# Elucidating the role of Dpb11 in replication initiation

By

Nalini Dhingra

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Biological Sciences

August, 2015

Nashville, Tennessee

Approved:

Daniel L. Kaplan, Ph.D.

Todd R. Graham, Ph.D.

Katherine L. Friedman, Ph.D.

Brandt F. Eichman, Ph.D.

William P. Tansey, Ph.D.

***to mom and dad for helping me pursue my dreams…….***

## ACKNOWLEDGEMNETS

I thank Vanderbilt University and Florida State University for providing me with a wonderful learning environment, where everyone is quick to help one another.

My thanks and appreciation to Dr. Daniel Kaplan for persevering with me as my advisor throughout the time it took me to complete this research and write the dissertation. I would like to thank you for encouraging my research and supporting me throughout my graduate career. Your advice on research as well as on my career has been invaluable. I am also very grateful to Dr. Irina Bruck, who generously shared her meticulous research and insights that supported and expanded my work. Thank you for your advice and many insightful discussions.

I am especially thankful to my thesis committee, Dr. Todd Graham, Dr. Katherine Freidman, Dr. Brandt Eichman and Dr. William Tansey, for their support, advice and insightful comments. I am especially thankful for your support during my time at Florida State University.

I will forever be thankful to my undergraduate research advisor, Dr. I. Prem Kumar. He was the reason I decided to pursue a career in research. His enthusiasm and motivation has always inspired me.

I thank all my labmates at Vanderbilt University and Florida State University, Dr. Diane Kanter, Boting Ning, Skye Smith and Dr. Patricia Perez for their support throughout my research work. I must acknowledge the many friends, colleagues and teachers who advised, and supported my efforts over the years. Especially, I need to express my gratitude to Krishna Mohanty, Pritha

Paul and Lakshmi Bhaskaran whose friendship, knowledge, and wisdom have supported, enlightened, and entertained me over the many years of our friendship.

A special thanks to my family. Words cannot express how grateful I am to my parents and my little brother, Nitish, for their infinite love and support. Your prayers for me were what sustained me so far. Finally, I would like to thank my loving husband, Sridhar, who always stood by me cheering me up. Thank you for your constant encouragement and support and most importantly for believing in me.

**TABLE OF CONTENTS**

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AAA+ | ATPases associated with diverse cellular activities |
| AAD | ATR activation domain |
| ACS | ARS Consensus Sequence |
| ALT | Alternative Lenghtening of Telomeres |
| APC | Anaphase Promoting Complex |
| ARS | Autonomously Replicating Sequence |
| ASF1 | Anti-Silencing Function 1 |
| ATM | Ataxia Telangiectasia Mutated |
| ATP | Adenosine Triphosphate |
| ATR | ATM and Rad3-related |
| BAH | Bromo Adjacent Homology |
| BER | Base Excision Repair |
| BIR | Break induced replication |
| bp | base pair |
| BRCT | BRCA1 C-terminus |
| BSA | bovine serum albumin |
| CAF-1 | Chromatin Assembly Factor-1 |
| CC | Coiled Coil |
| Cdc | Cell division cycle |
| CDK | Cyclin Dependent Kinase |
| Cdt 1 | Cdc10-dependent transcript 1 |

| | |
|---|---|
| CMG | Cdc45-Mcm-GINS |
| CTD | C-terminal domain |
| Dbf4 | Dumb bell forming 4 |
| DDK | Dbf4-Dependent Kinase |
| DDR | DNA Damage Response |
| DNA | Deoxyribonucleic Acid |
| dNTPs | deoxynucleotide triphosphates |
| Dpb11 | DNA polymerase B binding subunit 11 |
| ds | double stranded |
| DSB | Double Strand Break |
| DTT | Dithiothreitol |
| EDTA | Ethylenediaminotetraacetic acid |
| EM | Electron Microscopy |
| Fox | Forkhead box |
| GINS | Go Ichi Ni San |
| GST | Glutathione-S-transferase |
| HDAC | Histone Deacetylase |
| ID | Internal Domain |
| JM | Joint molecules |
| Mcm | Minichromosome maintenance |
| Mec1 | Mitosis entry checkpoint 1 |
| MGS | Meier-Gorlin Syndrome |
| NAP1 | Nucleosome Assembly Protein 1 |

| | |
|---|---|
| NASP | Nuclear Autoantigenic Sperm Protein |
| NER | Nucleotide Excision Repair |
| NHEJ | Non-Homologous End Joining |
| NTD | N-terminal domain |
| OB | Oligosaccharide/Oligonucleotide Binding |
| ORC | Origin Recognition Complex |
| ori | origin of replication initiation |
| PCNA | Proliferating Cell Nuclear Antigen |
| PIKK | Phosphatidylinositol 3-kinase-related protein kinase |
| PKA | Protein kinase A |
| PMSF | Phenylmethylsulfonyl fluoride |
| Pol-α | DNA polymerase -α primase |
| Pol-δ | DNA polymerase-δ |
| Pol-ε | DNA polymerase-ε |
| PP1 | Protein Phosphatase 1 |
| Pre-IC | Pre-Initiation Complex |
| Pre-LC | Pre-Loading Complex |
| Pre-RC | Pre-Replication Complex |
| PTM | Post Translational Modification |
| RFC | Replication Factor C |
| Rif1 | Rap1 interacting factor 1 |
| RNR | Ribonucleotide Reductase |
| ROS | Reactive Oxygen Species |

| | |
|---|---|
| RPA | Replication Protein A |
| SCF | Skp,Cullin, F-box containing |
| Sld | Synthetically lethal with *dpb11-1* |
| ss | single-stranded |
| SSBs | Single-strand breaks |
| SUMO | Small Ubiquitin-like Modifier |
| Tel1 | Telomere maintenance 1 |
| TopBP1 | Topoisomerase II Binding Protein I |
| UV | UltraViolet |

# CHAPTER I

## INTRODUCTION TO EUKARYOTIC DNA REPLICATION INITIATION


### Abstract

Every time a cell divides, a copy of its genomic DNA has to be faithfully copied to generate new genomic DNA for the daughter cells. The process of DNA replication needs to be precisely regulated to ensure that replication of the genome is complete and accurate, but that re-replication does not occur. Errors in DNA replication can lead to genome instability and cancer. The process of replication initiation is of paramount importance, because once the cell is committed to replicate DNA, it is optimal to complete replication with minimal errors. Furthermore, agents that inhibit DNA replication initiation are now being utilized for cancer therapy. A great deal of progress has been made in understanding how DNA replication is initiated in eukaryotic cells in the past ten years. This chapter will introduce how the position of replication initiation, called the replication origin, is chosen. This chapter will also introduce how replication initiation is integrated with the phases of the cell cycle, and how replication initiation is regulated in the case of damage to DNA. It is the cellular protein machinery that enables replication initiation to be activated and regulated. We now have an in-depth understanding of how cellular proteins cooperate to start DNA replication.

## Introduction

Eukaryotic replication is a highly controlled process and is tightly regulated to ensure that chromosomes duplicate only once per cell cycle and that the genomic stability of a cell is maintained. Replication occurs in three distinct steps: Initiation, Elongation and Termination. To ensure DNA replication occurs only once per cell cycle and that the entire DNA is faithfully replicated, a cell employs various control mechanisms at various steps that lead to the initiation of DNA replication. In general, the initiation process is divided into two distinct phases:

1. Origin licensing: process in late mitosis or early $G_1$ phase in which the Mcm2-7 complex (which forms the core of the replication fork helicase) is assembled onto replication origins as a double hexamer in the inactive form.
2. Origin activation: A process in S phase in which the Mcm2-7 complex is converted to an active replication fork helicase.

The Origin Recognition Complex (ORC) present at the replication origins is an initiator protein that facilitates the loading of Mcm2-7 double hexamer onto an origin. The loaded Mcm2-7 double hexamer encircles double-stranded DNA and then dissociates to form bi-directional replication forks. In the following chapter, I briefly discuss how origins are defined and activated in eukaryotes. I also discuss the mechanism of origin licensing and helicase formation. Finally, I mention the various mechanisms employed by a living cell in situations of DNA damage and replication stress.

## Origin Selection

Replication initiates at distinct DNA regions called the origins of replication initiation (*ori*). Replication initiator proteins bind at these replication origins. Origin sequences in budding

yeast are characterized by Autonomously Replicating Sequences (ARS), present at an average interval of 30 kbps throughout the chromosome (1). There are estimated to be approximately 400 ARSs in the yeast genome. Each ARS is 100-200 bps long and is characterized by the presence of A, B1, B2 and B3 elements. A and B1 elements are highly conserved and form the binding site for ORC. The B element consists of a region of helical instability that helps in the unwinding of DNA (2, 3). The A element also contains the ARS Consensus Sequence (ACS) which is an 11bp region rich in adenines and thymines and is required for ORC binding (2). However, a match to the ACS is not sufficient for origin function as there are more than 12,000 potential matches for ACS in the yeast genome, pointing out a need for additional sequence or chromatin requirements for defining replication origin (4). In contrast, origin sequences in higher eukaryotes including fission yeast are not defined by consensus sequences but are rather defined by chromatin structure and epigenetic modifications (5). Due to the lack of a consensus sequence, the ORC protein complex in metazoans may be targeted to specific sites (mainly the transcription start sites of actively transcribed genes) by various protein factors. In mammalian cells, many potential genetic and epigenetic determinants for replication origins have been reported using genome wide mapping techniques. (6). These studies also show the enrichment of origins near active promoter elements at CpG islands (7). Nucleosome positioning on chromatin is also a defining feature of replication origins. Various studies in yeast and higher eukaryotes show a decrease in nucleosome occupancy at origins (5). This nucleosome free region may be a determinant of ORC binding and may also facilitate the loading of Mcm2-7 complexes (8).

**Origin Activation**

The replication origins are further classified as early or late replicating origins based on their timing of replication. Various studies show strong correlation between replication timing and chromatin structure (9). Studies on replication timing in budding yeast have revealed early replication of origins in the centromeric region and late replication of origins in the sub-telomeric regions. This repression of origin activation at telomeres is attributed mainly to the local chromatin structure (8, 10). Centromeric regions also replicate early in fission yeast (10).

In higher eukaryotes, replication efficiency (defined by frequency of replication initiation) correlates with histone modification and transcriptional activity during development such that replication of gene-rich regions occurs earlier in S phase. Studies have shown that local changes in histone acetylation can also alter the replication program. For instance, sequences replicated in early S phase from HeLa cells exhibit hyperacetylation of histones H3 and H4 (5). Furthermore, depletion of Rpd3 (a histone deacetylase, HDAC) results in the early firing of normally late activating origins and an increase in the amount of Gcn5 (a histone acetyltransferase) around a late firing origin results in its earlier activation (10). Local chromatin environment may also regulate ORC recruitment and Pre-Replication Complex (pre-RC) assembly (7, 8). Replication timing is also regulated by various protein factors. Forkhead box (Fox) transcription factors, Fkh1 and Fkh2 have been shown to regulate replication timing in budding yeast (10). Advanced studies in budding yeast, fission yeast and mammalian cells have identified another protein factor Rif1 (Rap1 Interacting factor 1- originally identified as a telomere-associated protein in yeast), which has a broad role in replication timing control. In yeast, Rif1 directly acts to delay the origin firing of subtelomeric origins in budding yeast (11). However, in fission yeast and higher eukaryotic organisms, deletion of Rif 1 resulted in an

advanced timing of replication initiation of many late origins in the subtelomeric as well as internal chromosomal loci, while delaying the activation of many early origins. In fission yeast, Rif1 along with Taz1 also regulates the timing of Cdc45-Sld3 loading in G1 phase (10). Cdc45-Sld3 loading to replication origins has been suggested to be a limiting step controlling replication timing (12). Studies in several replication systems have shown that Cdc45 along with other initiation factors (Sld2, Sld3, Dpb11, DDK) are rate limiting for replication initiation (10, 13). Finally, the DNA replication checkpoint also regulates replication timing, as it is known to suppress the activation of late origins in response to replication stress.

In general, early replicating domains are euchromatic DNA regions localized in the interior of the nucleus and characterized by a high gene density and high GC content. Whereas, late replicating domains characterized by fewer genes are packaged into heterochromatin and localized to the nuclear periphery (6, 14). This temporal organization of genome replication, which is cell type specific, allows the cell to balance replication with limiting resources such as initiation factors and nucleotide pool and is conserved from yeast to humans, suggesting that timing of origin firing is regulated independently of origin selection (6, 14, 15), with replication timing largely being a consequence of genome architecture.

**Replication and Chromatin**

The basic unit of chromatin is the nucleosome that comprises of a histone octamer (consisting of two molecules each of histones H2A, H2B, H3 and H4) and 147 bps of DNA wrapped around the octamer 1.7 times. The epigenetic state of chromatin is defined by DNA methylation and post-translational modifications (PTMs) of histones such as acetylation, ribosylation, ubiquitination and SUMOylation (7). These histones and various histone variants

contribute to the diverse chromatin structure and are deposited in a replication-dependent or independent manner (8). It is well established that the diversity in chromatin structure and modifications influence the selection and activity of replication origins. However, replication fork progression leads to the disruption of existing chromatin structure by removal of nucleosomes from the DNA (16). Thus, it is essential to re-establish the epigenetic information on the newly synthesized chromatin. Chromatin reassembly on nascent strand occurs via two pathways, in the first, parental histones generated by the disruption of nucleosomes are recycled behind the fork and in the second pathway newly synthesized histones are deposited onto nascent DNA (16). Various biochemical and genetic studies have identified chromatin remodelers that contribute to the disruption and assembly of the chromatin structure during replication and also maintain its epigenetic states. Some of these remodelers include the ATP-utilizing chromatin assembly and remodeling factor (ACF) and the INO80 complex and its catalytic subunit SNF2. Biochemical studies have also identified histone chaperones that are responsible for the deposition of histones onto replicating DNA. Some of these chaperones are the human chromatin assembly factor-1 (CAF-1), antisilencing function 1 (ASF1), the nucleosome assembly protein 1 (NAP1) and the nuclear autoantigenic sperm protein (NASP) (8).

**Role of ORC in Replication Initiation**

ORC is a hetero-hexameric protein complex, consisting of the Orc1, Orc2, Orc3, Orc4, Orc5 and Orc6 subunits that is conserved among eukaryotes. It was first identified and purified from budding yeast as a factor that protected the ACS from digestion in a DNAseI footprinting assay (17). In *S.cerevisiae* ORC is present on the origin sequences throughout the cell cycle and binds DNA in an ATP dependent manner. In contrast, the fission yeast and metazoan ORC

complex binds replication origins periodically during the cell cycle. Orc1, Orc4 and Orc5 have AAA+ATPase domains (18). The ORC protein complex functions as a scaffold for the recruitment of Cdc6, Cdt1 and Mcm2-7 complex in $G_1$ phase. These proteins together with the ORC complex form the pre-RC. In addition to its role in recruiting the Mcm2-7 complex to origins, Orc1 protein has also been shown to interact with histone H4 via its conserved bromo-adjacent homology (BAH) domain in both yeast and human cells. This interaction may influence the local chromatin organization at some replication origins, affecting their activity (8). The Orc1 subunit of human ORC complex also associates with centrosomes and controls the cyclin E-CDK dependent reduplication of centrioles (19). Genome wide studies with budding yeast have classified ORC binding to the replication origins as either DNA dependent or chromatin dependent with chromatin dependent-ORC binding origins being associated with early activation (20). Studies in *Drosophila* and humans have shown that some of the ORC subunits interact with the heterochromatin protein (HP1) and maintain the heterochromatic environment (7). ORC along with various chromatin remodelers also facilitates the positioning of nucleosomes at the origins during their activation, thus remodeling the chromatin, which may be critical for the assembly of pre-RC (7).

Mutations in the *ORC1*, *ORC4* and *ORC6* subunits of human ORC complex have been associated with a rare autosomal recessive disorder called the Meier-Gorlin Syndrome (MGS). This disorder is characterized by postnatal growth retardation, dwarfism, microcephaly and developmental abnormalities in the ear and patella. Cells from these patients have a delayed cell cycle progression resulting in reduced cell number (21). Studies have identified mutations R105Q and E127G in Orc1, which affect the centriole copy number and cause centrosome reduplication in human cells, defects that may contribute to dwarfism and microcephaly (22).

Studies in zebrafish show that mutations in the H4K20me2 binding pocket of Orc1 also influence the recruitment of ORC onto replication origins, resulting in a diminished pre-RC assembly (23). Interestingly, depletion of Orc1 in zebrafish embryos resulted in abnormal body curvature and reduced viability. This defect might be a direct consequence of impaired origin licensing (24). A missense mutation in Tyr174 of human Orc4 was also found in patients with MGS. This residue is present in the highly conserved region of AAA+ATPase domain of Orc4. Mutation studies of the orthologous residue of Tyr174 in budding yeast (Tyr232), demonstrate that the strain exhibits a reduced growth rate with a defect in S phase progression (25). In *Drosophila*, a mutation in the C-terminal region of Orc6, which is implicated in MGS, impairs binding of Orc6 to the rest of the Orc complex thus preventing the loading of ORC onto replication origins (26). Additional mutations in MGS patients were also identified in *CDC6* and *CDT1* genes.

**Role of Mcm2-7 complex in Replication Initiation**

Mcm2-7 is a hexameric protein complex that consists of six distinct but evolutionarily related Mcm (Minichromosome maintenance) proteins having an ATPase domain at their C terminal end. These Mcm proteins were first isolated in a screen for yeast mutants defective in the maintenance of circular plasmids containing an ARS sequence (27). The six subunits of Mcm2-7 complex are assembled as a ring in the order Mcm3-Mcm5-Mcm2-Mcm6-Mcm4-Mcm7 forming the core of the eukaryotic replication fork helicase (28). The ATPase active sites in Mcm2-7 complex are formed at the dimer interfaces with one subunit contributing the Walker A motif and the adjoining subunit contributing an essential arginine (29) (Figure 1.1).

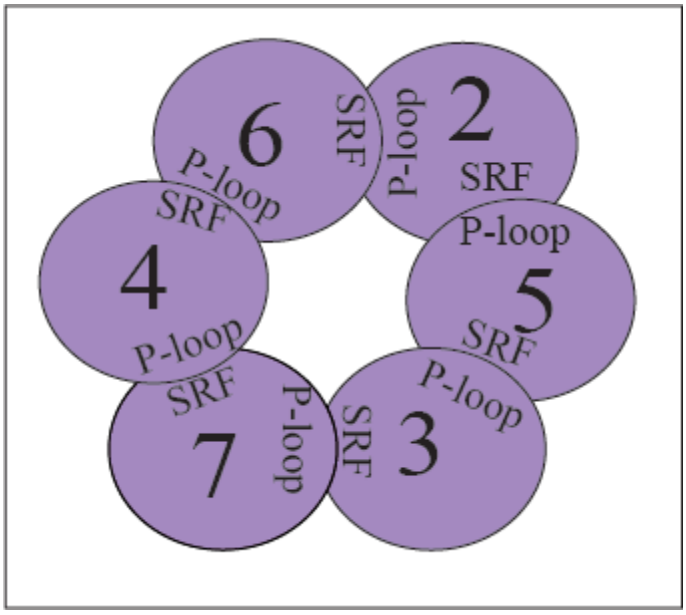


**Figure 1.1: Model of the Mcm2-7 hexamer.**

Adapted from Davey et al. 2003 (28). The orientation of the subunits around the ring is based on the view from the C-terminal face of multimeric AAA+ proteins, in which the catalytic arginine in one subunit is clockwise of the ATP site in the other. SRF represents the catalytic arginine motif and P-loop represents the ATP binding site.

A study with budding yeast proteins shows that Mcm2-7 complex by itself has a weak helicase activity *in vitro*, which depends on the specific buffer conditions (30). It was also demonstrated that the Mcm2-7 complex has an ATP-regulated gate at the Mcm2/Mcm5 interface that might facilitate the loading of Mcm2-7 at replication origins or extrusion of single stranded DNA during replication initiation (29). In addition, ATP binding plays a role in the stabilization of the Mcm complex. ATP hydrolysis by Mcm further facilitates the assembly of Mcm2-7 double hexamers on DNA (31).

The Mcm2-7 is loaded as a double hexamer in an ATP dependent manner to surround double stranded DNA, in a process called the licensing of replication origins (32). However, the

number of loaded Mcm2-7 complexes is much larger than actually required to establish replication forks. These additional copies of Mcm2-7 may be used to establish new forks in case of replication fork stalling due to DNA damage (33, 34).

**Licensing of replication origins**

Origin licensing is best studied in the budding yeast system. In the late M and $G_1$ phase, Cdc6 (also a AAA+ATPase protein) binds to the ORC protein complex and together they function with Cdt1 to load Mcm2-7 double hexamer onto origin sequences. These four factors together form the pre-RC. Earlier studies suggested a concerted loading of the Mcm2-7 double hexamer onto double stranded DNA (32), however recent studies support a step-by-step loading of the two hexamers (33, 35). ORC binds to Cdc6 in an ATP dependent manner and together they recruit Cdt1-Mcm2-7 to form an OCCM (Orc-Cdc6-Cdt1-Mcm2-7) complex in the absence of ATP hydrolysis. This recruitment of Mcm2-7 is facilitated by Mcm3 and Cdt1. The Cdt1-Mcm2-7 interacts with ORC-Cdc6 via the C-terminal region of the Mcm2-7 hexamer leaving its N-terminal region free to bind the second Mcm2-7 hexamer. Once the OCCM is formed, ATP hydrolysis of Orc1 and Cdc6 causes the release of Cdt1 from the OCCM complex to produce an OCM (Orc-Cdc6-Mcm2-7) complex. This OCM complex, which is a transient and salt sensitive intermediate, is capable of recruiting a second Mcm2-7 hexamer via the N-terminal domain (NTD) region of the first loaded Mcm2-7 hexamer. This results in the formation of another intermediate complex, the OCMM (Orc-Cdc6-Mcm2-7-Mcm2-7) complex in which the two hexamers are associated to each other head to head via their N terminal region. This loaded double hexamer appears to have a twisted structure that is relaxed in the active helicase (33, 36). Recent studies have also revealed that during licensing, the Mcm2-7 hexamer is loaded onto the

double stranded DNA via the interface between Mcm2 and Mcm5 at the stage of OCCM formation prior to ATP hydrolysis, thus separating the two events of helicase loading and double hexamer formation (37). The loaded Mcm2-7 double hexamer is then transformed into an active replication fork helicase during S phase by the action of various protein factors as described in detail below.

