *Cell 146(6)*, 931-941.

Gambus, A., Jones, R.C., Sanchez-Diaz, A., Kanemaki, M., van Deursen, F., Edmondson, R.D., & Labib, K. (2006). GINS maintain association of Cdc45 with MCM in replisome progression complexes at eukaryotic DNA replication forks. *Nature Cell Biology 8*, 358-366.

Gros, J., Kumar, C., Lynch, G., Yadav, T., Whitehouse, I., & Remus, D. (2015). Post-licensing specification of eukaryotic replication origins by facilitated Mcm2-7 sliding along DNA. *Molecular Cell 60*, 797-807.

Hartwell, L.H., Mortimer, R.K., Culotti, J., & Culotti, M. (1973). Genetic control of the cell division cycle in yeast: V. genetic analysis of cdc mutants. *Genetics 74*, 267-286.

Heller, R.H., Kang, S., Lam, W.M., Chen, S., Chan, C.S., & Bell, S.P. (2011). Eukaryotic origin-dependent DNA replication in vitro reveals sequential action of DDK and S-CDK kinases. *Cell 146(1)*, 80-91.

Hennessy, K.M., Lee, A., Chen, E., & Botstein, D. (1991). A group of interacting yeast DNA replication genes. *Genes & Development 5(6)*, 958-969.

Hofmann, J.F., & Beach, D. (1994). Cdt1 is an essential target of the Cdc10/Sct1 transcription factor: requirement for DNA replication and inhibition of mitosis. *EMBO 13*, 425-434.

Honey, S., & Futcher, B. (2007). Roles of the CDK phosphorylation sites of yeast Cdc6 in chromatin binding and rereplication. *Molecular Biology of the Cell 18(4)*, 1324-1336.

Hopwood, B., & Dalton, S. (1996). Cdc45p assembles into a complex with Cdc46p/Mcm5p, is required for minichromosome maintenance, and is essential for chromosomal DNA replication. *Proc. Natl. Acad. Sci. USA 93(22)*, 12309-12314.

Ilves, I., Petojevic, T., Pesavento, J.J., & Bothchan, M.R. (2010). Activation of the Mcm2-7 helicase by association with Cdc45 and GINS proteins. *Molecular Cell 37*, 247-258.

Kang, S., Warner, M.D., & Bell, S.P. (2014). Multiple functions for Mcm2-7 ATPase motifs during replication initiation. *Cell 55(5)*, 655-665.

Kanke, M., Kodama, Y., Takahashi, T. S., Nakagawa, T., & Masukata, H. (2012). Mcm10 plays an essential role in origin DNA unwinding after loading of the CMG components. *EMBO Journal 31(9)*, 2182-2194.

Kerzendorfer, C., Colnaghi, R., Abramowicz, I., Carpenter, G., & O'Driscoll, M. (2013). Meier-Gorlin syndrome and Wolf-Hirschhorn syndrome: two developmental disorders highlighting the importance of efficient DNA replication for normal development and neurogenesis. *DNA Repair 8*, 637-644.

Klemm, R. D., Austin, R.J. & Bell, S.P. (1997). Coordinate binding of ATP and origin DNA regulates the ATPase activity of the origin recognition complex. *Cell 88*, 493-502.

Kramer, E.R., Scheuringer, N., Podtelejnikov, A.V., Mann, M. & Peters, J. (2000). Mitotic regulation of the APC activator proteins CDC20 and CDH1. *Molecular Biology of the Cell 11*, 1555-1569.

Labib, K. (2010). How do Cdc7 and cyclin-dependent kinases trigger the initiation of chromosome replication. *Genes & Development 24*, 1208-1219.

Labib, K., Diffley, J.F.X., & Kearsey, S.E. (1999). G1-phase and B-type cyclins exclude the DNA replication factor Mcm4 from the nucleus. *Nature Cell Biology 1*, 415-422.