**Helicase activation**

The Mcm2-7 complex is activated in S phase by the action of two kinases, the Cyclin Dependent Kinase (S-CDK) and the Cdc7-Dbf4 dependent kinase (DDK) along with a number of other protein factors some of which also travel with the replication fork (38, 39). Studies in budding yeast have identified a number of initiation factors, namely, Cdc45, GINS [Go-Ichi-Ni-San, Japanese for 5-1-2-3, for Sld5, Psf1 (partner with Sld5), Psf2, Psf3], Sld7, Sld3, Sld2, Dpb11, Pol-ε, RPA and Mcm10 (40). Together, these proteins form the Pre-Initiation Complex (Pre-IC) that is finally converted to an active helicase composed of Cdc45, GINS and Mcm2-7 (CMG complex), through a series of highly regulated molecular events (35). Formation of the CMG complex constitutes two complex reactions. First, the Mcm2-7 double hexamer dissociates to form two single Mcm2-7 hexamers. Second, the Mcm2-7 ring opens for single-stranded DNA extrusion and then closes such that a single hexamer of Mcm2-7 is present around single-stranded DNA along with Cdc45 and GINS. Once the CMG complex is formed, the different polymerases are recruited to single-stranded DNA at the origins to start DNA synthesis and DNA replication proceeds bi-directionally (41). Pol-α synthesizes short DNA strands, while Pol-ε and Pol-δ elongate the leading and lagging strand, respectively (Figure 1.2).

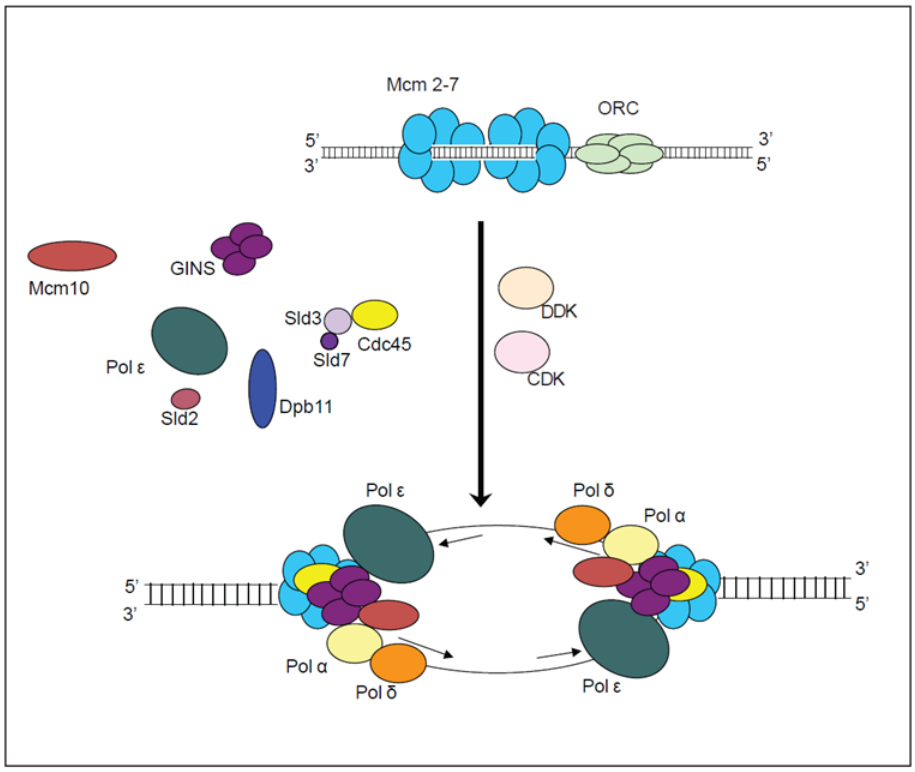


**Figure 1.2: A schematic of replication initiation in *Saccharomyces cerevisiae*.**

At replication origins, Mcm2-7 is present as a double hexamer encircling double-stranded DNA in $G_1$ phase. Along with CDK and DDK, the cell employs a complex machinery of protein factors that facilitate the formation of bi-directional replication forks in S-phase. Some of these factors become a part of the replication fork while others do not travel with the replication fork. Cdc45-Mcm2-7-GINS (CMG) forms the active replication fork helicase, unwinding double-stranded DNA to generate single-stranded templates for the replicative polymerases. Pol δ is devoted to replication of the lagging strain, and Pol ε is devoted to synthesis of the leading strand. Sld3, Sld2, and Dpb11 are required for initiation, but these three proteins do not travel with the replication apparatus. CDK phosphorylates Sld2 and Sld3 to form a ternary complex with Dpb11, while DDK phosphorylates subunits of the Mcm2-7 complex.

## THE Cdc7-Dbf4 DEPENDENT KINASE (DDK)

DDK consists of a catalytic subunit (Cdc7) and a regulatory subunit (Dbf4). The Cdc7 subunit is stable throughout the cell cycle, whereas the level of Dbf4 is regulated such that it remains high during S phase and then decreases in the late M and $G_1$ phase (38). Dbf4 becomes

ubiquitinated and is subjected to proteasomal degradation by the Anaphase Promoting Complex (APC/C) in the late M and $G_1$ phase. However, in the S phase, APC/C remains inactive allowing the accumulation of Dbf4 (35). Studies in budding yeast identified a mutant in MCM5 called *mcm5-bob1* that can bypass the requirement of DDK for replication initiation (42). The *mcm5-bob1* mutation causes a conformational change in the Mcm2-7 ring such that it allows for the binding of Cdc45 protein in the early $G_1$ phase, suggesting that DDK may be required for Cdc45 binding at the origins (43). It has also been shown that DDK phosphorylates Mcm2-7 complex at its Mcm2, Mcm4 and Mcm6 subunits at their amino terminals. Phosphorylation at the amino terminal serine/threonine domain (NSD) of Mcm4 has been shown to alleviate an inhibitory activity at Mcm4 (44, 45). These studies suggest that even though Mcm5 is not directly phosphorylated by DDK, the phosphorylation of other Mcm subunits is sufficient to cause a conformational change in Mcm5, such that Cdc45 binds Mcm2-7 (46, 47). A recent study in budding yeast elucidates how the *mcm5-bob1* mutation bypasses the requirement for DDK phosphorylation of Mcm subunits. This report shows that Mcm2 phosphorylation by DDK is essential for cell growth and DNA replication. Absence of Mcm2 phosphorylation by DDK also results in a decreased amount of origin single stranded DNA in S phase in contrast to the cells with the *mcm5-bob1* mutation. Dbf4-Cdc7 phosphorylation of Mcm2 weakens its interaction with Mcm5 and helps in the opening of Mcm2-7 ring at the Mcm2/Mcm5 gate, to allow for the extrusion of single stranded DNA from the central channel of Mcm2-7. Similarly, cells with the *mcm5-bob1* mutation also exhibit a weak interaction between Mcm2 and Mcm5, suggesting that the *mcm5-bob1* mutation bypasses the requirement of DDK phosphorylation of Mcm2 by an alternate mechanism that leads to the Mcm2-7 ring opening (48). Other independent studies have also shown that DDK facilitates the association of Sld3, Sld7 and Cdc45 with the Mcm2-7

complex (38). It has also been suggested that DDK might facilitate Mcm2-7 double hexamer dissociation to form Mcm2-7 single hexamer, prior to single stranded DNA extrusion (35).

As mentioned above, DDK dependent phosphorylation of Mcm4 is one of the key events for pre-RC formation. However, recent studies have shown that this event is under the control of Rif1- directed phosphatase action and loss of Rif1 partially compensates for impaired DDK function (49, 50). Rif1 was mentioned earlier as a factor that regulates replication timing. Budding yeast Rif1 contains a protein phosphatase 1 (PP1) docking motif. Rif1 binds Glc7 (the budding yeast PP1) and recruits it to telomeres and possibly to late origins of DNA replication. This is important to maintain the replication timing at telomeres, as the replication timing at budding yeast telomeres advanced when the ability of Rif1 to recruit Glc7 was compromised (50). It has been suggested that, in $G_1$ phase Rif1 recruits Glc7 onto chromatin and directs it to dephosphorylate Mcm4, thus preventing early initiation of replication. However, in S phase, when DDK levels are high, Rif1 gets phosphorylated by DDK and releases Glc7, thus favoring DDK dependent phosphorylation of Mcm4 and consequent origin activation. PP1 interaction motifs of Rif1 are conserved from yeast through higher eukaryotes. Studies in *Xenopus* and human cells also support the model, where Rif1 may counteract DDK-dependent phosphorylation of Mcm4 by targeting PP1 to dephosphorylate Mcm4 (49).

**S PHASE-CYCLIN DEPENDENT KINASE (S-CDK)**

S-CDK inhibits origin licensing and promotes DNA replication during S phase. Its concentration is regulated by APC/C mediated proteasomal degradation of the cyclin subuints (35). S-CDK inhibits origin licensing by phosphorylating Cdc6, which results in its SCF-dependent degradation. It also phosphorylates Mcm3, which causes the nuclear export of Mcm2-

7/Cdt1. S-CDK sterically inhibits ORC function by binding to the Orc6 RXL motif (a cyclin binding motif). Finally, it also phosphorylates Orc2 and Orc6 thus inhibiting the interaction of ORC with Cdt1 (51).

During replication initiation, budding yeast S-CDK (Clb5-Cdc28 and Clb6-Cdc28) phosphorylates Sld2 and Sld3 to facilitate their interaction with Dpb11. Dpb11 consists of two pairs of BRCT (BRCA1 C-terminus) domains that bind phosphorylated proteins. The N-terminal pair of BRCT domains binds phosphorylated Sld3; while the C-terminal pair binds phosphorylated Sld2 (52). Phosphorylation of Thr84 of Sld2 is essential for its association with Dpb11. Phosphorylation of Thr84 also stimulates Sld2 association with ssDNA (53). However, Thr84 phosphorylation requires prior phosphorylation of other Sld2 sites. Sld2 has a cluster of 11 CDK phosphorylation motifs. This pre-phosphorylation of Sld2 causes a conformational change in Sld2 protein to expose Thr84 to CDK activity, thereby facilitating its phosphorylation. The multisite phosphorylation of Sld2 also creates a high threshold for CDK activity that prevents premature replication. Sld3 has 12 CDK phosphorylation sites. However, binding of Sld3 to Dpb11 requires the simultaneous phosphorylation of Thr600 and Ser622, which may require high CDK activity (38). The S-CDK dependent formation of Dpb11-Sld3-Sld2 complex is an essential step during replication initiation. *In vivo* studies in budding yeast have shown that the fusion of Sld3-Dpb11 when combined with Sld2T84D (a phosphomimetic mutant of Sld2), bypasses the requirement for S-CDK (54). The phosphorylation dependent interaction of Sld2-Dpb11 is also important for the formation of a pre-loading complex (pre-LC), which consists of Sld2, Dpb11, GINS and Pol-ε. The pre-LC is formed transiently and is an important intermediate that may facilitate GINS loading to the origin (55).

**ROLES OF Sld2, Sld3 and Dpb11 IN REPLICATION INITIATION**

The budding yeast proteins, Sld2, Sld3 and Dpb11 are essential proteins required for the initiation of DNA replication. These proteins however, do not travel along the replication fork. Dpb11 is a 764 amino acid yeast protein and the *DPB11* (DNA Polymerase B binding protein subunit 11) gene was first isolated as a multicopy suppressor of mutations in the *DPB2* subunit of Pol-ε, indicating a strong genetic interaction between Dpb11 and Pol-ε. Dpb11 was shown to have a dual role in chromosomal replication and at the cell cycle checkpoint (56). Two-dimensional gel analysis further suggested that Dpb11 is required during the initiation step of DNA replication. Dpb11 was also shown to be associated with the ARS fragment using ChIP analysis (57). *SLD2* (synthetically lethal with *dpb11-1*) and *SLD3* were isolated in screens for identifying factors that interact with *DPB11*.

*In vitro* studies with purified budding yeast proteins have shown that Sld3 interacts with Cdc45 and help in its recruitment to the Mcm2-7 complex (12, 58, 59). Sld2 and Sld3 also associate with Mcm2-7 independently before the activation of S-CDK and this association prevents premature binding of GINS to Mcm2-7. This ensures that GINS does not associate with Mcm2-7-Cdc45 complex prior to the dissociation of Mcm2-7 double hexamer and extrusion of single-stranded DNA (60). However, once DDK and S-CDK are activated in S phase and single-stranded DNA is extruded from the central channel of Mcm2-7 complex, Sld2 and Sld3 dissociate from Mcm2-7 and bind origin single-stranded DNA. S-CDK phosphorylation of Sld2 and Sld3 results in the formation of Dpb11-Sld3-Sld2 complex that interacts tightly with origin single-stranded DNA via its three different binding sites. This may allow for the subsequent association of GINS with Mcm2-7-Cdc45 complex. Binding of GINS completes the formation of the CMG complex (60-62).

The orthologs of Dpb11, Sld3 and Sld2 in fission yeast are Cut5/Rad4, Sld3 and Drc1 respectively. In fission yeast, Sld3 associates with the origins in a DDK dependent manner however this association is independent of Cdc45 association. In fact, association of Sld3 with origins is essential for the subsequent recruitment of Cut5, Drc1, GINS and Cdc45. Similar to budding yeast, fission yeast Drc1 and Sld3 interact with Cut5 in a CDK dependent manner (38). In vertebrates, the functional homologs of Dpb11, Sld3 and Sld2 are reported to be TopBP1, Treslin/Ticrr and RecQL4 respectively even though they show very limited sequence similarity. TopBP1 is highly conserved in vertebrates. Vertebrate TopBP1 possesses nine BRCT domains (BRCT 0-8) and is required for cell survival and DNA replication similar to its yeast counterparts. *Xenopus* TopBP1 (Xmus101) has been shown to directly interact with Cdc45, thereby facilitating the loading of Cdc45 onto replication origins. This Cdc45 loading is however, dependent on ORC but independent of S-Cdk and Mcm2-7 (63). Human TopBP1 also interacts directly with Cdc45 and is required for the recruitment of Cdc45 onto origins of DNA replication (64). *Xenopus* Treslin/Ticrr also interacts with Cdc45 and is required for its association with chromatin. In addition, both human and *Xenopus* Treslin/Ticrr associate with the N-terminal BRCT domain of TopBP1 and this interaction is S-CDK dependent (65). Finally, RecQL4 has a very weak similarity to Sld2 and this similarity is restricted to the first 400 amino acids of the N-terminal region of RecQL4, which is essential for cell growth and DNA replication. The N-terminal region of *Xenopus* RecQL4 associates with TopBP1, however unlike budding yeast this interaction is CDK independent. The N-terminal region of human RecQL4 also binds TopBP1 and shows an interaction with ssDNA, dsDNA and Y-shaped DNA (66). *Xenopus* and human RecQL4 also binds to Mcm10 and associates with the CMG complex in Mcm10 dependent manner (35). Two additional metazoan protein factors GEMC1 and DUE-B

are also required for the recruitment of Cdc45 to chromatin and show binding to both Cdc45 and TopBP1. These two factors however have no identified homologs in lower eukaryotes suggesting that helicase activation is a more complicated process in vertebrates than in yeast.

These studies demonstrate that Dpb11, Sld3 and Sld2 play a critical role in replication initiation and their levels are significant for normal cell proliferation. Over-expression of these limiting factors leads to increased origin firing while their low levels result in low levels of replication initiation. Therefore, regulation of these essential protein factors is important for genome stability (67).

**ROLE OF Mcm10 IN REPLICATION**

Minichromosome maintenance protein 10 (Mcm10) is an essential replication protein present in eukaryotes and it has been shown to genetically interact with a wide array of proteins. These interacting proteins include replication initiation proteins, polymerases, replication checkpoint proteins, double strand break (DSB) repair proteins and proteins involved in the SUMO pathway. Structural studies on Mcm10 show the presence of a coiled coil (CC) motif in its N-terminal domain (NTD), an oligosaccharide/oligonucleotide binding (OB) fold in its Internal Domain (ID) and a variable C-terminal domain (CTD) which is absent in unicellular eukaryotes. The NTD of *S.pombe*, *Xenopus leavis* and humans has been implicated in self-interaction to form Mcm10 oligomers. A recent study has shown the interaction of NTD of Mcm10 with the Mec3 subunit of 9-1-1 checkpoint clamp (68). The OB fold present in the ID forms a DNA binding site and is also involved in interactions with Mcm2-7 complex, Pol-α and Proliferating Cell Nuclear Antigen (PCNA). Interaction with Pol-α is mediated through a conserved hydrophobic patch, known as the Hsp10-like domain and interaction with PCNA

occurs via the PIP (PCNA interacting peptide) box. The variable CTD provides an additional surface for interaction with proteins and DNA (69).

Mcm10 has been shown to be indispensible for CMG helicase activation (70, 71). It has also been shown to be involved in DNA unwinding since it has affinity for both single stranded (ss) and double stranded (ds) DNA (72). In addition to its role during replication initiation, Mcm10 is also required for polymerase loading and replication elongation. It has been identified as a component of replication forks and shown to recruit Pol-α to chromatin. It also interacts with PCNA (processivity factor for DNA polymerases) and this interaction in budding yeast is regulated by ubiquitylation of Mcm10 (69) . However, presence of Mcm10 at moving replication forks has recently been questioned (69, 70).

Mcm10 depletion in cells creates a requirement for checkpoint signaling and double-strand break repairs. Due to its essential role in genome maintenance, misregulation of Mcm10 expression correlates with cancer development. In addition, mutations within the conserved regions of Mcm10 have been identified during sequencing of various cancer genomes (73).

**The Cdc45-Mcm2-7-GINS (CMG) complex**

The CMG complex also called the active replication fork helicase is formed in the S phase around single stranded DNA and translocates in a 3' to 5' direction. The CMG complex catalyzes DNA unwinding during replication. It is composed of three essential proteins: Cdc45, Mcm2-7 and GINS, which are conserved throughout eukaryotes. The Cdc45 protein is conserved among eukaryotes and shows sequence similarity to archaeal proteins of the DHH family of phosphoesterases (74). Human Cdc45 has been predicted to have a strong structural similarity to the bacterial RecJ proteins (75). In addition, human Cdc45 also binds single stranded DNA (76,

77). GINS was identified as a heterotetrameric protein complex required for DNA replication in budding yeast, comprising of four subunits Psf1, Psf2, Psf3 and Sld5, which are highly conserved among eukaryotes (78). GINS complex was also purified from *Xenopus* egg extracts and was showed to have a ring like structure (79). Several independent studies have isolated the human GINS complex and described its crystal structure (80-82).

*In vitro* studies with *Drosophila* proteins show that *Drosophila* Mcm2-7 has a very minimal helicase activity. However, the helicase activity of Mcm2-7 increases by approximately 300 fold when it associates with Cdc45 and GINS to form the CMG complex. This complex also has a higher affinity for both single stranded DNA and forked DNA substrate than does the Mcm2-7 complex and this DNA binding is ATP dependent (30). Single particle EM studies using *Drosophila* proteins have shown that the Mcm2-7 by itself exists in 2 conformations, the planar notched-ring conformation and the spiral lock-washer conformation, with an opening present at the Mcm2/Mcm5 interface. The Mcm2-7 present within the CMG complex adopts a planar notched ring conformation with a gap between Mcm2 and Mcm5 subunits. However, the Mcm2-Mcm5 gate closes upon nucleotide binding (83).

In addition, Cdc45 and GINS were seen to form a handle like structure that also helps to bridge the gap between Mcm2-Mcm5 gate. This study using *Drosophila* proteins, also demonstrated that in the CMG complex, Cdc45 associates with the N-terminal of Mcm2 and the four subunits of GINS (Psf1, Psf2, Psf3 and Sld5) form extensive interactions with the N- and C-termini of Mcm3 and Mcm5. GINS and Cdc45 also make extensive contacts with each other (83). The association of Mcm2-7 proteins with Cdc45 and GINS provides stability to the Mcm2-7 ring and aids in its efficient functioning. The CMG complex was also isolated from human cells and was shown to have properties similar to that of the *Drosophila* CMG complex (84).

However, how this CMG complex aids in the unwinding of double stranded DNA still remains unclear.

**DNA Damage Response**

Cells are constantly exposed to various endogenous and exogenous DNA damaging agents, such as, reactive oxygen species (ROS) generated within a cell, ionizing or UV light mediated irradiation or enzymes involved in DNA compaction like DNA topoisomerases. As a result, DNA damage caused by these agents challenges the maintenance of cellular genome integrity. In order to maintain genomic integrity, eukaryotic cells activate the DNA damage response (DDR), which detects DNA lesions and coordinates various cellular processes important for recovery. Depending on the extent of DNA damage, DDR can either lead the cell to senescence or apoptosis, or activate specific mechanisms that repair the DNA damage or help the cell to tolerate DNA damage. Various repair mechanisms used by a cell in response to DNA lesions include, base excision repair (BER) to repair single strand breaks (SSBs) or subtle changes to DNA, nucleotide excision repair (NER) for bulkier single-strand lesions that distort the DNA helical structure, homologous recombination and non-homologous end joining (NHEJ) to cope with double strand breaks (DSBs), mismatch repair and finally translesion synthesis and template switching (85).

DDR causes a cell to arrest either in $G_1$ phase or in $G_2$ phase. In addition, replication fork associated DDR delays progression through S phase and controls initiation events (86). The signal transduction pathways of DDR that regulate cell cycle progression and activate the effector kinases in order to repair DNA lesions constitute the checkpoint machinery. Thus, DNA damage checkpoint is activated as a result of the initial processing of DNA damage.

The checkpoint signaling is mediated through two main kinases that belong to the phosphatidylinositol 3-kinase-related protein kinase (PIKK) family:

1. Mec1 (mitosis entry checkpoint 1) also called ATR (ATM and Rad3-related) in mammals, which is activated in response to ssDNA coated with RPA (replication protein A, a ssDNA binding protein) (87).
2. Tel1 (telomere maintenance 1) also called ATM (ataxia telangiectasia mutated) in mammals, which is activated in response to DSBs. At DSBs, Tel1/ATM is first recruited and activated by the MRN complex (Mre11-Rad50-Xrs2 in budding yeast or Mre11-Rad50-Nbs1 in mammals). This promotes resection at DSBs, generating ssDNA, which then activates Mec1/ATR kinase (87).

Mec1/ATR is recruited onto chromatin via its regulatory subunit called Ddc2 (or ATRIP, ATR interacting protein, in mammals). Mec1/ATR activation requires activator proteins, which are the 9-1-1 checkpoint clamp (Rad9-Hus1-Rad1 in mammals or Ddc1-Rad17-Mec3 in budding yeast) and Dpb11 (or TopBP1 I mammals) (88). The 9-1-1 checkpoint clamp is loaded onto chromatin via the clamp loader Rad24-RFC (replication factor C, Rad17-RFC in humans). In budding yeast, Mec1 also phosphorylates Ddc1/Rad9 (component of the 9-1-1 complex), which then recruits Dpb11 to stimulate Mec1 kinase activity. This Dpb11-Ddc1 interaction is conserved in higher eukaryotes (89). Activation of Mec1 results in the phosphorylation of various proteins in the cell including effector kinases Chk1 and Rad53 (Chk2 in humans) (87). These effector kinases undergo trans-autophosphorylation with the aid of mediator proteins like Rad9 (53BP1/MDC1/BRCA1 in mammals) or Mrc1 (Claspin in mammals). The hyper-phosphorylated effector kinases finally regulate various downstream processes by transmitting the checkpoint

response to a range of effector proteins. Mec1 and Tel1 also phosphorylate chromatin bound proteins like the histone variant H2A (H2AX in mammals) to cause local chromatin changes (90, 91).

Studies in budding yeast have demonstrated that during $G_1$ phase, the DNA damage checkpoint is mediated through the Ddc1 subunit of the 9-1-1 complex which directly activates Mec1, while Dpb11 was shown to be dispensable during the $G_1$ phase. However, the $G_2$/M DNA damage checkpoint requires both the 9-1-1 and Dpb11 dependent activation of Mec1 (92, 93).

In addition to DNA damage checkpoint signaling, DNA damage induced sumoylation (DDIS) of several protein factors also forms an integral part of DDR and enhances the cell's ability to replicate and repair damaged DNA. Sumoylation involves covalent addition of small ubiquitin-like modifier (SUMO) to one or more lysines of the target protein. SUMO targets proteins involved in DNA replication and in DNA repair pathways like recombination, base excision repair, nucleotide excision repair and non-homologous end joining. Some of these proteins identified in budding yeast are Dpb11, Mcm2, Mcm4, Orc2, Orc6, Pol1, Rad 52, Rad59, Apn1, Rad1 and Rad2. In addition, sumoylation of some DNA lesion sensor proteins may also contribute to achieve checkpoint activation (94).

**Replication Checkpoint Signaling**

The progressing replication fork can encounter obstacles mainly DNA breaks, that partially block the progression of replication fork and disturb its stability. Replication fork stalling can also occur in situations of replication stress like nucleotide depletion. Under such conditions that threaten DNA replication and cause replication fork stalling, the cell activates a replication or S-phase checkpoint-signaling pathway. The replication checkpoint signaling

regulates cell cycle progression through S phase in response to DNA damage or replication stress. This is important to maintain genome integrity and to ensure error free duplication of the entire genome. The replication checkpoint promotes DNA repair and stabilizes the stalled replication fork by the activation of a signal transduction cascade involving various protein factors. It also inhibits origin firing and slows down DNA synthesis to facilitate DNA repair (95).

During conditions of replicative stress, the helicase uncouples itself from DNA polymerases. As a result, the helicase keeps unwinding DNA, while DNA synthesis halts, creating an excess of ssDNA bound with RPA (replication protein A, a ssDNA binding protein). This ssDNA generated at stalled replication forks activates Mec1/ATR mediated replication checkpoint signaling (90). Studies in budding yeast show that Mec1 activation in S-phase checkpoint is regulated by three activator proteins, which act in a redundant manner. These three proteins are the 9-1-1 checkpoint clamp, Dpb11 and Dna2 (a conserved nuclease, essential for Okazaki fragment maturation), which activate Mec1 by a similar mechanism that ultimately phosphorylates Rad53. In addition to the Mec1 mediated checkpoint signaling, the replication checkpoint also has a secondary pathway for Rad53 phosphorylation that involves Tel1. Thus, the complete elimination of S phase checkpoint signaling can be achieved only by the elimination of Mec1 activation function of all the three activators (9-1-1 complex, Dpb11 and Dna2) and the elimination of Tel1 mediated Rad53 phosphorylation (95, 96).

An activated replication checkpoint regulates cell cycle progression and blocks the $G_2$/M transition. It also phosphorylates various components of the replication machinery to facilitate stabilization of stalled replication forks and also blocks further origin firing (97). Studies in budding yeast identified Dbf4 (regulatory subunit of DDK) and Sld3 as Rad53 substrates,

demonstrating the direct regulation of DNA replication machinery by checkpoints. Rad53 dependent phosphorylation of Sld3 prevents its interaction with Cdc45 and Dpb11, which is essential for activation of replication origins. Sld3 and Dbf4 phosphorylation thus interferes with the CDK and DDK dependent activation of origins (98). Similarly, phosphorylation of Treslin by Chk1 interferes with its interaction with TopBP1 BRCT1+2. Another key feature of replication checkpoint response is the regulation of ribonucleotide reductase (RNR) in order to maintain the optimum level of dNTPs, since too little or too much dNTP can be mutagenic (97). Mec1/ATR activation also prevents chromosome breakage at fragile sites, which experience slow movement of replication fork (95). However, some studies argue that replisome stability might not be a key feature of checkpoint response. Studies using *Xenopus* egg extracts indicated that replication could resume even in the absence of checkpoint kinases under certain circumstances (97). Another study in budding yeast showed the stable association of replisome with replication forks during replication stress even in the absence of Mec1 or Rad53.This suggests that checkpoint kinases might regulate the function of replisome proteins rather than its stability during conditions of replication fork stalling (99).

**Break Induced DNA-replication**

Break Induced Replication (BIR) is a DSB repair pathway that is used by a cell in situations where only one end of the DSB shares homology with a template. BIR contributes to replication restart at stalled or collapsed replication forks. It also plays an important role in telomere maintenance in the absence of telomerase. In eukaryotes, BIR is best studied in the budding yeast model system. BIR initiates when a single strand invades into the homologous DNA template and forms a displacement loop (D-loop). This process is mediated by Rad51 and

is followed by the assembly of a unidirectional replication fork and extensive DNA synthesis. However, formation of a replication fork from the D-loop is not very well understood (100). Studies in budding yeast have shown that BIR requires almost all the components of normal DNA replication including, Mcm2-7, Cdt1, Cdc45, GINS, DDK, Dpb11, Sld3, Pol α-primase, Mcm10 and Ctf4. Whereas, Cdc6 and ORC are not necessary for BIR (101). BIR initiation requires Pol δ, and Pol ε is required to continue DNA synthesis later. However, how the replication fork is established outside S-phase, in the $G_2$ phase still remains an important question in the field. Replication during BIR has a much higher mutation rate than normal replication. BIR may also result in various chromosomal rearrangements like template switching, copy number variation or non-reciprocal translocations. In humans, BIR is mainly involved in alternative lengthening of telomeres (ALT) or chromosomal rearrangements, which cause genetic instability and are particularly associated with several human cancers (102).

**Role of Dpb11/TopBP1 in DDR**

Dpb11 and its homologs, Rad4 in fission yeast, Mus101 in Drosophila and TopBP1 in metazoans play an important role in the maintenance of genome stability. In addition to its essential function in replication initiation, Dpb11 is also involved in DNA damage response and replication checkpoint pathway. Budding yeast Dpb11 interacts with phosphorylated Ddc1 (a subunit of the 9-1-1 complex) after DNA damage, which subsequently activates Mec1 (89, 103). This interaction is conserved in yeast and vertebrates and is mediated via the C-terminal tail of Ddc1. Both the 9-1-1 complex and Dpb11 can also directly activate the Mec1/Ddc2 kinase independently of each other (88). Mec1 activation is mediated via the interaction between Ddc2 and the unstructured C-terminal region of Dpb11. This region of Dpb11 is however dispensable

for replication initiation (93). Similarly TopBP1 also activates ATR via the region between its sixth and seventh BRCT domains called the ATR activation domain (AAD). However, there is no sequence conservation between the Dpb11 C-terminal domain and the AAD in TopBP1(104). Studies using Rad4 (Dpb11 homolog in fission yeast) have identified Rad4 BRCT4 as the region that interacts with phosphorylated Rad9 (budding yeast Ddc1). In contrast, metazoan TopBP1 binds the phosphorylated tail of Rad9 via its BRCT1, 2 (105). Dpb11 also interacts with CDK-phosphorylated Rad9 via its BRCT1,2 for an efficient checkpoint activation. This interaction is also evolutionarily conserved (106).

In addition to its role in ATR activation, studies using *Xenopus* egg extracts have shown TopBP1 interaction with MRN complex and MRN-associated proteins. This recruitment is independent of the 9-1-1 complex and requires BRCT3-6 of TopBP1. However, these interactions have not yet been shown in yeast (107). A recent study showed Dpb11/TopBP1 to bind Slx4, a DNA repair scaffold protein, and this interaction is mediated through CDK-dependent phosphorylation of Slx4. This Slx4-Dpb11 complex regulates replication fork stalling when the progressing replication fork encounters DNA lesions and promotes Mus81-Mms4 dependent JM (Joint molecules) resolution, during bypass of DNA damage via template switch recombination (108). Dpb11/TopBP1 forms nuclear foci at sites of double strand breaks in the $G_1$, S and $G_2$ phase. Dpb11 foci formation is dependent on the 9-1-1 complex and requires the C-terminal region of Dpb11 (109). TopBP1 has also been shown to interact directly with the tumor suppressor p53. Alterations in TopBP1 expression levels have been linked to breast cancer and have also been reported in Glioblastoma (107).

Though a lot is known about the role of Dpb11 in DNA damage response and S-phase checkpoint, the biochemical function of Dpb11 in initiation is still unclear. It is known that

Dpb11 is an essential protein required during replication initiation, but it does not travel along the replication fork. It forms a ternary complex with phosphorylated Sld2 and Sld3 and the formation of this complex is also essential for replication initiation. Studies in the Kaplan lab have shown that both Sld2 and Sld3 function independently before S-CDK activation. These proteins bind Mcm2-7 independently and prevent the premature binding of GINS to Mcm2-7 thus regulating the timing of CMG complex formation. However, after the activation of DDK and S-CDK, both Sld2 and Sld3 bind ssDNA extruded from Mcm2-7 and allow GINS to bind Mcm2-7 after the extrusion of ssDNA at the origin. The role of Dpb11 is still not clear in this model of replication initiation since here it is only described as a scaffold protein for the binding of phosphorylated Sld2 and Sld3. The following chapters elucidate a novel biochemical function of the yeast Dpb11 protein in replication initiation in the formation of an active replication fork helicase. In the end, I also discuss the revised model for replication initiation, which includes the function of Dpb11, Sld2 and Sld3 proteins independently and also suggests the role of Dpb11-Sld3-Sld2 ternary complex.

# CHAPTER II

## Dpb11 DIRECTLY INTERACTS WITH Mcm2-7 AND ssDNA*


### Abstract

Dpb11 is an essential protein and is required for the initiation of DNA replication in budding yeast. Dpb11 binds to S-CDK phosphorylated Sld2 and Sld3 to form a ternary complex during S phase and is considered as a scaffold protein for Sld2 and Sld3 binding. The replication fork helicase in eukaryotes is composed of Cdc45, Mcm2-7 and GINS. I show here using purified proteins from budding yeast that Dpb11 directly binds to Mcm2-7, and Dpb11 also competes with GINS for binding to Mcm2-7. Furthermore, Dpb11 binds directly to single-stranded DNA (ssDNA), and ssDNA inhibits Dpb11 interaction with Mcm2-7. I propose a mechanism, wherein Dpb11 binds Mcm2-7 and blocks the interaction between GINS and Mcm2-7. Upon the extrusion of ssDNA from the central channel of Mcm2-7, Dpb11 dissociates from Mcm2-7 and Dpb11 binds to ssDNA, thereby allowing GINS to bind to Cdc45-Mcm2-7.


### Introduction

Dpb11 is required for the initiation of DNA replication in budding yeast (56). The homologs of Dpb11 include Cut5 (*S. pombe*), Mus101 (*Xenopus*), and TopBP1 (human). In budding yeast, Sld2 (synthetic lethal with *dpb11-1*) and Sld3 bind to Dpb11 in a cyclin-dependent kinase (CDK)-dependent manner (52, 54, 110). Dpb11 (DNA polymerase B

* The work presented in this chapter is published in Dhingra N, Bruck I, Smith S, Ning B, Kaplan D (2015) JBC.

associated protein) binds to CDK-phosphorylated Sld3 and Sld2, and the CDK- phosphomimetic mutant of Sld2, Sld2T84D, binds to Dpb11 (52, 54, 110). The fusion of Sld3 with Dpb11, when combined with the CDK-phosphomimetic mutant of Sld2 (Sld2T84D), bypasses the requirement for CDK in the budding yeast cell (54). These data suggest that formation of the Dpb11-Sld3-Sld2 ternary complex is essential for cell growth. Thus, Dpb11 is currently viewed as a scaffolding protein for binding Sld2 and Sld3, but the molecular function of Dpb11 in replication initiation is not known.

Mcm2-7 forms a hexameric ring, and the subunit composition of this ring is known (Figure 2.1 C) (28, 111). Mcm2-7 loads onto double-stranded DNA origins during late M phase and $G_1$. The molecular details of this loading process have been elucidated (32, 112). The Mcm2-7 complex is loaded as a double hexamer, and the Mcm2-7 ring is cracked open at the Mcm2/Mcm5 interface during this process to encircle double-stranded DNA (36, 37). Mcm2-7, by itself is a very weak ATPase and helicase (28, 29, 111). In S phase, critical events function at the Mcm2-7 complex to activate the replication fork helicase.

During S phase, the Dbf4-dependent kinase phosphorylates components of the Mcm2-7 complex, and Cdc45 is recruited to Mcm2-7 (45, 113, 114). Cdc45 binds to the Mcm2 subunit of Mcm2-7 (Figure 2.1 C) (83). Also, during S phase, the Mcm2-7 ring transitions from surrounding double-stranded DNA to encircling single-stranded DNA (83, 115). Thus, the Mcm2-7 cracks open once again in S phase, also at the Mcm2-Mcm5 interface, to allow for the extrusion of single-stranded DNA (48). This event is mediated by phosphorylation of Mcm2 by the Dbf4-dependent kinase (48)

During S phase, GINS associates with Cdc45-Mcm2-7, thereby completing the assembly of the Cdc45-Mcm2-7-GINS (CMG complex), the replication fork helicase in eukaryotes (30,

116-118). GINS binds to the Mcm3 and Mcm5 subunits of Mcm2-7 (Figure 2.1 C) (83). The association of GINS with Cdc45-Mcm2-7 may require the activity of the S-phase cyclin-dependent kinase (S-CDK) (119-121). Furthermore, the addition of Cdc45 and GINS to Mcm2-7 substantially stimulates the ATPase and helicase activities of Mcm2-7, suggesting that the assembly of the CMG complex is critical for activating the replication fork helicase (30). Finally, whereas the Mcm2-7 complex can crack open to encircle double-stranded DNA or single-stranded DNA, the CMG complex is a locked ring that encircles only single-stranded DNA (83).

It was previously demonstrated that Sld2 or Sld3 can each bind directly to Mcm2-7, and Sld2 or Sld3 compete with GINS for binding to Mcm2-7 (58, 61). However, in the presence of single-stranded DNA, Sld2 or Sld3 dissociate from Mcm2-7, allowing GINS to bind Mcm2-7 (61, 62). Furthermore, GINS disrupts the interaction between Sld3 and Cdc45, suggesting an elegant mechanism to disengage Sld2 and Sld3 from Cdc45-Mcm2-7 (58).

In this chapter, I show using purified proteins that Dpb11 can bind directly to Mcm2-7, or single-stranded DNA. Furthermore, Dpb11 competes with GINS for interaction with Mcm2-7. In the presence of single-stranded DNA (ssDNA), the interaction between Dpb11 and Mcm2-7 is disrupted and this allows GINS to bind Mcm2-7. I propose a mechanism, where Dpb11 binds Mcm2-7 thus preventing the premature association of GINS with Mcm2-7. However, extrusion of ssDNA acts as a trigger to dissociate Dpb11 from Mcm2-7 and aids in the binding of GINS to Mcm2-7.

## Experimental Procedures

### Cloning and purification of proteins

Full length *DPB11* PCR product was cloned into SpeI/XhoI sites of pET 41a vector and NdeI/XhoI sites of pET 33b vector to contain an N-terminal GST tag or a PKA tag respectively as described (53). The cloning of the *DPB11* into pET 41a generates a two-tagged protein—a GST tag at the N-terminus, and a His-tag at the C-terminus. Purification of GST-Dpb11 uses sequential nickel and glutathione resins. The details of purification of GST-Dpb11 and PKA-Dpb11 are described (53). Fragments of Dpb11 were each cloned into the NdeI/XhoI sites of pET 33b vector, and purified as described (53). GST alone was purified as described (53). Full length *MCM3* PCR product was cloned into SpeI/XhoI site of pET 41a vector and full length *MCM5* PCR product was cloned into NcoI/XhoI site of pET 41a vector to contain a GST tag at the N-terminus of the protein. Full length *MCM3* PCR product was cloned into the pET 33b vector and full-length *MCM5* PCR product was cloned into the NdeI/BglI sites of the pET 33b vector to contain a PKA tag at the N-terminus of the protein. *MCM2* was likewise cloned into the pET41a vector to obtain GST-tagged Mcm2. The purification of Mcm2-7 proteins are described in detail elsewhere (28, 122). PKA-GINS, GST-Mcm2-7, PKA-Mcm2-7 were purified as described (58).[†]

### Kinase labeling of proteins

Kinase labeling was performed as described (53). Proteins with a PKA tag at the N-terminus (Dpb11, Mcm2-7 (Mcm3) and GINS (Psf1)) were labeled in a reaction volume of 100 µl that contained 3 µM of PKA tagged protein with 0.017 mg/ml PKA and ATP in kinase

[†] GINS and Mcm2-7 were purified by Dr. Irina Bruck.

reaction buffer (25mM Tris-HCl pH 7.5, 5 mM DTT, 50 mM magnesium chloride). The reaction was incubated at 30°C for 1 hour and stopped with the addition of 2 µl of 0.5 M EDTA. The kinase was removed from the mixture by affinity purification before the pulldown assay was performed.

**Radiolabeling DNA**

Single stranded (ss) DNA was end labeled with T4 polynucleotide kinase (NEB) and radiolabeled ssDNA was purified over G-25 Sephadex Columns for Radiolabeled DNA Purification (Roche). To make double stranded (ds) DNA substrates with radiolabeled ssDNA, 4 µl of 500 nM radiolabeled ssDNA was incubated with 4 µl of 500 nM complementary DNA in 4 µl of reaction buffer (20 mM Tris HCl, 4% glycerol, 0.1 mM EDTA, 40 µg/ml BSA, 5 mM DTT and 10 mM magnesium acetate) in a final volume of 12 µl. The reaction was incubated overnight at 37°C. The reaction was then diluted with 20 mM Tris-HCl, 0.1 mM EDTA to a final volume of 40 nM radiolabeled DNA.

**GST pull-down assays**

The GST pulldown assays were performed as described (53). GST- tagged protein attached to prepared Glutathione Sepharose was incubated with varying concentrations of radiolabeled protein or DNA (as described in each figure) in GST binding buffer (40 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 10 % glycerol, 0.1% Triton X-100, 1 mM DTT, 0.7 µg/ml pepstatin, 0.1 mM PMSF and 0.1 mg/ml BSA) in a final reaction volume of 100 µl. The reactions were incubated at 30°C for 10 minutes. Following incubation, the reactions were shifted to room temperature and glutathione sepharose beads were allowed to settle. The

supernatant was removed and the beads were washed two times with GST binding buffer. After the last wash, beads were heated at 90°C for 10 minutes in solution containing 2% SDS, 4% glycerol, 4 mM Tris-HCl, 2 mM DTT and 0.01% bromophenol blue. The reactions were then analyzed by SDS/PAGE followed by phosphor-imaging and quantification.

**Biotin pull-down assays**

Biotinylated DNA (4 pmoles) conjugated to streptavidin-agarose magnetic beads was incubated with different concentrations of radiolabeled protein in a solution containing 20 mM Tris-HCl pH 7.5, 100 μM EDTA, 10% glycerol, 40 μg/ml BSA, 10 mM magnesium acetate and 200 μM DTT in a final reaction volume of 25 μl. The reactions were incubated at 30°C for 10 minutes. After the incubation, the magnetic beads were collected at room temperature using a magnet (Dynal). The supernatant was removed and the beads were washed twice with a solution containing 20 mM Tris-HCl pH 7.5, 100 μM EDTA, 10% glycerol and 40 μg/ml BSA. After the second wash, the beads were collected and heated at 90°C for 10 minutes in a solution containing 2% SDS, 4% glycerol, 4 mM Tris-HCl, 2 mM DTT and 0.01% bromophenol blue. The reactions were analyzed by SDS/PAGE. The gel was dried at 80°C for 1 hour and then exposed to phosphor-imaging screen for 30 minutes.

**Results**

**Dpb11 binds to Mcm2-7**

Dpb11 is required for the initiation of DNA replication, and Dpb11 acts at a replication origin. Activation of the helicase is a critical step in the initiation process, and I investigated if

Dpb11 binds directly to the Mcm2-7 complex. GST-Mcm2-7 was purified by incubating GST-Mcm3 with native versions of the other five Mcm proteins, and then assembled and purified the GST-Mcm2-7 complex by established methods (28, 122). I also purified Dpb11 with an N-terminal protein kinase A tag (amino acids LLRASV), followed by radiolabeling with Protein Kinase A and $\gamma$-$^{32}$P-ATP. The PKA tag is not physiologic; it is a tool to radiolabel the Dpb11 protein with $^{32}$P.