Lascaris, R.F., Groot, E., Hoen, P.B., Mager, W.H., & Planta, R.J. (2000). Different roles for Abf1p and a T-rich promoter element in nucleosome organization of the yeast RPS28A gene. *Nucleic Acids Research 28*, 1390-1396.

Lee, D., & Bell, S.P. (1997). Architecture of the yeast origin recognition complex bound to origins of DNA replication. *Molecular and Cellular Biology 17(12)*, 7159-7168.

Li, J.J., & Herskowitz, I. (1993) Isolation of Orc6, a component of the origin recognition complex by a one-hybrid system. *Science 17*, 1870-1874.

Li, N., Zhai, Y., Zhang, Y., Li, W., Yang, W., Lei, J., ... Gao, N. (2015). Structure of the eukaryotic MCM complex at 3.8Å. *Nature 524*, 186-191.

Li, X., Romero, P., Rani, M., Dunker, A. K., & Obradovic, Z. (1999). Predicting protein disorder for N-, C-, and internal regions. *Genome Informatics 10*, 30-40.

Liku, M.E., Nguyen, V.Q., Rosales, A.W., Irie, K., Li, J.J. (2005). CDK phosphorylation of a novel NLS-NES module distributed between two subunits of the Mcm2-7 complex prevents chromosomal rereplication. *Molecular Bilogy of the Cell 16*, 5026-5039.

Lipford, J.R., & Bell, S. P. (2001). Nucleosomes positioned by ORC facilitate the initiation of DNA replication. *Molecular Cell 7(1)*, 21-30.

Liu, S., Balasov, M., Wang, H., Wu, L., Chesnokov, I.N., & Liu, Y. (2011). Structural analysis of human Orc6 protein reveals a homology with transcription factor TFIIB. *Proc. Natl. Acad. Sci. USA 108*, 7373-7378.

Lõoke, M., Kristjuhan, K., Värv, S., & Kristjuhan, A. (2013). Chromatin-dependent and –independent regulation of DNA replication origin activation in budding yeast. *EMBO Reports 14(2)*, 191-198.

Lõoke, M., Maloney, M., & Bell, S. P. (2017). Mcm10 regulates DNA replication elongation by stimulating the CMG replicative helicase. *Genes and Development 31*, 291-305.

Maine, G.T., Sinha, P., & Tye, B.K. (1984). Mutants of S. cerevisiae defective in the maintenance of minichromosomes. *Genetics 106*, 365-385.

Mantiero, D., Mackenzie, A., Donaldson, A., & Zegerman, P. (2011). Limiting replication initiation factors execute the temporal programme of origin firing in budding yeast. *EMBO Journal 30*, 4805-4814.

Marahrens, Y., & Stillman, B. (1992) A yeast chromosomal origin of DNA replication defined by multiple functional elements. *Science 255*, 817-823.

Masuda, T., Mimura, S., & Takisawa, H. (2003). CDK- and Cdc45-dependent priming of the MCM complex on chromatin during S-phase in Xenopus egg extracts: possible activation of MCM helicase by association with Cdc45. *Genes to Cells 8(2)*, 145-161.

Mimura, S., & Takisawa, H. (1998). Xenopus Cdc45-dependent loading of DNA polymerase alpha onto chromatin under the control of S-phase Cdk. *EMBO 17 (19)*, 5699-5707.

Moir, D., Stewart, S.E., Osmond, B.C., & Botstein, D. (1982). Cold-sensitive cell-division-cycle mutants of yeast: isolation, properties, and pseudoreversion studies. *Genetics 100(4)*, 547-563.

Moyer, S. E., Lewis, P. W., & Botchan, M.R., (2006). Isolation of the Cdc45/Mcm2-7/GINS (CMG) complex, a candidate for the eukaryotic DNA replication fork helicase. *Proc. Natl. Acad. Sci. USA 103*, 10236-10241.