Thirty pmoles of GST-Mcm2-7 or GST was incubated with varying amounts of radiolabeled PKA-Dpb11 (Figure 2.1 A). The reaction was incubated with glutathione sepharose, and the material bound to beads was analyzed by SDS/PAGE. I found that roughly one-sixth of the input Dpb11 was bound to GST-Mcm2-7, more than five-times the signal for GST alone (Figure 2.1 B). These data suggest that Dpb11 binds directly to the Mcm2-7 complex. I postulated that Dpb11 interaction may be important for assembly of the CMG complex, since Dpb11 initiates DNA replication initiation and binds directly to Mcm2-7. Cdc45 binds to Mcm2, and GINS to Mcm3 and Mcm5 (Figure 2.1 C) (83). Since Mcm2, Mcm3, and Mcm5 are adjacent to each other on the Mcm2-7 complex (Figure 2.1 C) (28), I focused on this region of Mcm2-7 for further analysis.

I next investigated whether Mcm2, Mcm3 or Mcm5 alone bind directly to Dpb11. I cloned, overexpressed, and purified GST-Mcm2, GST-Mcm3, or GST-Mcm5. I then incubated each purified subunit individually with radiolabeled PKA-Dpb11. I quantified the Dpb11 bound by SDS/PAGE, and found that GST-Mcm3 binds to the greatest fraction of input Dpb11, followed by Mcm5 (Figure 2.1 D). Binding to Mcm2 was equivalent to GST background.

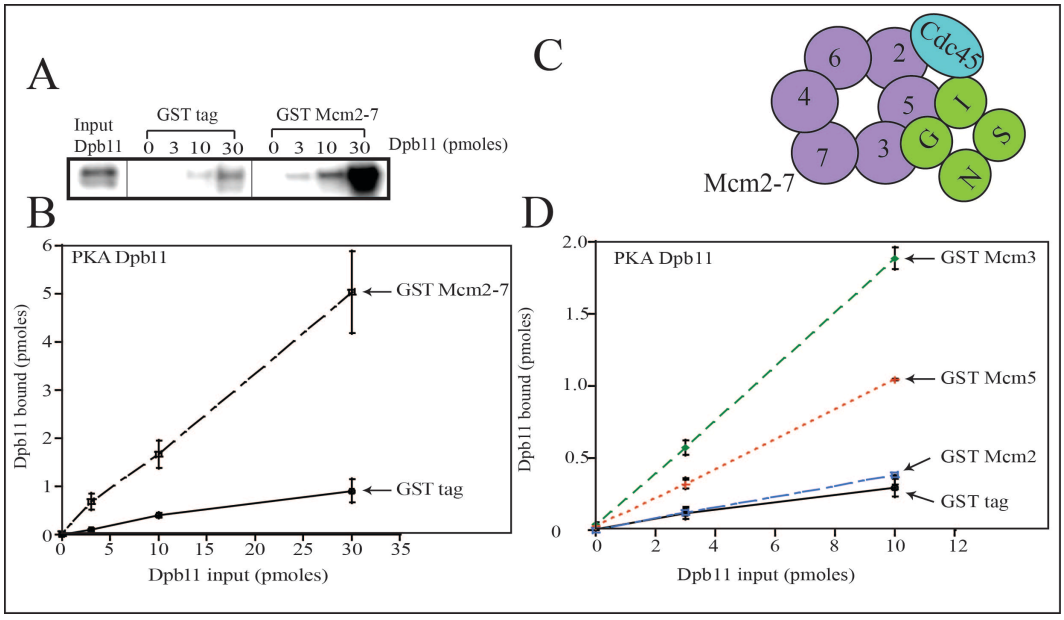


**Figure 2.1: Dpb11 binds to Mcm2-7.**

(A) 30 pmoles of GST-Mcm2-7 (contains GST at the N-terminus of Mcm3) or GST alone was incubated with glutathione sepharose and increasing concentrations of radiolabeled PKA-Dpb11 at 30°C for 10 minutes in a GST pulldown assay as described in the Experimental Procedures. The bound radioactive Dpb11 was analyzed by SDS/PAGE followed by phosphorimaging. (B) The results from experiments similar to (A) were then quantified, averaged and plotted as pmoles of Dpb11 bound versus the pmoles of Dpb11 input. (C) Arrangement of Mcm subunits forming the Mcm2-7 hexamer, along with the position of Cdc45 and GINS in the CMG complex (28, 83). (D) 30 pmoles of GST-Mcm2, GST-Mcm3, or GST-Mcm5 was incubated with increasing concentrations of radiolabeled Dpb11 in a GST pulldown assay.

### The BRCT4 motif of Dpb11 binds to Mcm3 or Mcm5‡

I wanted to determine which domain of Dpb11 binds to Mcm3 or Mcm5. Dpb11 has four BRCT motifs, BRCT1-4 (Figure 2.2 A). The region C-terminal to the BRCT motifs is predicted to be unstructured, and it is not required for cell growth under normal conditions. I incubated GST-Mcm3 with full-length and three different fragments of Dpb11 (253-615, 1-253, and 616-764) (Figure 2.2 B). GST-Mcm3 pulled down similar levels of full-length Dpb11 and Dpb11-253-615, whereas GST-Mcm3 pulled down a substantially smaller fraction of Dpb11 1-253 or

‡ I performed these experiments with the help of an undergraduate student Boting Ning.

Dpb11-616-764. (Figure 2.2 B). These data suggest that Mcm3 binds to the region of Dpb11 encompassing amino acids 253-615. I next performed a similar analysis with GST-Mcm5 and fragments of Dpb11 (Figure 2.2 C). Similar to GST-Mcm3, GST-Mcm5 also pulled down similar levels of full-length Dpb11 and Dpb11-253-615. I then subdivided Dpb11-253-615 into two smaller fragments, 253-430 and 430-615. Both GST-Mcm3 and GST-Mcm5 pulled down Dpb11-253-430 equivalent to the GST background. However, binding of GST-Mcm3 and GST-Mcm5 to Dpb11-430-615 was significantly higher when compared to the GST background, suggesting that the region 430-615 of Dpb11 binds to both Mcm3 and Mcm5 (Figure 2.2 D and 2.2 E). This region (amino acids 430-615) of Dpb11 encompasses BRCT domain 4 (Figure 2.2 A).

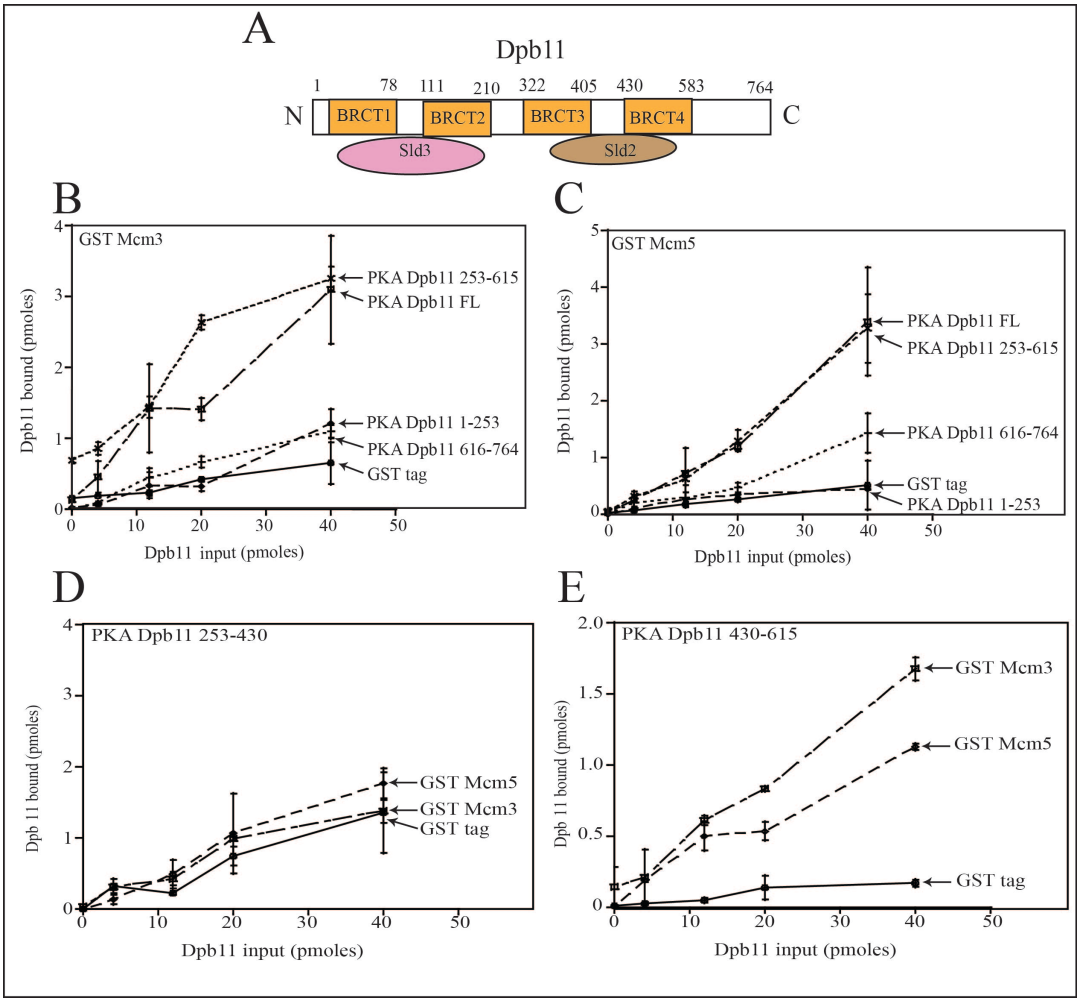


**Figure 2.2: Dpb11 binds Mcm3 and Mcm5 via its BRCT4 region.**

(A) A schematic of budding yeast Dpb11 protein indicating the four BRCT domains and the binding region of Sld3 and Sld2. (B) Different fragments of PKA-Dpb11 were incubated with 30 pmoles of GST-Mcm3 in a GST pulldown assay to determine the region of Dpb11 involved in Dpb11-Mcm3 interaction. (C) Different fragments of PKA-Dpb11 were incubated with 30 pmoles of GST-Mcm5 in a GST pulldown assay to determine the region of Dpb11 involved in Dpb11-Mcm5 interaction. (D) Radiolabeled PKA-Dpb11 (253-430) was incubated with 30 pmoles of GST-Mcm3, GST-Mcm5 or GST alone to determine whether amino acids 253-430 of Dpb11 bind GST-Mcm3 or GST-Mcm5. (E) Radiolabeled PKA-Dpb11 (430-615) was incubated with 30 pmoles of GST-Mcm3, GST-Mcm5 or GST alone to determine whether amino acids 430-615 of Dpb11 bind GST-Mcm3 or GST-Mcm5.

**Dpb11 binds to ssDNA**

Since Dpb11 acts at a replication origin, I next investigated whether Dpb11 can bind directly to origin DNA. I initially studied the *ARS1* origin because it is a well-characterized yeast origin. *ARS1* contains four elements: the A, B1, B2 and B3 elements. The A element contains the ARS consensus sequence (ACS) and the B region (also called the DNA Unwinding Element, DUE) consists of sequences that can be easily unwound. I incubated GST-Dpb11 with four different regions from *ARS1*, each 80 nucleotides in length. The regions are *ARS1*-1 and its complement *ARS1*-2, which encompass the A and B1 elements, *ARS1*-3 and its complement *ARS1*-4, which encompass the B2 and B3 elements (Figure 2.3 A). The sequences for these regions are given in Table 2.1. GST-Dpb11 pulled down a greater fraction of single-stranded *ARS1*-3. Weak interactions were also detected for ss*ARS1*-2 (Figure 2.3 B). These data suggest that Dpb11 binds more tightly to ss*ARS1*-3 than to the other regions tested in this assay. I next performed a similar analysis with a different yeast origin, *ARS305* (Figure 2.3 C). I incubated GST-Dpb11 with four different regions from *ARS305*, each 80 nucleotides in length (Table 2.1). GST-Dpb11 pulled down a greater fraction of *ARS305*-1. Weak interactions were also detected with the other three regions (Figure 2.3 D). These data suggest that Dpb11 binds more tightly to ss*ARS305*-1 than to the other regions of *ARS305*.

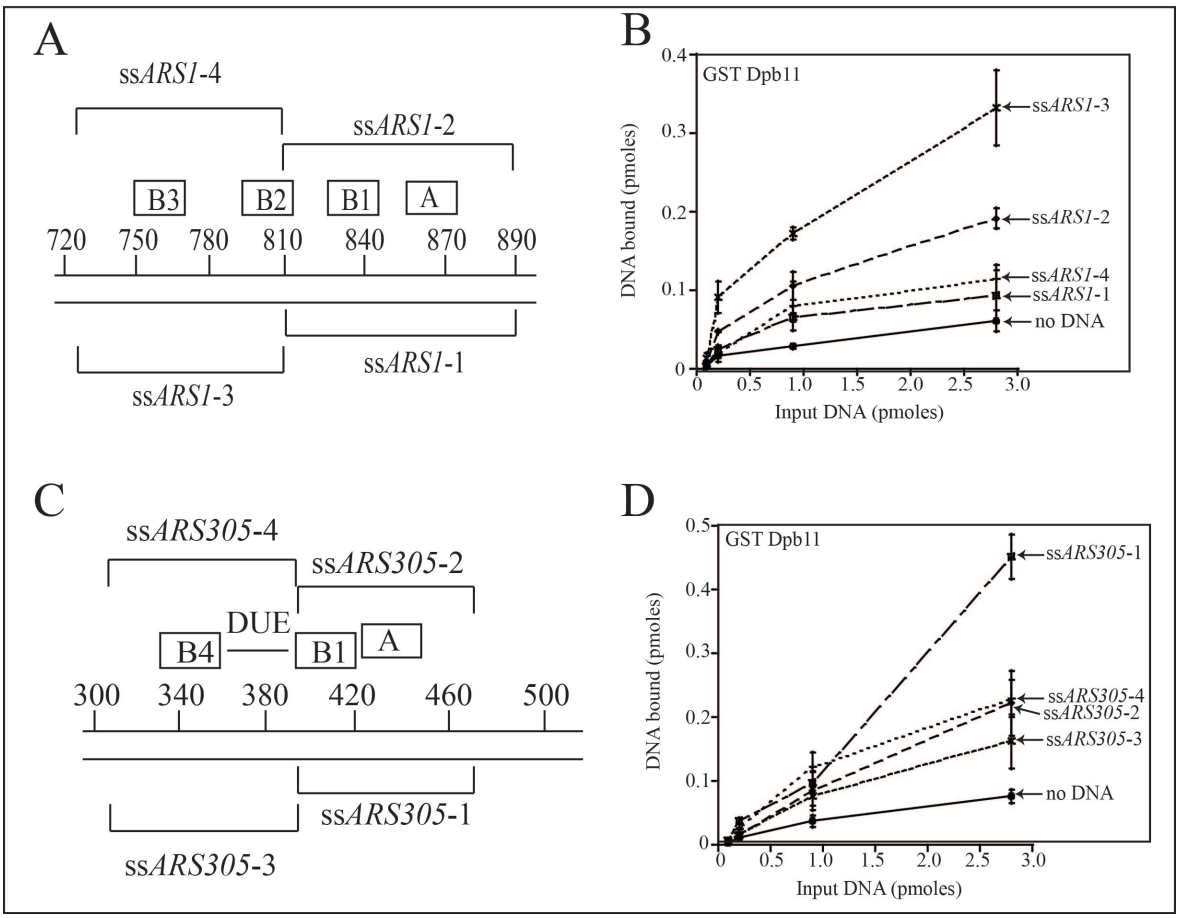


**Figure 2.3: Dpb11 binds origin ssDNA.**

(A) Schematic of the *ARS1* yeast origin indicating the various oligomers used. (B) Purified GST-Dpb11 or GST were studied for interaction with radiolabeled single stranded (ss) sequences from the *ARS1* origin in budding yeast. 13 pmoles of the GST-Dpb11 was incubated with DNA and glutathione sepharose for 10 minutes at 30°C. The glutathione sepharose beads were washed and analyzed as described in Experimental Procedures. The results from the experiment were quantified, averaged and plotted as pmoles of DNA bound versus pmoles of input DNA. (C) Schematic of the *ARS305* origin indicating the various oligomers used. (D) Purified GST-Dpb11 or GST were studied for interaction with radiolabeled single stranded (ss) sequences from the *ARS305* origin in budding yeast. 13 pmoles of the GST-Dpb11 was incubated with DNA and glutathione sepharose for 10 minutes at 30°C. The glutathione sepharose beads were washed and analyzed as described in Experimental Procedures. The results from the experiment were quantified, averaged and plotted as pmoles of DNA bound versus pmoles of input DNA.

To test if Dpb11 binds single stranded DNA and does not bind double stranded DNA, I incubated GST-Dpb11 with ss*ARS1*-3, which encompasses the B2 and B3 elements of the

replication origin (53) and a double-stranded sequence *ARS1*-3/*ARS1*-4. GST-Dpb11 pulled down a substantially greater fraction of single-stranded *ARS1*-3 compared to the double-stranded *ARS1*-3/*ARS1*-4 (Figure 2.4 A). I then performed the reverse reaction, by incubating biotinylated DNA with $^{32}$P-labeled-Dpb11, and pulling down with streptavidin beads (Figure 2.4 B). Again, I found that single-stranded *ARS1*-3 pulled down a greater fraction of Dpb11 compared to the double-stranded *ARS1*-3/*ARS1*-4 (Figure 2.4 B). I then performed the same experiment with a sequence from *ARS305*, *ARS305*-1, encompassing the A and B1 elements of the replication origin (sequence shown in Figure 2.3 C), and found that single-stranded *ARS305*-1 pulls down a greater fraction of Dpb11 compared to the double-stranded *ARS305*-1/*ARS305*-2 (Figure 2.4 C). These data suggest that Dpb11 binds directly to ssDNA containing an origin sequence.

To determine the sequence specificity for Dpb11-ssDNA interaction, i incubated GST-Dpb11 with radiolabeled *ARS1*-3 with the following nucleotide substitutions: T to A, C to A, A to C, or G to C. The sequences are shown in Table 2.1. Substitution of *ARS1*-3 with T to A resulted in a substantial decrease in ssDNA binding (Figure 2.4 D). Substitution of C to A resulted in a modest decrease in ssDNA binding. In contrast, the A to C and G to C substitutions had only a slight effect on ssDNA binding. Dpb11 may be recognizing the thymine bases, or simply the more flexible structure of a pyrimidine-rich sequence. Future studies will investigate the nature of Dpb11-ssDNA sequence dependence more extensively.

Since I found that Dpb11 binds thymine-rich ssDNA, I constructed an artificial 3'-biotinylated 80dT sequence, and incubated it with PKA-Dpb11. I then performed a biotin pulldown assay, and found that 80dT bound to Dpb11 (Figure 2.4 E). I then incubated different lengths of ssdT with PKA-Dpb11, and found that when matched for oligomer concentration, the 80dT pulled down the greatest fraction of Dpb11 (Figure 2.4 E). I then repeated the experiment

of Figure 2.4 E, but matched for nucleotide concentration (Figure 2.4 F). When matched for nucleotide concentration, 40dT, 60dT, and 80dT each bound Dpb11, but 20dT binding was close to background. These data suggest that Dpb11 binds to 21-40 nucleotides of ssdT.[§]

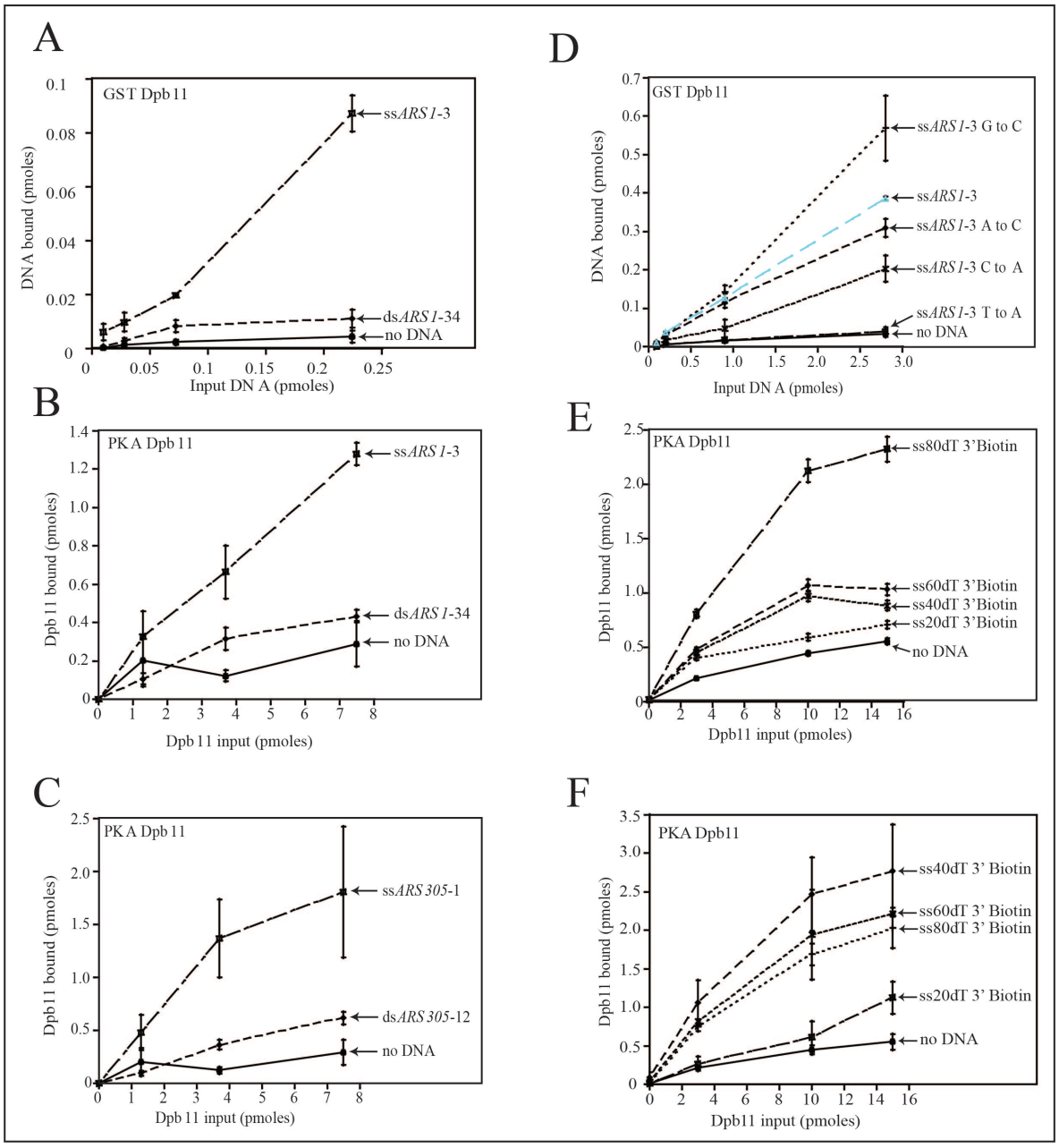


**Figure 2.4: Dpb11 binds to T-rich single stranded (ss) DNA of 40 nucleotides.**

[§] I performed these experiments with the help of an undergraduate student Skye Smith.

(A) Purified GST-Dpb11 or GST were studied for interaction with radiolabeled single stranded (ss) or double stranded (ds) sequences from the *ARS1* origin in budding yeast. 13 pmoles of the GST-Dpb11 was incubated with DNA and glutathione sepharose for 10 minutes at 30°C. The glutathione sepharose beads were washed and analyzed as described in Experimental Procedures. The results from the experiment were quantified, averaged and plotted as pmoles of DNA bound versus pmoles of input DNA. (B), (C) PKA-Dpb11 was radiolabeled and used in a biotin pulldown assay. Single-stranded or double-stranded DNA from yeast origins (*ARS1* or *ARS305*) with a 3' biotin tag was conjugated to magnetic streptavidin beads. 4 pmoles of this biotinylated DNA was incubated with radiolabeled PKA-Dpb11 at 30°C for 10 minutes. As a control beads without DNA were incubated with PKA-Dpb11. The beads were washed and analyzed as described in Experimental Procedures. The results from the experiment were quantified, averaged and plotted as pmoles of Dpb11 bound versus the pmoles of input Dpb11. (D) GST Dpb11 or GST was studied for interaction with radiolabeled DNA (ss*ARS1*-3 or modifications of ss*ARS1*-3) as indicated in the graph. 13 pmoles of GST Dpb11 or GST was incubated with glutathione sepharose and varying amounts of DNA at 30°C for 10 minutes. The glutathione sepharose beads were washed and analyzed as described in Experimental Procedures. The results from the experiment were quantified, averaged and plotted as pmoles of DNA bound versus pmoles of input DNA. (E) PKA-Dpb11 was radiolabeled and used in a biotin pulldown assay. Single-stranded DNA of varying lengths (ss80dT, ss60dT, ss40dT and ss20dT) with a 3' biotin tag was conjugated to magnetic streptavidin beads. 4 pmoles of this biotinylated DNA was incubated with radiolabeled PKA-Dpb11 at 30°C for 10 minutes. As a control beads without DNA were incubated with PKA-Dpb11. The beads were washed and analyzed as described in Experimental Procedures. The results from the experiment were quantified, averaged and plotted as pmoles of Dpb11 bound versus the pmoles of input Dpb11. (F) Similar to (E), except matching nucleotides, instead of matching pmoles of DNA. Thus, 4 pmoles of ss80dT, 5.3 pmoles of ss60 dT, 8 pmoles ss40dT, or 16 pmoles ss20dT were used in the pulldowns.

| Name | Sequence |
|---|---|
| ss*ARS1*-1 | 5’-TTA CAT CTT GTT ATT TTA CAG ATT TTA TGT TTA GAT CTT TTA TGC TTG CTT TTC AAA AGG CCT GCA GGC AAG TGC ACA AA-3’ |
| ss*ARS1*-2 | 5’-TTT GTG CAC TTG CCT GCA GGC CTT TTG AAA AGC AAG CAT AAA AGA TCT AAA CAT AAA ATC TGT AAA ATA ACA AGA TGT AA-3’ |
| ss*ARS1*-3 | 5’- CAA TAC TTA AAT AAA TAC TAC TCA GTA ATA ACC TAT TTC TTA GCA TTT TTG ACG AAA TTT GCT ATT TTG TTA GAG TCT TT-3’ |
| ss*ARS1*-4 | 5’-AAA GAC TCT AAC AAA ATA GCA AAT TTC GTC AAA AAT GCT AAG AAA TAG GTT ATT ACT GAG TAG TAT TTA TTT AAG TAT TG- 3' |
| ss*ARS1*-3 (T to A) | 5’- CAA **A**AC **AA**A AA**A** AAA **A**AC **A**AC **A**CA G**A**A AA**A** ACC **AAA AA**C **AA**A GCA **AAA AA**G ACG AAA **AAA** GC**A** A**AA AA**G **AA**A GAG **ACA AA**-3’ |
| ss*ARS1*-3 (C to A) | 5’- **A**AA TA**A** TTA AAT AAA TA**A** TA**A** T**A**A GTA ATA A**AA** TAT TT**A** TTA G**A**A TTT TTG A**A**G AAA TTT G**A**T ATT TTG TTA GAG T**A**T TT-3’ |
| ss*ARS1*-3 (A to C) | 5’- C**C**C T**C**C TT**C C**CT **CCC** T**C**C T**C**C TC**C**GT**C C**T**C C**CC T**C**T TTC TT**C** GC**C** TTT TTG **C**CG **CCC** TTT GCT **C**TT TTG TT**C** G**C**G TCT TT-3’ |
| ss*ARS1*-3 (G to C) | 5’- CAA TAC TTA AAT AAA TAC TAC TCA **C**TA ATA ACC TAT TTC TTA **C**CA TTT TT**C** AC**C** AAA TTT **C**CT ATT TT**C** TTA **CAC** TCT TT-3’ |
| ss*ARS305*-1 | 5'-TAA TGA GTA TTT GAT CCT TTT TTT TAT TGT GTT GGT TTT TAT ATG TTT TGT TAT GTA TTG TTT ATT TTC CCT TTA ATT TT-3' |
| ss*ARS305*-2 | 5'-AAA ATT AAA GGG AAA ATA AAC AAT ACA TAA CAA AAC ATA TAA AAA CCA ACA CAA TAA AAA AAA GGA TCA AAT ACT CAT TA-3' |
| ss*ARS305*-3 | 5'-GGA TAT GAA AAC AAG AAT TTA TCA AAG AAA AAA ATC TTA GCT TTA AGA ACT ACA AAG TAA ATA AAA AAT AAT AAA TCA CA-3' |
| ss*ARS305*-4 | 5'-TGT GAT TTA TTA TTT TTT ATT TAC TTT GTA GTT CTT AAA GCT AAG ATT TTT TTC TTT GAT AAA TTC TTG TTT TCA TAT CC-3' |

**Table 2.1: Name and Sequence of DNA Oligonucleotides**

**Regions of Dpb11 that bind to single-stranded DNA**

To determine the regions of Dpb11 that bind ssDNA, I first incubated biotinylated *ssARS1*-3 with full length PKA-Dpb11 and two different fragments of Dpb11 (1-253 and 253-764) (Figure 2.5 A). Single-stranded *ARS1*-3 pulled down similar levels of full length PKA-Dpb11 and PKA-Dpb11 (253-764) (Figure 2.5 A). I then subdivided Dpb11 (253-764) into three fragments (Dpb11 253-430 encompassing the BRCT3 region of Dpb11, Dpb11 430-615 encompassing the BRCT4 region of Dpb11 and Dpb11 616-764 that includes the unstructured C terminal region of Dpb11) and incubated them with biotinylated *ssARS1*-3. PKA-Dpb11 253-430 bound to biotinylated *ssARS1*-3 with an affinity similar to that of mock biotin beads without any DNA sequence (Figure 2.5 B). However, PKA-Dpb11 430-615 and PKA-Dpb11 616-764 each bound to biotinylated *ssARS1*-3 with an affinity higher than the mock biotin beads without DNA (Figure 2.5 C and 2.5 D). This data suggests that the region encompassing BRCT4 and also the unstructured C-terminal region of Dpb11, bind to ssDNA.

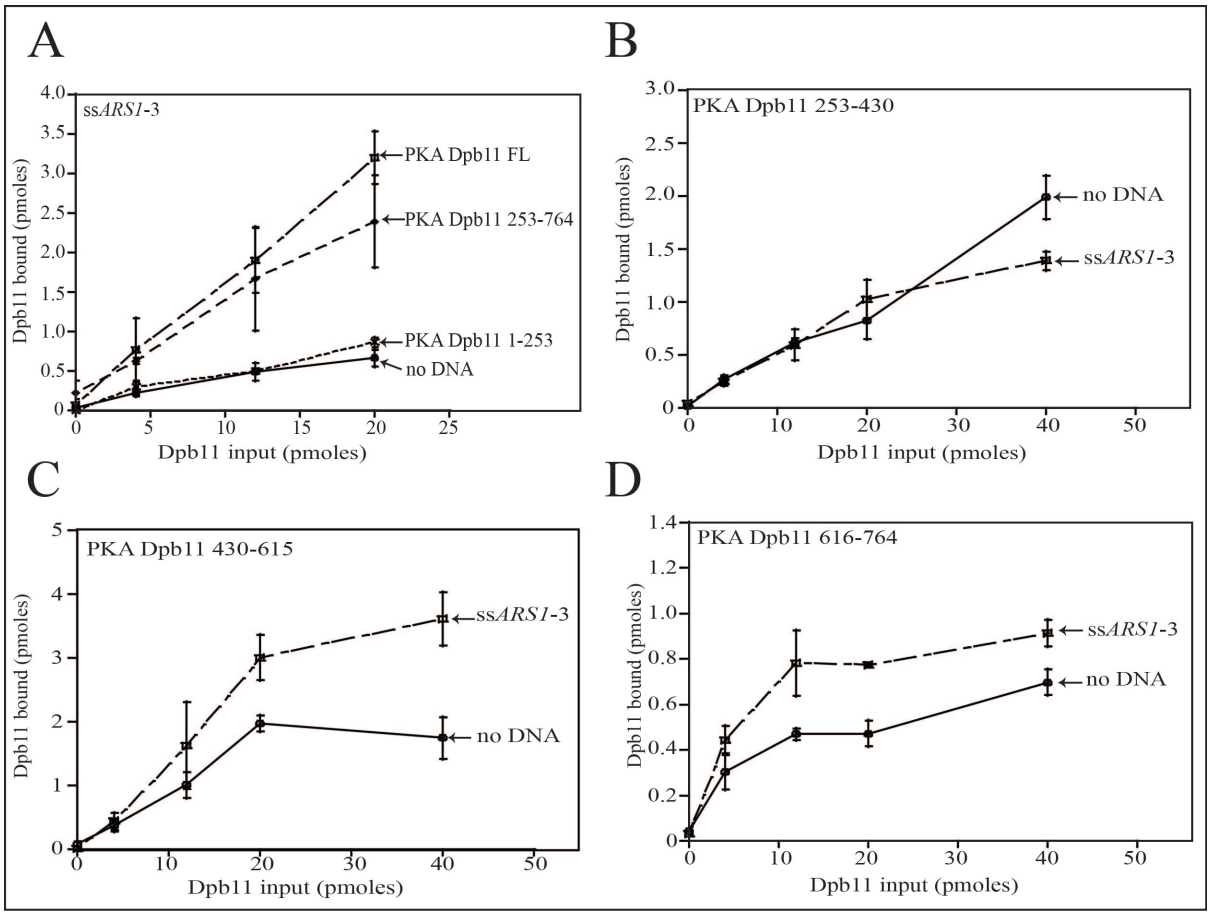


**Figure 2.5: The BRCT 4 motif or the C-terminal region of Dpb11 binds to single-stranded (ss) DNA.**

(A) (B), (C), (D) To determine the region of Dpb11 involved in Dpb11-ssDNA interaction, single-stranded DNA from yeast origins (*ARS1*-3) with a 3' biotin tag was conjugated to magnetic streptavidin beads. 4 pmoles of this biotinylated DNA was incubated with the different fragments of radiolabeled PKA-Dpb11 (as indicated in each figure) at 30°C for 10 minutes. As a control beads without DNA were incubated with radiolabeled PKA-Dpb11 fragments. The beads were washed and analyzed as described in Experimental Procedures. The results from the experiment were quantified, averaged and plotted as pmoles of Dpb11 bound versus the pmoles of input Dpb11.

**Dpb11 substantially inhibits GINS binding to Mcm2-7**

Dpb11 binds to the Mcm3 and Mcm5 subunits of Mcm2-7 (Figure 2.1), and the Mcm3 and Mcm5 subunits also bind to GINS (30). Thus, I next investigated whether Dpb11 competes

with GINS for binding to Mcm2-7. I incubated radiolabeled PKA-GINS with GST-Mcm2-7, and found a direct interaction between GINS and Mcm2-7 (Figure 2.6 A). I next added unlabeled Dpb11 to the reaction, and found that the addition of Dpb11 substantially inhibited the interaction between GINS and Mcm2-7 (Figure 2.6 A). These data suggest that Dpb11 competes with GINS for binding to Mcm2-7 (Figure 2.6 B for illustration).

**Single-stranded DNA competes with Dpb11 for binding to Mcm2-7**

Since the BRCT4 motif of Dpb11 binds to ssDNA and also to Mcm3 and Mcm5, I next investigated whether single-stranded DNA can displace Dpb11 from Mcm2-7 (Figure 2.6 C). I incubated GST-Dpb11 with radiolabeled PKA-Mcm2-7 and found a direct interaction between Dpb11 and Mcm2-7, as expected. Next, I added different concentrations of ss40dT and found that ss40dT disrupted the interaction between Dpb11 and Mcm2-7 (Figure 2.6 C). Similar results were found using the origin sequence *ARS1*-3 (not shown). This data suggests that ssDNA specifically disrupts the interaction between Dpb11 and Mcm2-7 (Figure 2.6 D for illustration).

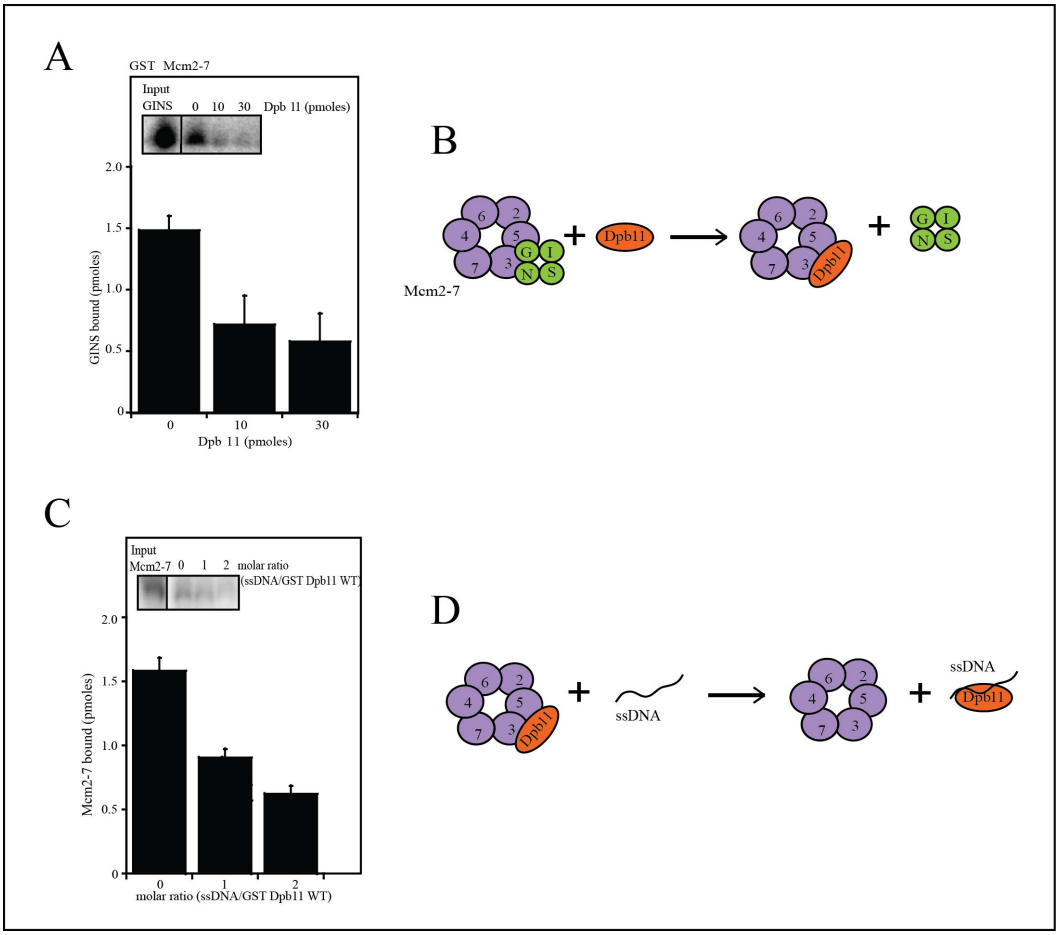


**Figure 2.6: Dpb11 competes with GINS for binding to Mcm2-7, and single-stranded DNA inhibits Dpb11 interaction with Mcm2-7.**

(A) 30 pmoles of GST-Mcm2-7 (contains GST at the N-terminus of Mcm3) was incubated with 30 pmoles of radiolabeled PKA GINS and different amounts of Dpb11 in a GST pulldown assay. The beads were washed and analyzed as described in Experimental Procedures. The results from similar experiments were quantified, averaged and plotted as pmoles of GINS bound versus pmoles of Dpb11 added. (B) A schematic showing the competition between Dpb11 and GINS for Mcm2-7 binding is also shown. (C) 30 pmoles of GST Dpb11 was incubated with 30 pmoles of radiolabeled PKA Mcm2-7 and varying amounts ss40dT in a GST pulldown assay. The beads were washed and analyzed as described in Experimental Procedures. Results from similar experiments were quantified, averaged and plotted as pmoles of Mcm2-7 bound versus the molar ratio of ss40dT/ GST Dpb11. (D) A model representing the inhibition of Dpb11-Mcm2-7 interaction by single-stranded DNA is also shown.

**Single-stranded DNA releases Dpb11 from Mcm2-7, allowing GINS to bind Mcm2-7.**

Since Dpb11 inhibits GINS binding to Mcm2-7 and ssDNA disrupts the interaction between Dpb11 and Mcm2-7, I next investigated whether the addition of ssDNA to a reaction with Dpb11, GINS and Mcm2-7 can rescue the binding of GINS to Mcm2-7. I incubated radiolabeled PKA-GINS with GST Mcm2-7 and found a direct interaction between GINS and Mcm2-7 (Figure 2.7 A). Next, I added unlabeled Dpb11 to the reaction, and found that the addition of Dpb11 inhibited the interaction between GINS and Mcm2-7 (Figure 2.7 A). To this, I further added ss40dT and found a substantial increase in the binding of GINS to Mcm2-7 (Figure 2.7 A). This data suggests that ssDNA releases Dpb11 from Mcm2-7, allowing GINS to bind Mcm2-7 (Figure 2.7 B for illustration).

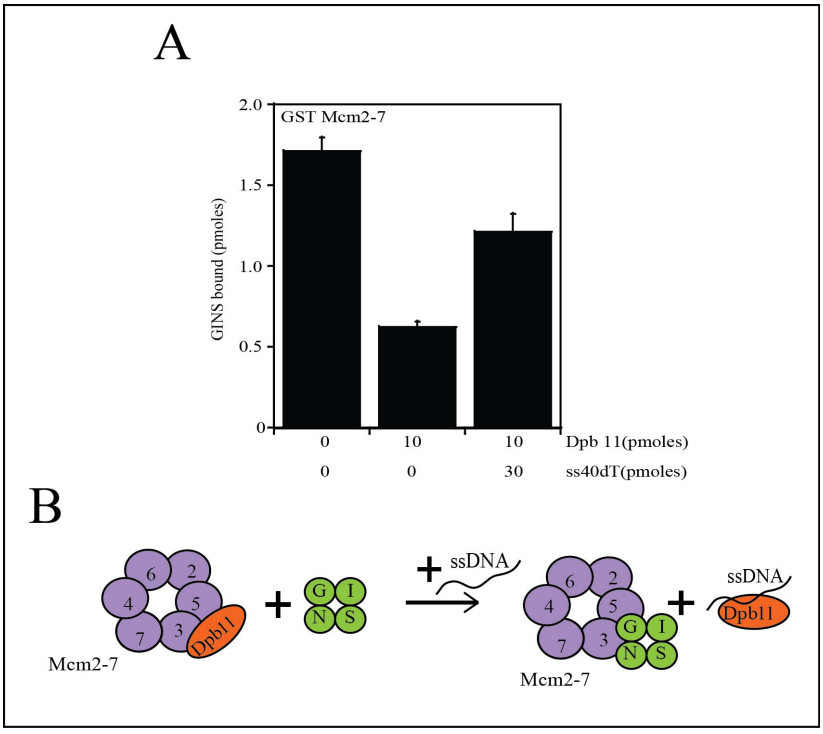


**Figure 2.7: Single-stranded DNA triggers the release of Dpb11 from Mcm2-7 and allows GINS to bind Mcm2-7.**

(A) 30 pmoles of GST-Mcm2-7 (contains GST at the N-terminus of Mcm3) was incubated with 30 pmoles of radiolabeled PKA GINS and different amounts of Dpb11 (0 and 10 pmoles) at

30°C for 10 minutes in a GST pulldown assay. Following this, 30 pmoles of ss40dT was added to the reaction containing Mcm2-7, GINS and Dpb11 and further incubated at 30°C for 10 minutes. The beads were washed and analyzed as described in Experimental Procedures. The bound radioactive GINS was analyzed by SDS/PAGE followed by phosphorimaging. These results were then quantified, averaged and plotted as pmoles of GINS bound versus pmoles of Dpb11 and pmoles of ss40dT added. (B) A model representing the release of Dpb11 from Mcm2-7 on addition of ssDNA and allowing GINS to bind Mcm2-7 is also shown.

## Conclusion

### Summary of results

I found using purified proteins that Dpb11 binds directly to Mcm2-7, or single-stranded DNA. Dpb11 inhibits the interaction between GINS and Mcm2-7. Interestingly, ssDNA disrupts the interaction between Dpb11 and Mcm2-7. Furthermore, the inhibition of GINS-Mcm2-7 interaction caused by Dpb11 can be overcome by the addition of ssDNA. Amino acids 430-615 of Dpb11 bind to Mcm3/Mcm5 and also ssDNA, suggesting a mechanism for competitive interaction between ssDNA and Mcm3/Mcm5 for binding to Dpb11. The region aa430-615 of Dpb11 is poorly conserved from yeast to humans, making it difficult to determine if the residues involved in ssDNA binding are conserved from yeast to human. Dpb11 prefers single-stranded DNA binding to blunt duplex binding. Furthermore, Dpb11 prefers long ssDNA (40 nucleotides) containing thymidines. Yeast origins typically contain a strand that is thymine-rich. In fact, it is the thymine-rich strand that is extruded from the central channel of Mcm2-7 during origin melting (123), suggesting a mechanism for Dpb11 interaction with origin ssDNA.