Muramatsu, S., Hirai, K., Tak, Y.S., Kamimura, Y., & Araki, H. (2010). CDK-dependent complex formation between replication proteins Dpb11, Sld2, Pol epsilon, and GINS in budding yeast. *Genes & Development 24*, 602-612.

Nguyen, V.Q., Co, C., & Li, J.J. (2001). Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms. *Nature 411(6841)*, 1068-1073.

Nguyen, V.Q., Co, C., Irie, K., & Li, J. (2000). Clb/Cdc28 kinases promote nuclear export of the replication initiator proteins Mcm2-7. *Current Biology 10*, 195-205.

Nurse, P., & Thuriaux, P. (1980). Regulatory genes controlling mitosis in the fission yeast *Schizosaccharomyces pombe*. *Genetics 96(3)*, 627-637.

Owens, J.C., Detweiler, C.S., & Li, J.J. (1997). CDC45 is required in conjunction with CDC7/DBF4 to trigger the initiation of DNA replication. *Proc. Natl. Acad. Sci. USA 94 (23)*, 12521-12526.

Pacek, M., Tutter, A.V., Kubota, Y., Takisawa, H., & Walter J.C. (2006). Localization of MCM2-7, Cdc45, and GINS to the site of DNA unwinding during eukaryotic DNA replication. *Molecular Cell 21(4)*, 581-587.

Piatti S., Lengauer,C., & Nasmyth,K. (1995). Cdc6 is an unstable protein whose *de novo* synthesis in G1 is important for the onset of S phase and for p reventing a 'reductional' anaphase in the budding yeast *Saccharomyces cerevisiae. EMBO Journal 14*, 3788-3799.

Quan, Y., Xia, Y., Liu, L., Cui, J., Li, Z., Cao, Q., ... & Lou, H. (2015). Cell-cycle-regulated interaction between Mcm10 and double hexameric Mcm2-7 is required for helicase splitting and activation during S Phase. *Cell Reports 13*, 2576-2586.

Randell, J.C.W., Bowers, J.L., Rodriguez, H.K., & Bell, S.P. (2006). Sequential ATP hydrolysis by Cdc6 and ORC directs loading of the Mcm2-7 helicase. *Molecular Cell 21*, 29-39.

Randell, J.C.W., Fan, A., Chan, C., Francis, L.I. Heller, R.C., Galani, K., & Bell, S.P. (2010). Mec1 is one of the multiple kinases that prime the Mcm2-7 helicase for phosphorylation by Cdc7. *Molecular Cell 40*, 353-363.

Rao, H., & Stillman, B. (1995). The origin recognition complex interacts with a bipartite DNA binding site within yeast replicators. *Proc. Natl. Acad. Sci. USA 92*, 2224-2228.

Remus, D., Beuron, F., Tolun, G., Griffith, J.D., Morris, E.P., & Diffley, J.F.X. (2009). Concerted loading of Mcm2–7 double hexamers around DNA during DNA replication origin licensing. *Cell 139*, 719-730.

Romero, P., Obradovic, Z., Kissinger, C. R., Villafranca, J. E., Garner, E., Guilliot, S., & Dunker, A. K. (1998) Thousands of proteins likely to have long disordered regions. *Pacific Symposium on Biocomputing*, 437-448.

Samel, S. A., Fernandez-Cid, A., Sun, J., Riera, A., Tognetti, S., Herrera, M. C., ... & Speck, C., (2014) A unique DNA entry gate serves for regulated loading of the eukaryotic replicative helicase MCM2-7 onto DNA. *Genes and Development 28*, 1653-1666.

Sanchez-Pulido, & Ponting, C.P. (2011). Cdc45: the missing RecJ ortholog in eukaryotes? *Bioinformatics 27(14)*, 1885-1888.

Schlecht, U., Erb, I., Demougin, P., Robine, N., Borde, V., Nimwegen, E., ... Primig, M. (2008). Genome-wide expression profiling, in vivo DNA binding analysis, and probabilistic motif prediction reveal novel Abf1 target genes during fermentation, respiration, and sporulation in yeast. *Mol. Biol. Cell. 19*, 2193-2207.

Sengupta, S., van Deursen, F., de Piccoli, G., & Labib, K. (2013). Dpb2 integrates the leading-strand DNA polymerase into the eukaryotic replisome. *Current Biology 23*, 543-552.

Siow, C. C., Nieduszynska, S. R., Müller, C. A., Nieduszynski, C. A. (2012). OriDB, the DNA replication origin database updated and extended. *Nucleic Acids Research 40 (D1)*, D682-D686.

Speck, C., Chen, Z., Li, H., & Stillman, B. (2005). ATPase-dependent cooperative binding of ORC and Cdc6 to origin DNA. *Nature Structural and Molecular Biololgy 12*, 965-971.

Speck, C., & Stillman, B. (2007). Cdc6 ATPase activity regulates ORC-Cdc6 stability and the selection of specific DNA sequences as origins of DNA replication. *J. Biol. Chem. 282*, 11705-11714.

Sheu, Y., & Stillman, B. (2010). The Dbf4-Cdc7 kinase promotes S Phase by alleviating and inhibitory activity in Mcm4. *Nature 463*, 113-117.

Stinchcomb, D.T., Struhl, K., & Davis, R.W. (1979). Isolation and characterisation of a yeast chromosomal replicator. *Nature 282*: 39-43.

Sun, J., Evrin, C., Samel, S.A., Fernandez-Cid, A., Riera, A., Kawakami, H., ... Li, H. (2013). Cryo-EM structure of helicase loading intermediate containing ORC-Cdc6-Cdt1-Mcm2-7 bound to DNA. *Nature Structural and Molecular Biology 20*, 944-951.

Sun, J., Shi, Y., Georgescu, R. E., Yuan, Z., Chait, B. T., Li, H., O'Donnell, M. E. (2015). The architecture of a eukaryotic replisome. *Nature Structural and Molecular Biology 22*, 976-982.

Takahashi, K., Yamada, H., & Yanagida, M. (1994). Fission yeast minichromosome loss mutants mis cause lethal aneuploidy and replication abnormality. *Molecular Biology of the Cell 5(10)*, 1145-1158.

Takara, T. J., & Bell, S.P. (2011). Multiple Cdt1 molecules act at each origin to load replication-competent Mcm2–7 helicases. *EMBO Jornal 30*, 4885-4896.

Tanaka, S., & Diffley, J.F.X. (2002). Interdependent nuclear accumulation of budding yeast Cdt1 and Mcm2–7 during G1 phase. *Nature Cell Biology 4*, 198-207.

Tanaka, S., Komeda, Y., Umemori, T., Kubota, Y., Takisawa, H., & Araki, H. (2013). Efficient initiation of DNA replication in eukaryotes requires Dpb11/TopBP1-GINS interaction. *Molecular and Cellular Biology 33(13)*, 2614-2622.

Tanaka, S., Nakato, R., Katou, Y., Shirahige, K., & Araki, H. (2011). Origin association of Sld3, Sld7 and Cdc45 proteins is a key step for determination of origin-firing timing. *Cell 21(24)*, 2055-2063.

Tanaka, S., Umemori, T., Hirai, K., Muramatsu, S., Kamimura, Y., & Araki, H. (2007). CDK-dependent phosphorylation of Sld2 and Sld3 initiates DNA replication in budding yeast. *Nature 445(7125)*, 328-332.

Tercero, J.A., Labib, K., & Diffley, J.F.X. (2000). DNA synthesis at individual replication forks requires the essential initiation factor Cdc45p. *EMBO 19(9)*, 2082-2093.

Ticau, S., Freidman, L.J., Ivica, N.A., Gelles, J., & Bell, S.P. (2015). Single-molecule studies of origin licensing reveal mechanism ensuring bidirectional helicase loading. *Cell 161(3)*, 513-525.