### Dpb11 prevents premature binding of GINS to Mcm2-7

Based on the *in vitro* data presented in this chapter, I hypothesize that Dpb11 actively regulates helicase assembly. Previous studies show that both Sld2 and Sld3 directly interact with Mcm2-7 and inhibit GINS interaction with Mcm2-7 (53, 58, 60). Similar to Sld2 and Sld3,

Dpb11 also binds directly to Mcm2-7 (Figure 2.1 A and 2.1 B) and prevents GINS binding to Mcm2-7 (Figure 2.6 A). The data from this chapter and from previous publications further suggests that Dpb11, Sld3, Sld2, and GINS all share an overlapping binding site on the Mcm2-7 complex, presumably at the Mcm3/Mcm5 subunits (83). Thus, I speculate that the cell has designed redundant mechanisms for preventing the premature binding of GINS to Cdc45-Mcm2-7 complex.

**Single-stranded DNA triggers the dissociation of Dpb11, Sld3, or Sld2 from Mcm2-7, thereby allowing GINS to bind Mcm2-7**

A recent study shows that DDK phosphorylation of Mcm2 weakens the interaction between Mcm2 and Mcm5 subunits of the Mcm2-7 complex. This results in the opening of the gate between Mcm2-Mcm5 subunits, thus facilitating the extrusion of T-rich ssDNA from the central channel of Mcm2-7 (48). Dpb11, Sld3, or Sld2 each bind individually to ssDNA (Figure 2.3) (53, 62). Furthermore, ssDNA disrupts the interaction between Dpb11 and Mcm2-7 (Figure 2.6), Sld2 and Mcm2-7 (61), or Sld3 and Mcm2-7 (62). These data suggest that once ssDNA is extruded from the central channel of Mcm2-7 during S phase, Dpb11, Sld3, and Sld2 will bind to ssDNA instead of Mcm2-7. This allows for GINS to bind Mcm2-7. The cell employs this elegant mechanism to ensure that GINS binds Mcm2-7 only after the extrusion of ssDNA, as premature binding of GINS to Mcm2-7-Cdc45 complex would seal the opening between Mcm2-Mcm5 subunits thus resulting in the formation of a CMG complex encircling double-stranded DNA and inhibiting replication initiation. Disengagement of Dpb11, Sld3, and Sld2 from Mcm2-7-Cdc45 complex in the presence of ssDNA, also suggests a mechanism to ensure that Dpb11, Sld3, and Sld2 do not travel with the replication fork.

**Acknowledgements**

I thank Dr. Susan Taylor for supplying purified PKA. I thank Dr. Mike O'Donnell for providing expression constructs for GINS. This work was supported by the National Science Foundation Grant 1265431 to D.L.K.

# CHAPTER III

## Dpb11 DIRECTLY INTERACTS WITH Cdc45[**]


### Abstract

Cdc45 and GINS bind to Mcm2-7 single hexamer in a regulated process to form the active CMG complex. The formation of an active CMG complex is highly regulated to ensure that it is formed only after single-stranded DNA is extruded from the central channel of Mcm2-7, as binding of GINS and Cdc45 to Mcm2-7 seals the opening between Mcm2-Mcm5, preventing the extrusion of ssDNA. This regulation requires the action of various replication factors along with DDK and CDK kinases. Dpb11 is a yeast protein that does not travel with the replication fork but its function is essential for replication initiation. In the previous chapter, I showed that Dpb11 directly binds Mcm2-7 and prevents the premature binding of GINS to Mcm2-7. However, in the presence of ssDNA, GINS can bind Mcm2-7 while Dpb11 binds ssDNA. I show here using purified proteins that Dpb11 also binds directly to Cdc45 and helps in its recruitment onto Mcm2-7. I also found that ssDNA inhibits the interaction of Dpb11 with Cdc45. This data supports our hypothesis that Dpb11 actively regulates the formation of the CMG helicase.


### Introduction

Mcm2-7 is a six-subunit protein complex (28) that is loaded onto replication origins as a double hexamer in $G_1$ phase and encircles double-stranded DNA. This loading of Mcm2-7

[**] The work presented in this chapter is published in Dhingra N, Bruck I, Smith S, Ning B, Kaplan D (2015) JBC.

double hexamer requires the action of ORC, Cdc6 and Cdt1 proteins (36, 37). However in the early S-phase Mcm2-7 is present as a single hexamer encircling single-stranded DNA (115). This single hexamer binds Cdc45 and GINS to form an active CMG complex (35, 83). The transition of an inactive Mcm2-7 double hexamer to an active CMG complex requires the activity of Dbf4-depnedent kinase and cyclin-dependent kinases along with other replication factors (119-121, 124). GINS interacts with the Mcm3 and Mcm5 subunits while Cdc45 interacts with the Mcm2 subunit of the Mcm2-7 complex (83) and together the CMG complex has a 300 fold higher ATPase activity and a higher helicase activity than the Mcm2-7 alone (30).

Dpb11 is an essential yeast protein that is required for the initiation of replication (56). Dpb11 binds CDK-phosphorylated Sld2 and Sld3 (52, 54, 110), which facilitates the formation of a Pre-Initiation Complex (Pre-IC) and recruitment of other replication factors. However, Dpb11, Sld3 and Sld2 do not travel along the replication fork. Previous published data using *Xenopus* Mus101 and human TopBP1 (functional homologs of Dpb11) has suggested a role of Dpb11 in the recruitment of Cdc45 onto chromatin (63, 64). In addition, Cdc45 was isolated as *SLD4* in *sld* (synthetically lethal with Dpb11) screening to identify factors interacting with Dpb11, suggesting that Cdc45 genetically interacts with Dpb11 (125).

It was previously demonstrated that Sld3 helps recruit Cdc45 to Mcm2-7 (12, 58, 126). Furthermore GINS disrupts the interaction between Sld3 and Cdc45 suggesting a mechanism for disengaging Sld3 from the replication fork helicase since Sld3 does not travel with the replication fork (58). In the previous chapter I have shown that Dpb11 directly binds Mcm3 and Mcm5 subunits of the Mcm2-7 complex and single-stranded DNA inhibits this interaction. Dpb11 also competes with GINS for Mcm2-7 binding. Here I show using purified proteins that yeast Dpb11 can also bind directly to Cdc45. Furthermore, Dpb11 can recruit Cdc45 to Mcm2-7

and single-stranded DNA also disrupts Dpb11-Cdc45 interaction. I propose a mechanism wherein Dpb11 first binds Mcm2-7 to help recruit Cdc45 onto Mcm2-7. Once single-stranded DNA is extruded from the central channel of Mcm2-7, Dpb11 dissociates from Mcm2-7 and Cdc45 and binds ssDNA. This allows for the formation of Mcm2-7-Cdc45 complex that can bind GINS to form the replication fork helicase. Dissociation of Dpb11 from the Mcm2-7-Cdc45 complex is important since Dpb11 does not travel with the replication fork.

## Experimental Procedures

### Cloning and purification of proteins

Full length GST tag, GST-Dpb11, PKA-Dpb11 and PKA Cdc45 were cloned and purified as described (53, 58).[††]

### Kinase labeling of proteins

Kinase labeling of PKA-tagged Dpb11, Mcm2-7 (Mcm3) and Cdc45 was performed as described (53).

### GST pull-down assays

The GST pulldown assays were performed as described (53). GST- tagged protein (GST-Dpb11 or GST Cdc45) attached to prepared Glutathione Sepharose was incubated with varying concentrations of radiolabeled protein or DNA (as described in each figure) in GST binding buffer (40 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 10 % glycerol, 0.1% Triton X-

[††] Cdc45 was purified by Dr. Irina Bruck.

100, 1 mM DTT, 0.7 µg/ml pepstatin, 0.1 mM PMSF and 0.1 mg/ml BSA) in a final reaction volume of 100 µl. The reactions were incubated at 30°C for 10 minutes. Following incubation, the reactions were shifted to room temperature and glutathione sepharose beads were allowed to settle. The supernatant was removed and the beads were washed two times with GST binding buffer. After the last wash, beads were heated at 90°C for 10 minutes in solution containing 2% SDS, 4% glycerol, 4 mM Tris-HCl, 2 mM DTT and 0.01% bromophenol blue. The reactions were then analyzed by SDS/PAGE followed by phosphor-imaging and quantification.

## Results

### Dpb11 binds directly to Cdc45

Dpb11 binds to Mcm5, which is adjacent to Mcm2 in the Mcm2-7 ring (Figure 2.1 C). Furthermore, Cdc45 binds to Mcm2 (Figure 2.1 C). Thus, Dpb11 is positioned to recruit Cdc45 to the Mcm2-7 ring. To investigate whether Dpb11 recruits Cdc45 to Mcm2-7, I first determined whether Dpb11 binds directly to Cdc45 (Figure 3.1). GST-Dpb11 was incubated with radiolabeled PKA-Cdc45, and a direct interaction was observed by a pulldown assay. (Figure 3.1)

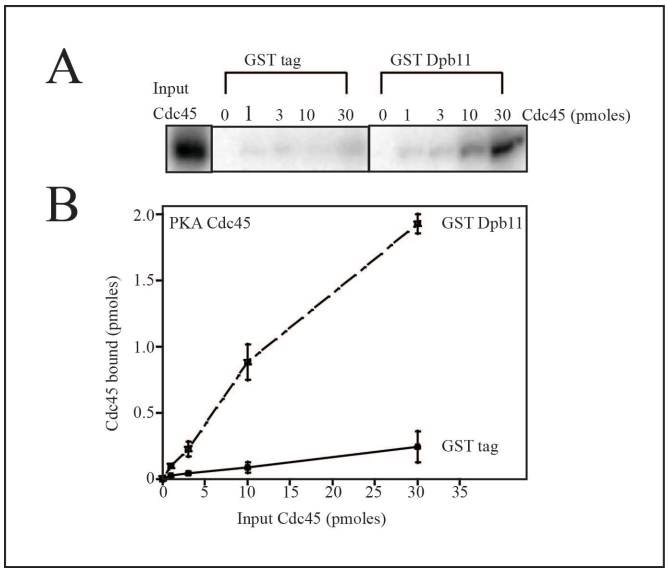


**Figure 3.1: Dpb11 directly binds to Cdc45.**

(A) 30 pmoles of GST tag or GST Dpb11 was incubated with glutathione sepharose and varying amounts of radiolabeled PKA-Cdc45 in a GST pulldown assay. The beads were washed and analyzed as described in Experimental Procedures. (B) The results from experiments similar to (A) were quantified, averaged and plotted as pmoles of Cdc45 bound versus pmoles of Cdc45 input.

**Dpb11 recruits Cdc45 onto Mcm2-7**

To test whether Dpb11 can recruit Cdc45 to Mcm2-7, I next incubated GST-Cdc45 with radiolabeled PKA-Mcm2-7 and increasing concentrations of radiolabeled PKA-Dpb11 (Figure 3.2). I observed a direct, weak interaction between Cdc45 and Mcm2-7, as expected, but as Dpb11 was added in increasing concentrations, the amount of Cdc45 bound to Mcm2-7 substantially increased (Figure 3.2). Furthermore, since both Dpb11 and Mcm2-7 were radiolabeled in this reaction, I observe a simultaneous increase in binding of Dpb11 and Mcm2-7 to Cdc45 (Figure 3.2). These data suggest that Dpb11 can recruit Cdc45 to Mcm2-7 (Figure 3.2 C for illustration).

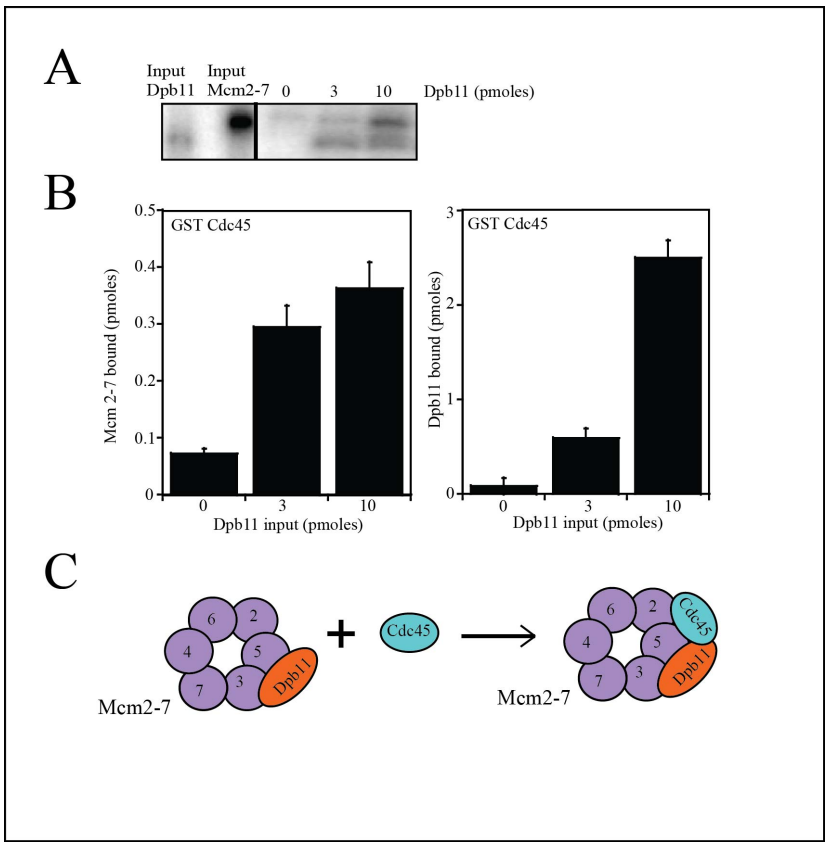


**Figure 3.2: Dpb11 recruits Cdc45 to the Mcm2-7 complex.**

(A) 10 pmoles of GST-Cdc45 was incubated with 10 pmoles of radiolabeled PKA-Mcm2-7 and varying amounts of radiolabeled PKA-Dpb11 in a GST pulldown assay. (B) Results from experiments similar to (A) were quantified, averaged and plotted as pmoles of Mcm2-7 bound versus pmoles of Dpb11 input and pmoles of Dpb11 bound versus pmoles of Dpb11 input. (C) A model representing the recruitment of Cdc45 to Mcm2-7 with the help of Dpb11 is also shown.

**Single-stranded DNA competes with Cdc45 for Dpb11 interaction.**

Cdc45 and Dpb11 interaction must be transient, since Cdc45 travels with the replication fork and Dpb11 does not (126). Thus, some factor must dislodge Cdc45 from Dpb11. To determine if single-stranded DNA disrupts Cdc45-Dpb11 interaction, I incubated GST-Dpb11 with radiolabeled PKA-Cdc45 and increasing concentrations of ss40dT. I found that ssDNA disrupts the interaction between Cdc45 and Dpb11 (Figure 3.3 A), suggesting a mechanism for

dissociation *in vivo* (Figure 3.3 B for illustration). Similar results were found using *ssARS1*-3 (not shown).

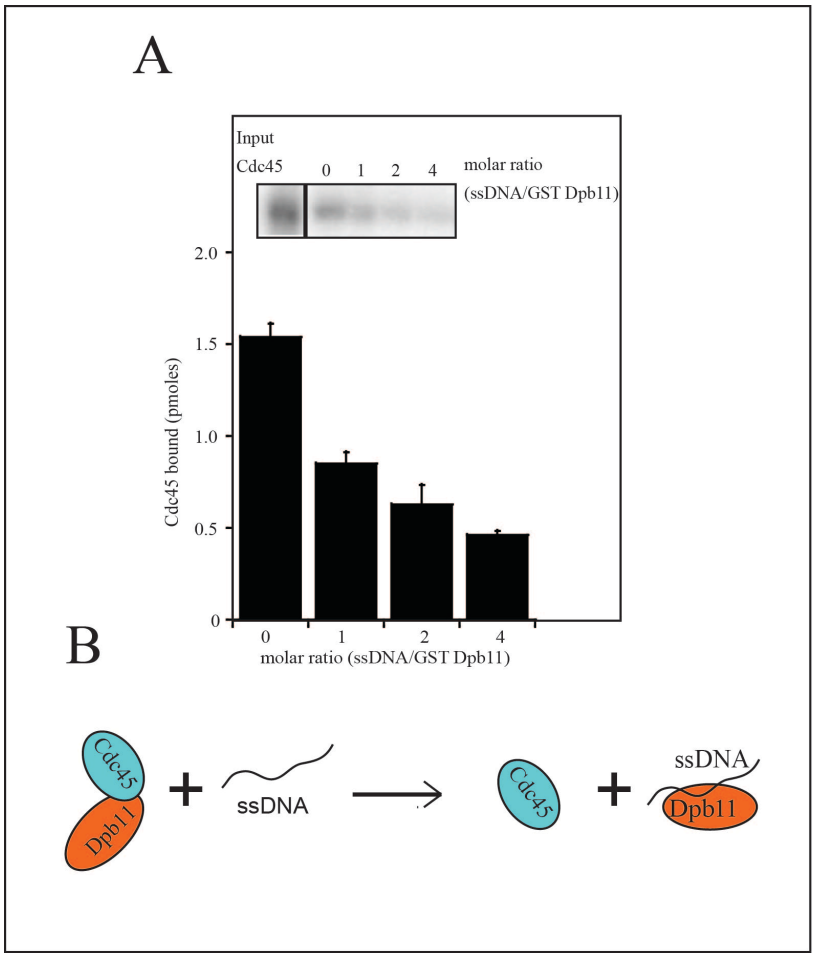


**Figure 3.3: Single-stranded DNA disrupts the interaction between Cdc45 and Dpb11.**

(A) 30 pmoles of GST Dpb11 was incubated with 30 pmoles of radiolabeled PKA-Cdc45 and varying amounts ss40dT in a GST pulldown assay. The beads were washed and analyzed as described in Experimental Procedures. The results from similar experiments were quantified, averaged and plotted as pmoles of Cdc45 bound versus the molar ratio of ss40dT/GST-Dpb11. (B) A model showing the inhibition of Dpb11-Cdc45 interaction by single stranded DNA is also shown.

## Conclusion

### Summary of results

I found using purified proteins that Dpb11 binds directly to Cdc45. Dpb11 can recruit Cdc45 to the Mcm2-7 complex. The recruitment of Cdc45 to Mcm2-7 may be conserved in higher organisms, since it has been shown that Xmus101 may recruit Cdc45 to replication origins in *Xenopus* extract assays (63). Interestingly, ssDNA disrupts the interaction between Dpb11 and Cdc45.

### Cdc45 is recruited to Mcm2-7 by Sld3, and possibly by Dpb11 and Sld7

Previous data suggests that Sld3 is responsible for the recruitment of Cdc45 to Mcm2-7 (12). I show here that Dpb11 can also function to recruit Cdc45 to Mcm2-7 *in vitro*, suggesting an additional mechanism for Cdc45 recruitment to Mcm2-7 *in vivo*. A recent study also shows that high salt stable Cdc45 recruitment onto Mcm2-7 requires the presence of Dpb11 (124). Sld7 may also play a role in the recruitment of Cdc45 to Mcm2-7 (127). This redundancy may underscore the critical importance of recruitment of Cdc45 to Mcm2-7 complex. It has also been shown that DDK phosphorylates Mcm4, relieving an inhibitory function for the N-terminus of Mcm4 (44). DDK phosphorylation of Mcm4 may also be important for the binding of Cdc45 to loaded Mcm2-7 (45, 113). Furthermore, DDK phosphorylation of Mcm6 may also be important for cell growth (113, 128). Cdc45 binding to Mcm2-7 may mark the Mcm2-7 complex as one that will be subsequently activated, since Cdc45, Sld3, Dpb11 and even Dbf4 (DDK subunit) are limiting, while loaded Mcm2-7 complexes are in excess of active Mcm2-7 complexes (13, 34, 127). Future work may help resolve how DDK stimulates Cdc45 recruitment to Mcm2-7.

**Dpb11 actively regulates helicase assembly**

The data presented in this chapter supports our hypothesis that Dpb11 plays a vital role in the formation of Cdc45-Mcm2-7-GINS complex, as Dpb11 helps in the recruitment of Cdc45 onto Mcm2-7 in addition to regulating GINS interaction with Mcm2-7. Interestingly, single-stranded DNA inhibits Dpb11 interaction with both Mcm2-7 and Cdc45. Thus, the extrusion of ssDNA from the central channel of Mcm2-7 could act as a trigger to finalize the assembly of the CMG complex. The ability of ssDNA to displace Dpb11 from Mcm2-7 and Cdc45 thus fulfills a sophisticated regulatory role to ensure that the CMG complex can only assemble once a single-strand is extruded from the central channel of Mcm2-7.

**Acknowledgements**

I thank Dr. Susan Taylor for supplying purified PKA. This work was supported by the National Science Foundation Grant 1265431 to D.L.K.

# CHAPTER IV

## Dpb11 ACTIVELY REGULATES THE ASSEMBLY OF Cdc45-Mcm2-7-GINS COMPLEX[‡‡]


### Abstract

Dpb11, Sld2, and Sld3 are required for the initiation of DNA replication, but these three proteins do not travel with the replication fork. During S phase, S-CDK phosphorylates Sld2 and Sld3, resulting in the formation of an Sld2-Sld3-Dpb11 complex. In chapter II, I showed that Dpb11, like Sld2 and Sld3, blocks the association of GINS with Mcm2-7. Here I identify a mutant of the BRCT4 motif of Dpb11 that remains bound to Mcm2-7 in the presence of ssDNA (dpb11- *m1,m2,m3,m5),* and this mutant exhibits a DNA replication defect when expressed in budding yeast cells. Expression of this mutant results in increased interaction between Dpb11 and Mcm2-7 during S phase, impaired GINS interaction with Mcm2-7 during S phase, and decreased RPA interaction with origin DNA during S phase. I propose a model for helicase regulation wherein Sld2, Sld3, and Dpb11 inhibit the interaction of GINS with Mcm2-7 when no ssDNA is present. However, in the presence of ssDNA, Sld2, Sld3, and Dpb11 dissociate from Mcm2-7-Cdc45 complex, allowing GINS to bind Cdc45-Mcm2-7. Thus, Sld2, Sld3, and Dpb11 have similar roles in replication initiation, forming a triad for helicase regulation.