Ticau, S., Friedman, L.J., Champasa, K., Corrêa, Gelles, J., & Bell, S.P. (2017). Mechanism and timing of Mcm2-7 ring closure during DNA replication origin licensing. *Nature Structural & Molecular Biology 24(3)*, 309-315.

Tocilj, A., Fan On, K., Yuan, Z., Sun, J. Elkayam, E., Li, H., Stillman, B., & Joshua-Tor, L. (2017). Structure of the active form of human origin recognition complex and its ATPase motor module. *Elife 6*, e208818.

Verma, R., Annan, R.S., Huddleston, M.J., Carr, S.A., Reynard, G., & Deshaies, R.J. (1997). Phosphorylation of Sic1p by G_1 Cdk required for its degradation and entry into S phase. *Science 278*, 455-459.

Walters, J., & Newport, J. (2000). Initiation of eukaryotic DNA replication: origin unwinding and sequential chromatin association of Cdc45, RPA and DNA polymerase. *Molecular Cell 5*, 617-627.

Watson, J. D., Crick, F. H. C. (1953) Molecular structure of nucleic acids. *Nature 4356 (171)*, 737-738.

Wilmes, G.M., Archambault, V., Austin, R.J., Jacobson, M.D., Bell, S.P., & Cross, F.R. (2004). Interaction of the S-phase cyclin Clb5 with an "RXL" docking sequence in the initiator protein Orc6 provides an origin-localized replication control switch. *Genes & Development 18(9)*, 981-991.

Wilmes, G.M., & Bell, S.P. (2002). The B2 element of the Saccharomyces cerevisiae ARS1 origin of replication requires specific sequences to facilitate pre-RC formation. *Proc. Natl. Acad. Sci. USA 99*, 101-106.

Yeeles, J. T., Deegan, T. D., Janska, A., Early, A. and Diffley, J. F. X. (2015). Regulated eukaryotic DNA replication origin firing with purified proteins. *Nature 519*, 431-435.

Yuan, Z., Bai, L., Sun, J., Georgescu, R., Liu, J., O'Donnell, M. E., & Li, H. (2016). Structure of the eukaryotic replicative CMG helicase suggests a pumpjack motion for translocation. *Nature Structural and Molecular Biology 23(3)*, 217-224.

Yuan, Z., Riera, A., Bai, L., Sun, J., Nandi, S., Spanos, C., ..., & Li, H. (2017). Structural basis of Mcm2-7 replicative helicase loading by ORC-Cdc6 and Cdt1. *Nature Structural and Molecular Biology 24(3)*, 316-324.

Zegerman, P., & Diffley, J.F.X. (2010). Checkpoint-dependent inhibition of DNA replication initiation by Sld3 and Dbf4 phosphorylation. *Nature 467*, 474-478.

Zhai, Y., Cheng, E., Wu, H., Li, N., Yuk Kwong Yung, P., Gao, N., & Tye, B. (2017). Open-ringed structure of the Cdt1-Mcm2-7 complex as a precursor of the MCM double hexamer. *Nature Structural & Molecular Biology 24(3)*, 300-308.

Zou, L., Mitchell, J., & Stillman, B. (1997). CDC45, a novel yeast gene that functions with the origin recognition complex and Mcm proteins in initiation of DNA replication. *Molecular Cell Biology 17(2)*, 553-563.

Zou, L., & Stillman, B. (1998). Formation of a preinitiation complex by S-phase cyclin CDK-dependent loading of Cdc45p onto chromatin. *Science 280*, 593-596.

Zou, L., & Stillman, B. (2000). Assembly of a complex containing Cdc45p, replication protein A, and Mcm2p at replication origins controlled by S-phase cyclin-dependent kinases and Cdc7p-Dbf4p kinase. *Molecular and Cellular Biology 20(9)*, 3086-3096.