[‡‡] The work presented in this chapter is published in Dhingra N, Bruck I, Smith S, Ning B, Kaplan D (2015) JBC.

## Introduction

Eukaryotic replication is a highly controlled process and is tightly regulated to ensure that chromosomes duplicate only once per cell cycle and that genomic integrity is maintained. Initiation of DNA replication is defined by the presence of an active helicase, unwinding of DNA helix and loading of DNA polymerase and the rest of the DNA synthesis machinery. Mcm2-7 is loaded as a double hexamer encircling double-stranded DNA. However, in S-phase Mcm2-7 is present as a single hexamer encircling single-stranded DNA. Electron microscopy studies have shown that Mcm2-7 complex is present in equilibrium between an open and a closed form, while the CMG complex exists in the closed form and exhibits helicase activity (83). The CMG complex was also shown to have 300-fold increase of ATPase activity over Mcm2-7 alone. The CMG complex thus adopts a locked conformation that encircles only ssDNA (29, 30, 83).

*DPB11*, an essential *Saccharomyces cerevisiae* gene, functions in the initiation of DNA replication and the DNA damage checkpoint (56). CDK phosphorylation of Sld2 and Sld3 promotes their interaction with the C and N terminal BRCT domains of Dpb11, respectively. Though Dpb11 does not travel with the replication fork, it plays an essential role in the initiation of replication (52, 54, 125, 129). It was previously demonstrated that Sld2 and Sld3 independently associate with Mcm2-7 and block the Mcm2-7 interaction with GINS (58, 61). Sld3 and phosphorylated Sld2 also independently associate with single-stranded (ss) DNA and that this association plays an important role in the formation of the CMG complex (Cdc45-Mcm2-7-GINS), the eukaryotic replication fork helicase (53, 60, 62).

Our data presented in chapters II and III shows that Dpb11 also binds Mcm2-7, Cdc45 and ssDNA directly. Dpb11-Mcm2-7 interaction prevents GINS binding to Mcm2-7 while ssDNA triggers the dissociation of Dpb11 from Mcm2-7-Cdc45 complex. This results in GINS

binding to the Mcm2-7-Cdc45 complex. This data suggests that Dpb11 functions like Sld2 and Sld3 in the formation of an active replication fork helicase.

To support our hypothesis, I identify mutations in Dpb11 BRCT4 that regulate Dpb11-Mcm2-7 interaction. I constructed a mutant Dpb11 (*dpb11-m1, m2, m3, m5*) with mutations in the BRCT4 region, which partially binds ssDNA. However, unlike the wild type Dpb11 that dissociates from Mcm2-7 in the presence of ssDNA (Figure 2.6 C), the mutant *dpb11-m1, m2, m3, m5* protein remains bound to Mcm2-7 even in the presence of ssDNA. When mutant *dpb11-m1,m2,m3,m5* (BRCT4 motif mutant of Dpb11 that remains bound to Mcm2-7 in the presence of ssDNA) is expressed in yeast cells, the cells suffer severe growth and DNA replication defects. Expression of the *dpb11-m1,m2,m3,m5* mutant results in decreased GINS-Mcm2-7 interaction during S phase, and increased Dpb11 interaction with Mcm2-7 during S phase. These data suggest that Dpb11 functions to regulate assembly of the CMG complex. Expression of *dpb11-m1,m2,m3,m5* also results in decreased RPA interaction with origin DNA during S phase. I propose a model wherein Dpb11 directly binds to Mcm2-7 and blocks the premature formation of the CMG complex by inhibiting GINS interaction with Mcm2-7. However, once single-stranded DNA is extruded from the central channel of Mcm2-7, Dpb11 dissociates from Mcm2-7 and Cdc45 to bind ssDNA, thereby allowing for the formation of the CMG complex.

## Experimental Procedures

### Cloning and purification of proteins

Full length *DPB11* with PKA or GST tag was cloned and purified as described (53). DPB11 constructs with single or multiple mutations in the BRCT4 region were cloned in full

length Dpb11 and purified similar to the wild-type Dpb11. PKA-Mcm2-7, PKA-Mcm3, PKA-Mcm5, PKA-Cdc45 and PKA-Sld2T84D were purified as described (53, 58).

**Kinase labeling of proteins**

Kinase labeling was performed as described (53).

**GST pull-down assays**

The GST pulldown assays were performed using GST-tagged WT-Dpb11 and Dpb11 mutants as described (53).

**Biotin pull-down assays**

Biotinylated DNA (4 pmoles) conjugated to streptavidin-agarose magnetic beads was incubated with different concentrations of radiolabeled Dpb11 in a solution containing 20 mM Tris-HCl pH 7.5, 100 μM EDTA, 10% glycerol, 40 μg/ml BSA, 10 mM magnesium acetate and 200 μM DTT in a final reaction volume of 25 μl. The reactions were incubated at 30°C for 10 minutes. After the incubation, the magnetic beads were collected at room temperature using a magnet (Dynal). The supernatant was removed and the beads were washed twice with a solution containing 20 mM Tris-HCl pH 7.5, 100 μM EDTA, 10% glycerol and 40 μg/ml BSA. After the second wash, the beads were collected and heated at 90°C for 10 minutes in a solution containing 2% SDS, 4% glycerol, 4 mM Tris-HCl, 2 mM DTT and 0.01% bromophenol blue. The reactions were analyzed by SDS/PAGE. The gel was dried at 80°C for 1 hour and then exposed to phosphorimaging screen for 30 minutes.

**Yeast strains and plasmids**

Degron strain *dpb11-td* [YJT70, MAT a ade2-1 ura3-1 his 3-11, 15 trp 1-1 leu2-3, 112 can 1-100 dpb11-td (DPB11 5'upstream -100 to -1 is replaced with kanMX-tTA tetR-VP16-$tetO_2$-Ub-DHFRts-HA-linker) UBR1::GAL-Ubiquitin-M-lacI fragment-Myc-UBR1 (HIS3) leu2-3,112::pCM244 (tetR'-SSN6, LEU2)] was a generous gift from John F.X. Diffley (London Research Institute, Cancer Research UK, London, UK) (54). The degron strain *dpb11-td* was transformed with a PRS416 vector containing an empty vector, *DPB11* wild type, or *dpb11-m1,m2,m3,m5* (BRCT4 motif mutant that remains bound to Mcm2-7 in the presence of ssDNA) under the control of native *DPB11* promoter. Positive transformants were selected on CSM-Ura plates.

**Serial dilution analysis**

Serial dilution was performed as described (61). Yeast strains in overnight culture (CSM-Ura containing raffinose, 30°C) were transferred into YPGal media containing 50 µg/ml doxycycline and incubated for 2 hrs at 37°C. The 10 fold serial dilution was performed and spotted onto a plate containing CSM-Ura, which was incubated at 30°C (permissive conditions) and a plate containing CSM-Ura+Gal+50 µg doxycycline, which was incubated at 37°C (restrictive conditions) for two days.

**Fluorescence Activated Cell Sorting (FACS)**

FACS was performed as described (61). The strains were grown overnight in CSM-Ura media containing raffinose at 30°C. For $G_1$ arrest $6x10^6$ cells/ml were treated with α-factor (Zymo Research) for 3 hrs at 37°C in YPGal media containing 50 µg/ml doxycycline. Following

extensive washes and addition of 50 µg/ml Pronase (Calbiochem) to fresh YPGal+Doxycycline, cells were further incubated at 37°C. Cells were collected at indicated time intervals and stained with propidium iodide. Cell cycle progression data was obtained using the BD FACS Canto Ruo Special Order System and analyzed using the FACS Diva Software.

**Antibodies**

Antibody directed against RPA was purchased (RPA-Pierce MA1-25889). Antibodies against Cdc45, GINS, Sld2, Dpb11 and Mcm2 were generated and purified as described (48, 60, 61, 76).

**Chromatin immunoprecipitation (ChIP)**

For $G_1$ arrest and release $6x10^6$ cells/ml were treated with α-factor (Zymo Research) for 3 hrs at 37°C in YPGal media containing 50 µg/ml doxycycline. Following extensive washes and addition of 50 µg/ml Pronase (Calbiochem) to fresh YPGal+Doxycycline, cells were further incubated at 37°C for the indicated time. Chromatin immunoprecipitation was performed as described (76). I performed PCR with [$^{32}$P-α]-dCTP as a component of the PCR reaction to quantify the amplified product. Formaldehyde cross-linked cells were lysed with glass beads in a Bead Beater. DNA was fragmented by sonication (Branson 450). RPA antibody and magnetic protein A beads (Dynabeads protein A, Invitrogen 100.02D) were added to the cleared lysate to immunoprecipitate the DNA. Immunoprecipitates were washed extensively to remove nonspecific DNA. Eluted DNA was then subjected to PCR analysis using primers directed against *ARS306* or a site midway between *ARS306* and *ARS305* as described (70). The radioactive band in the native gel, representing specific PCR amplified DNA product was

quantified by phosphorimaging and normalized by a reference standard run in the same gel. The reference standard was a PCR reaction with a known quantity of template DNA replacing the immunoprecipitate.

**Co-immunoprecipitation**

For $G_1$ arrest and release $6x10^6$ cells/ml were treated with α-factor (Zymo Research) for 3 hrs at 37°C in YPGal media containing 50 µg/ml doxycycline. Following extensive washes and addition of 50 µg/ml Pronase (Calbiochem) to fresh YPGal+Doxycycline, cells were further incubated at 37°C for the indicated time. Co-immunoprecipitation was performed as described (60). Cells were collected and lysed at 4°C with glass beads in IP buffer (100mM HEPES-KOH pH 7.9, 100mM potassium acetate, 10mM magnesium acetate, 2mM sodium fluoride, 1mM PMSF, 0.1 mM Na3VO4, 20mm β-gycerophosphate, 1% Triton X-100, leupeptin, pepstatin, 1x complete protease inhibitor cocktail without EDTA (Roche)). Lysed material was treated with 200U of Benzonase nuclease (Novagen 70746-3) at 4°C for 1 hour. Clarified extract was then mixed with 2 µl of specified antibody and rotated for 2 hours at 4°C. Following this, 7 µl of Dynabeads Protein A (Invitrogen 100.01D) beads equilibrated with IP buffer were added to the extract and further rotated for 1 hr at 4°C. Beads were washed twice with 500 µl of IP buffer and finally resuspended in SDS-sample buffer. Western analysis was performed and blots were scanned using the LI-COR Odyssey Infrared Imager and analyzed in the Image Studio 4.0 Software.

## Results

### Identification of Dpb11 mutant that is defective in ssDNA binding

To study the effect of Dpb11-ssDNA binding *in vivo,* I identified mutations that disrupt Dpb11-ssDNA binding. Since I observed that Dpb11-430-615 fragment bound to *ssARS1*-3, I further did a fungal alignment and found that there are 8 conserved positively charged residues in the region 430-615 of Dpb11 (not shown). Anticipating that a charge reversal mutation of a positively charged residue may inhibit interaction with the negatively charged ssDNA, I engineered 6 full-length Dpb11 mutants (m1, m2, m3, m4, m5 and m6) that contain one or more charge reversal mutations (Figure 4.1 A). I incubated biotinylated *ssARS1*-3 with the different mutants of Dpb11 and found that mutations in m1, m2, m3 and m5 partially decreased the interaction between Dpb11 and *ssARS1*-3 DNA (Figure 4.1 B). In contrast, incubation with dpb11-m4 or dpb11-m6 resulted in similar pulldown efficiency compared to wild-type Dpb11 (not shown). In order to find a Dpb11 mutant that is defective in ssDNA binding, I combined mutations m1, m2, m3 and m5 to form a quadruple mutant. In addition, I also deleted the unstructured C-terminal region of Dpb11 (Dpb11-616-764), since I observed that Dpb11-616-764 also binds to ssDNA. This Dpb11 mutant (dpb11-m1,m2,m3,m5,Δ CTD, quadruple mutant plus C-terminal deletion) is defective in binding to ssDNA when compared with the wild type Dpb11, as observed in a biotin pulldown assay using biotinylated *ssARS1*-3 with radiolabeled Dpb11 (Figure 4.1 C and 4.1 D). However, the Dpb11 mutant (dpb11-m1,m2,m3,m5,ΔCTD, quadruple mutant plus C-terminal deletion) binds to Sld2T84D, Mcm2-7 and Cdc45 like the wild type full length Dpb11 (Figure 4.1 E, 4.1 F and 4.1 G).

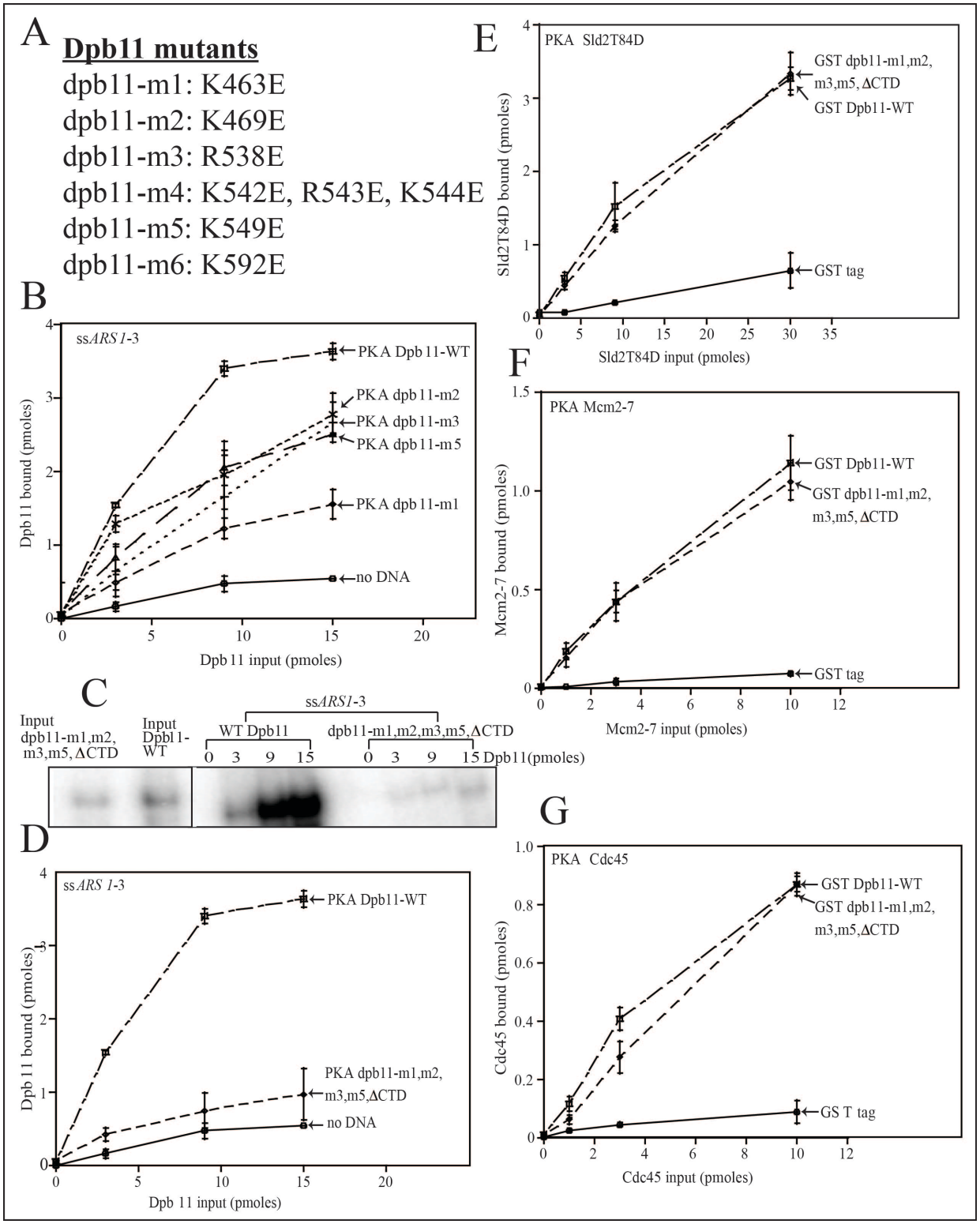
A
Dpb11 mutants
dpb11-m1: K463E
dpb11-m2: K469E
dpb11-m3: R538E
dpb11-m4: K542E, R543E, K544E
dpb11-m5: K549E
dpb11-m6: K592E
B
ssARS1-3
PKA Dpb11-WT
PKA dpb11-m2
PKA dpb11-m3
PKA dpb11-m5
PKA dpb11-m1
no DNA
Dpb11 bound (pmoles)
Dpb11 input (pmoles)
C
Input dpb11-m1,m2, m3,m5,ΔCTD
Input Dpb11-WT
ssARS1-3
WT Dpb11
dpb11-m1,m2,m3,m5,ΔCTD
0 3 9 15
0 3 9 15 Dpb11(pmoles)
D
ssARS 1-3
PKA Dpb11-WT
PKA dpb11-m1,m2, m3,m5,ΔCTD
no DNA
Dpb11 bound (pmoles)
Dpb 11 input (pmoles)
E
PKA Sld2T84D
GST dpb11-m1,m2, m3,m5, ΔCTD
GST Dpb11-WT
GST tag
Sld2T84D bound (pmoles)
Sld2T84D input (pmoles)
F
PKA Mcm2-7
GST Dpb11-WT
GST dpb11-m1,m2, m3,m5, ΔCTD
GST tag
Mcm2-7 bound (pmoles)
Mcm2-7 input (pmoles)
G
PKA Cdc45
GST Dpb11-WT
GST dpb11-m1,m2, m3,m5,ΔCTD
GS T tag
Cdc45 bound (pmoles)
Cdc45 input (pmoles)

**Figure 4.1: A Dpb11 mutant (dpb11-m1,m2,m3,m5,ΔCTD) does not bind ssDNA, but it binds Sld2T84D, Mcm2-7 and Cdc45 like wild type Dpb11.**

(A) Charge reversal mutations in the BRCT 4 region of Dpb11 (amino acids 430-615) which were studied for interaction with ss*ARS1*-3. (B) Various concentrations of radiolabeled full length PKA-Dpb11-wild type, dpb11-m1, dpb11-m2, dpb11-m3, dpb11-m4 (not shown, binds like Dpb11-wild-type), dpb11-m5 or dpb11-m6 (not shown, binds like Dpb11-wild-type) were incubated with 4 pmoles of ss*ARS1*-3 in a biotin pulldown assay. The beads were washed and analyzed as described in Experimental Procedures. The results from the experiment were quantified, averaged and plotted as pmoles of Dpb11 bound versus the pmoles of input Dpb11. (C) Different concentrations of PKA-Dpb11 and PKA-dpb11-m1,m2,m3,m5,ΔCTD were incubated with 4 pmoles of ss*ARS1*-3 in a biotin pulldown assay. The beads were washed and analyzed as described in Experimental Procedures. (D) The results from experiments similar to (C) were quantified, averaged and plotted as pmoles of Dpb11 bound versus pmoles of Dpb11 input. (E) Different concentrations of radiolabeled PKA-Sld2T84D were incubated with 30 pmoles of GST-Dpb11 wild type, GST-dpb11-m1,m2,m3,m5,ΔCTD or GST tag alone in a GST pulldown assay as described in the Experimental Procedures. The bound radioactive Sld2T84D was analyzed by SDS/PAGE followed by phosphorimaging. These results were then quantified, averaged and plotted as pmoles of Sld2T84D bound versus the pmoles of Sld2T84D input. (F) Different concentrations of radiolabeled PKA-Mcm2-7 was incubated with 10 pmoles of GST-Dpb11 wild type, GST-dpb11-m1,m2,m3,m5,ΔCTD or GST tag alone in a GST pulldown assay as described in the Experimental Procedures. The bound radioactive Mcm2-7 was analyzed by SDS/PAGE followed by phosphorimaging. These results were then quantified, averaged and plotted as pmoles of Mcm2-7 bound versus the pmoles of Mcm2-7 input. (G) Different concentrations of radiolabeled PKA-Cdc45 was incubated with 10 pmoles of GST-Dpb11 wild type, GST- dpb11-m1,m2,m3,m5,ΔCTD or GST tag alone in a GST pulldown assay as described in the Experimental Procedures. The bound radioactive Cdc45 was analyzed by SDS/PAGE followed by phosphorimaging. These results were then quantified, averaged and plotted as pmoles of Cdc45 bound versus the pmoles of Cdc45 input.

**Expression of a BRCT4 motif mutant of Dpb11 that remains bound to Mcm2-7 in the presence of ssDNA exhibits severely inhibited cell growth and DNA replication**

Since the BRCT4 motif of Dpb11 binds to ssDNA and also to Mcm3 and Mcm5, I hypothesized that this BRCT4 region of Dpb11 may be responsible for mediating competition between ssDNA and Dpb11 for Mcm2-7. To investigate if the ssDNA binding residues of the BRCT4 motif are regulating Dpb11-Mcm2-7 interaction, I constructed a mutant with charge reversal mutations of the BRCT4 motif that are specifically defective in ssDNA binding (dpb11-

m1,m2,m3,m5). This mutant is not completely defective in ssDNA as it binds partially to ssDNA (Figure 4.2) because the C-terminal region of Dpb11 also binds to ssDNA.

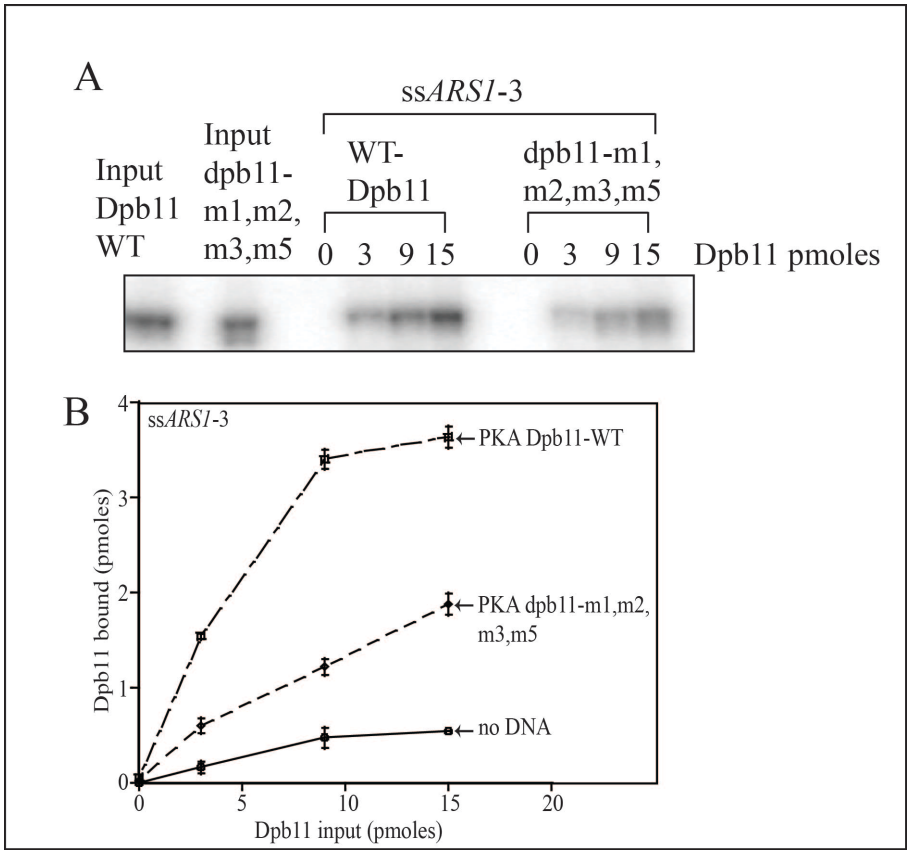


**Figure 4.2: A BRCT4 motif mutant of Dpb11 (dpb11-m1,m2,m,m5) partially binds ssDNA.**

(A) Different concentrations of PKA-Dpb11 and PKA-dpb11-m1,m2,m3,m5 were incubated with 4 pmoles of ss*ARS1*-3 in a biotin pulldown assay. The beads were washed and analyzed as described in Experimental Procedures. (B) The results from experiments similar to (A) were quantified, averaged and plotted as pmoles of Dpb11 bound versus pmoles of Dpb11 input.

However, this mutant (dpb11-m1,m2,m3,m5) binds to Mcm2-7, Mcm3, and Mcm5 like wild-type Dpb11 (Figures 4.3 A, 4.3 B, and 4.3 C). Furthermore, unlike wild-type Dpb11, mutant Dpb11 (dpb11-m1,m2,m3,m5) remains bound to Mcm2-7 in the presence of single-

stranded DNA (Figure 4.3 D). In addition, mutant Dpb11 (dpb11-m1,m2,m3,m5) also binds to Sld2T84D and Cdc45 like the wild type full length Dpb11 (Figure 4.3 E and 4.3 F), suggesting that the observed defect is specific.

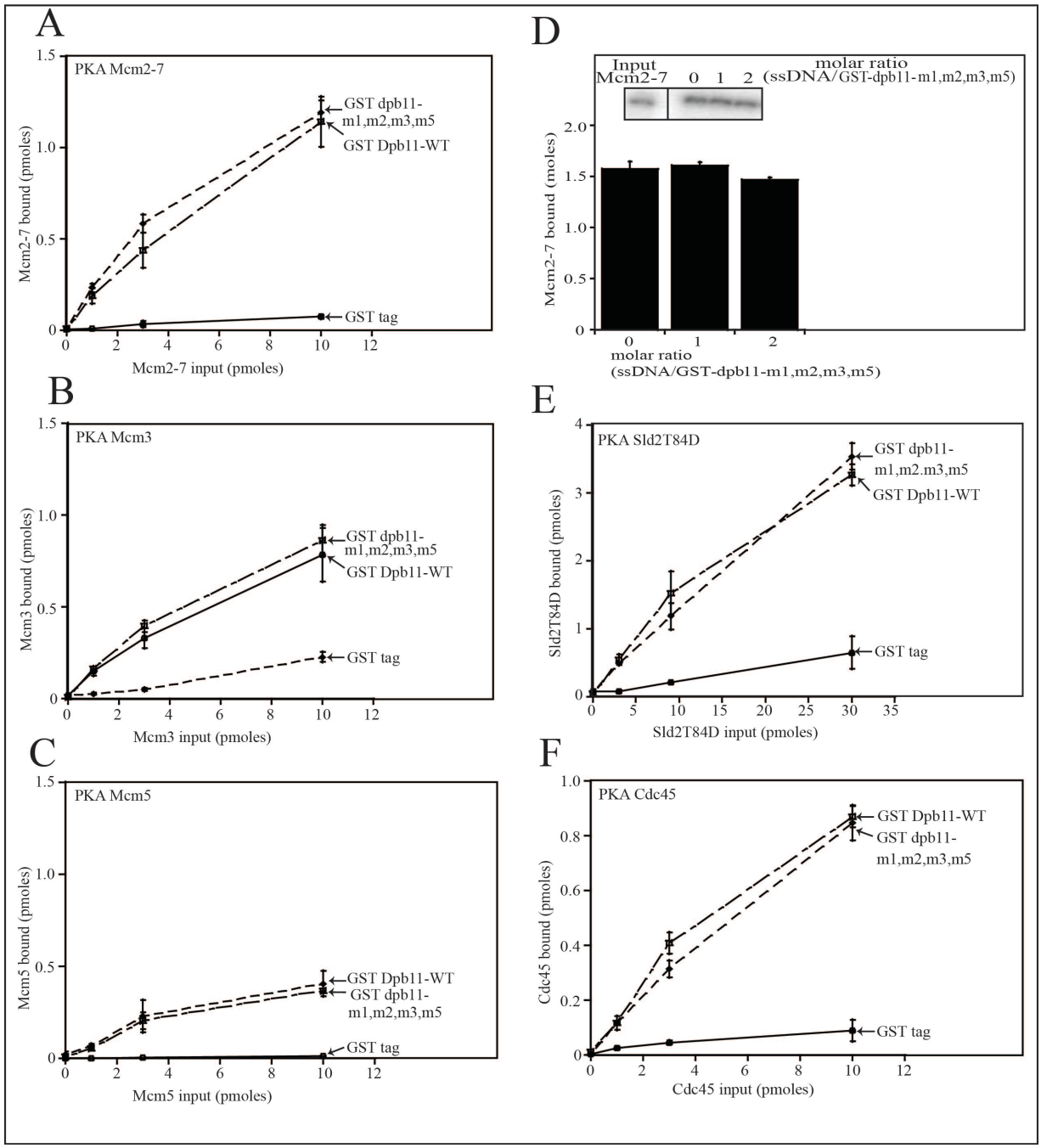

**Figure 4.3: A BRCT4 motif mutant of Dpb11 (dpb11-m1,m2,m3,m5) remains bound to Mcm2-7 in the presence of single-stranded DNA.**

(A) Different concentrations of radiolabeled PKA-Mcm2-7 was incubated with 10 pmoles of GST-Dpb11 wild type, GST-dpb11-m1,m2,m3,m5 or GST tag alone in a GST pulldown assay as described in the Experimental Procedures. The bound radioactive Mcm2-7 was analyzed by SDS/PAGE followed by phosphorimaging. These results were then quantified, averaged and plotted as pmoles of Mcm2-7 bound versus the pmoles of Mcm2-7 input. (B) Different concentrations of radiolabeled PKA-Mcm3 was incubated with 10 pmoles of GST-Dpb11 wild type, GST-dpb11-m1,m2,m3,m5 or GST tag alone in a GST pulldown assay as described in the Experimental Procedures. The bound radioactive Mcm3 was analyzed by SDS/PAGE followed by phosphorimaging. These results were then quantified, averaged and plotted as pmoles of Mcm3 bound versus the pmoles of Mcm3 input. (C) Different concentrations of radiolabeled PKA-Mcm5 was incubated with 10 pmoles of GST-Dpb11 wild type, GST- dpb11-m1,m2,m3,m5 or GST tag alone in a GST pulldown assay as described in the Experimental Procedures. The bound radioactive Mcm5 was analyzed by SDS/PAGE followed by phosphorimaging. These results were then quantified, averaged and plotted as pmoles of Mcm5 bound versus the pmoles of Mcm5 input. (D) 30 pmoles of GST-dpb11-m1,m2,m3,m5 was incubated with 30 pmoles of radiolabeled PKA Mcm2-7 and varying amounts ss40dT in a GST pulldown assay. The beads were washed and analyzed as described in Experimental Procedures. Results from similar experiments were quantified, averaged and plotted as pmoles of Mcm2-7 bound versus the molar ratio of ss40dT/GST-dpb11-m1,m2,m3,m5. (E) Different concentrations of radiolabeled PKA-Sld2T84D were incubated with 30 pmoles of GST-Dpb11 wild type, GST-dpb11-m1,m2,m3,m5 or GST tag alone in a GST pulldown assay as described in the Experimental Procedures. The bound radioactive Sld2T84D was analyzed by SDS/PAGE followed by phosphorimaging. These results were then quantified, averaged and plotted as pmoles of Sld2T84D bound versus the pmoles of Sld2T84D input. (F) Different concentrations of radiolabeled PKA-Cdc45 was incubated with 10 pmoles of GST-Dpb11 wild type, GST-dpb11-m1,m2,m3,m5 or GST tag alone in a GST pulldown assay as described in the Experimental Procedures. The bound radioactive Cdc45 was analyzed by SDS/PAGE followed by phosphorimaging. These results were then quantified, averaged and plotted as pmoles of Cdc45 bound versus the pmoles of Cdc45 input.

To investigate *in vivo* the mutant of Dpb11 that remains bound to Mcm2-7 in the presence of ssDNA, I constructed plasmids with wild type and mutant *DPB11 (dpb11-m1,m2,m3,m5*) under the control of its native promoter. These plasmids were then transformed into temperature sensitive Dpb11 degron yeast strain (*dpb11-td*, obtained from John Diffley (54)). I then studied these strains for cell growth by performing a 10-fold serial dilution and spotting onto CSM-Ura plates at 30°C (permissive conditions) and CSM-Ura+Gal+Doxycycline

at 37°C (restrictive conditions). At the permissive conditions, *DPB11-WT*, *dpb11-m1,m2,m3,m5* and the vector only control all grow to similar levels. However, at the restrictive conditions, there is a severe growth defect in the *dpb11- m1,m2,m3,m5* and vector only control compared to the *DPB11-WT* (Figure 4.4 A) This data suggests that the mutant Dpb11 protein cannot support yeast growth in the absence of wild type *DPB11*. However, Dpb11 protein is expressed at equivalent levels in both the wild type and mutant strains (Figure 4.4 B), suggesting that the mutant protein is properly transcribed and expressed.

I further studied the cell cycle progression into S phase in a FACS analysis experiment. I arrested the cells in $G_1$ with α-factor at restrictive temperature (37°C) for 3 hours and then released them into growth media (YPGal+50μg/ml doxycycline) lacking α-factor at restrictive temperature for 15 to 220 minutes. Cells expressing wild type *DPB11* accumulated 2C DNA within 45 minutes after α-factor release. However, cells with the vector only control and *dpb11-m1,m2,m3,m5* exhibited a severe defect in progression into S phase (Figure 4.4 C), suggesting that *dpb11-m1,m2,m3,m5* is defective in DNA replication initiation.

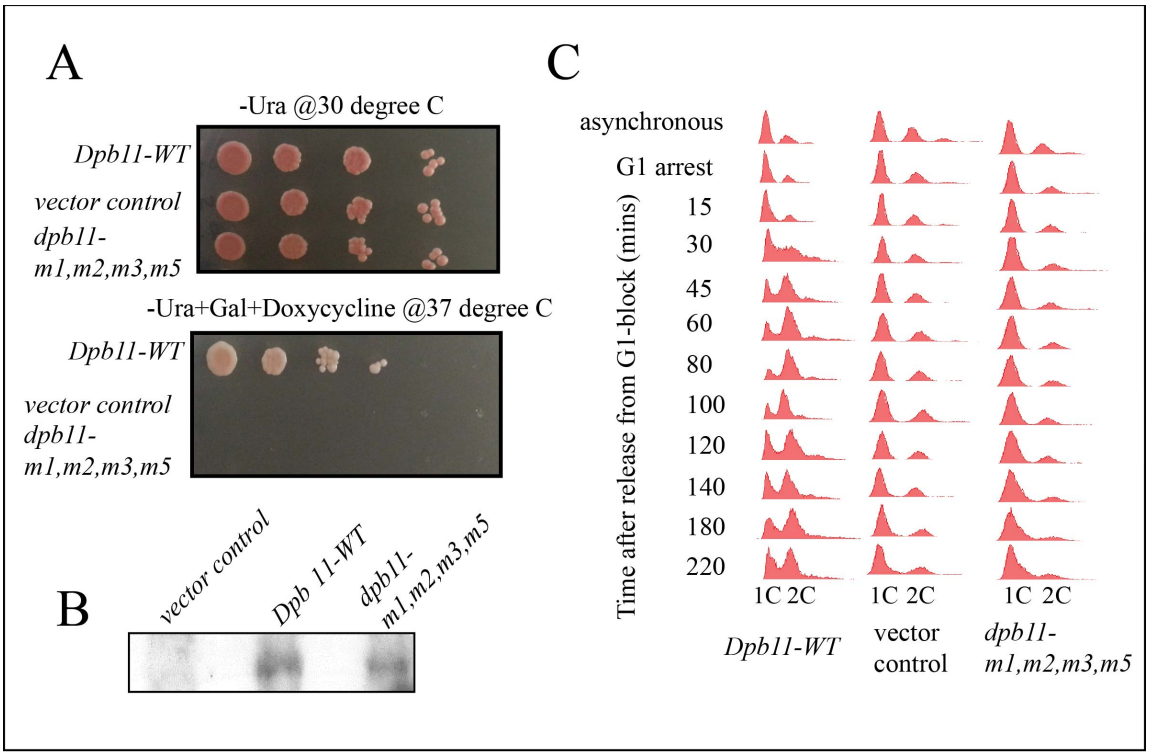


**Figure 4.4: Cells expressing *dpb11-m1,m2,m3,m5* exhibit a defect in cell growth and DNA replication.**

(A) Ten-fold serial dilutions of *dpb11-td* cells expressing *DPB11*-wild type, vector only control and *dpb11-m1,m2,m3,m5* at the permissive conditions (CSM-Ura, 30°C) or restrictive conditions (CSM-Ura+gal+doxycycline, 37°C). (B) Western analysis of *DPB11-WT*, *dpb11-m1,m2,m3,m5* and vector only control whole cell extracts showing equivalent levels of Dpb11 expression under restrictive conditions. (C) FACS analysis was performed as described in Experimental Procedures on *dpb11-td* cells expressing *DPB11*-wild type, vector only control and *dpb11-m1,m2,m3,m5*. Cells were synchronized in $G_1$ with α-factor and then released into medium lacking α-factor for the time points indicated.

**Cells expressing dpb11-m1,m2,m3,m5 exhibit a decreased RPA interaction with origin DNA during S phase**

I next examined whether *dpb11-m1,m2,m3,m5* is impaired in the accumulation of origin ssDNA coated by RPA, the yeast single-stranded binding protein. For this experiment, I used chromatin immunoprecipitation with antibodies directed against RPA (Figure 4.5). I then subjected the immunoprecipitate to quantitative PCR using oligonucleotides directed against the

early origin *ARS306*, or a non-origin region located midway between the *ARS305* and *ARS306* origins (non-origin sequence). I arrested the cells at the restrictive temperature in $G_1$ with α-factor, and then released cells into medium lacking α-factor for 15 minutes to collect cells in S phase. No hydroxyurea was added to the medium. For wild-type cells, there is a substantial increase in origin PCR signal for S phase cells compared to $G_1$ phase cells (Figure 4.5). There is also a slight increase in non-origin PCR signal for S phase cells compared to $G_1$ phase cells. The increase in signal at origin sequence may predominantly reflect the extrusion of ssDNA from the central channel of Mcm2-7 during S phase, and it may also include some signal for the initiation of DNA unwinding. For cells expressing *dpb11-m1,m2,m3,m5*, there is a substantially diminished signal for origin sequence during S phase. There is also a slightly decreased signal for non-origin sequence, consistent with the mutant slow progression through S phase. The decrease in RPA-ChIP signal at origin sequence may reflect the importance of these BRCT4 residues (K463,K469,R538,K549) for stabilizing the extruded strand of origin DNA. Alternatively, the decrease in RPA-ChIP signal may reflect that the mutant is defective in release of Dpb11 from Mcm2-7. A third possibility is that the decrease in signal for the mutant at an origin may reflect a decrease in unwinding during initiation.

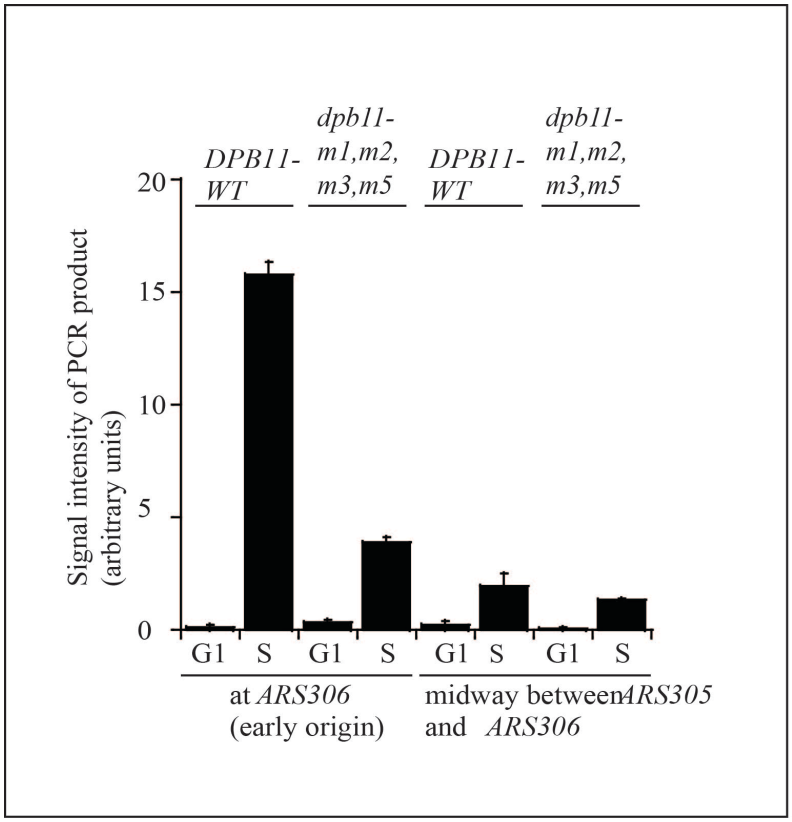


**Figure 4.5: Cells expressing *dpb11-m1,m2,m3,m5* exhibit a decreased RPA interaction with origin DNA during S phase.**

The *dpb11-td* cells expressing *DPB11-WT* or *dpb11-m1,m2,m3,m5* at the restrictive conditions were arrested with α-factor and then released into medium lacking α-factor for 15 minutes. Chromatin Immunoprecipitation was performed as described in Experimental Procedures. PCR primers were used that target the early yeast origin *ARS306* or a region positioned midway between *ARS305* and *ARS306*. [$^{32}$P-α]-dCTP was included in the PCR reaction for quantification. Radioactive PCR bands were quantified and plotted.

**Cells expressing dpb11-m1,m2,m3,m5 exhibit decreased GINS-Mcm2-7 interaction**

I next performed co-immunoprecipitation (co-IP) analysis to study the assembly of proteins required for replication initiation in strains expressing the Wild type-*DPB11* and *dpb11-m1,m2,m3,m5* at the restrictive conditions. Using the approach to isolate loaded Mcm2-7 complexes on chromosomal DNA as described (70), I studied the interaction between loaded

Mcm2-7 and Dpb11, and also between Sld2 and Dpb11 (Figure 4.6 A). I arrested cells in $G_1$ with α-factor at the restrictive temperature (37°C) for 3 hours and then released them into growth media (YPGal+50µg/ml doxycycline) lacking α-factor at the restrictive temperature for 0, 15, 30 or 45 minutes. I did not use any crosslinking reagent in these experiments, and no hydroxyurea was added to the medium. I made whole-cell extracts and probed them with antibodies against Mcm2 and Sld2, and found similar levels of Mcm2 and Sld2 proteins in wild type compared to mutant cells (Figure 4.6 A). However, for cell extracts precipitated with antibodies against Dpb11 and then probed with Mcm2, I observed an increased Dpb11-Mcm2 interaction in the *dpb11-m1,m2,m3,m5* cells at 15 and 30-minute time points compared to *DPB11*-Wild Type cells (Figure 4.6 A). Mcm2 precipitates as part of the loaded Mcm2-7 complex in these experiments, and the Dpb11-Mcm2 co-IP signal therefore reflects interaction between Dpb11 and loaded Mcm2-7. These data suggest that Dpb11-ssDNA interaction is required for the release of Dpb11 from loaded Mcm2-7 as cells progress into S phase. I next probed these Dpb11 immunoprecipitates with Sld2 antibody and found there is a very weak signal at 0 minutes for both wild type and mutant cells. However, at 15, 30 and 45 minutes I observed a similar Dpb11-Sld2 interaction for wild type and mutant cells. This is consistent with the CDK dependent binding of Sld2 with Dpb11 and suggests that *dpb11-m1,m2,m3,m5* binds normally with Sld2, similar to *DPB11-WT* (Figure 4.6 A).

I next probed for the interaction between loaded Mcm2-7 and Cdc45 or GINS using a similar approach (Figure 4.6 B). The whole cell extracts showed similar levels of Mcm2, Cdc45 or GINS proteins in the cells expressing *DPB11-WT* or *dpb11-m1,m2,m3,m5* forms of Dpb11 protein (Figure 4.6 B). These cell extracts were then precipitated with antibodies against Mcm2 and probed with Cdc45 or GINS antibodies. The interaction between loaded Mcm2 and Cdc45 is

slightly affected in *DPB11-WT* and *dpb11-m1,m2,m3,m5* cells (Figure 4.6 B), suggesting that these residues play some role in Cdc45-Mcm2-7 interaction. However, there is a substantially diminished interaction between loaded Mcm2 and GINS in *dpb11-m1,m2,m3,m5* cells compared to the wild type cells where GINS is associated with loaded Mcm2 at 15 and 30 minutes after $G_1$ release (Figure 4.6 B). This suggests that release of Dpb11 from Mcm2-7 is important for GINS to bind loaded Mcm2-7 in S phase.

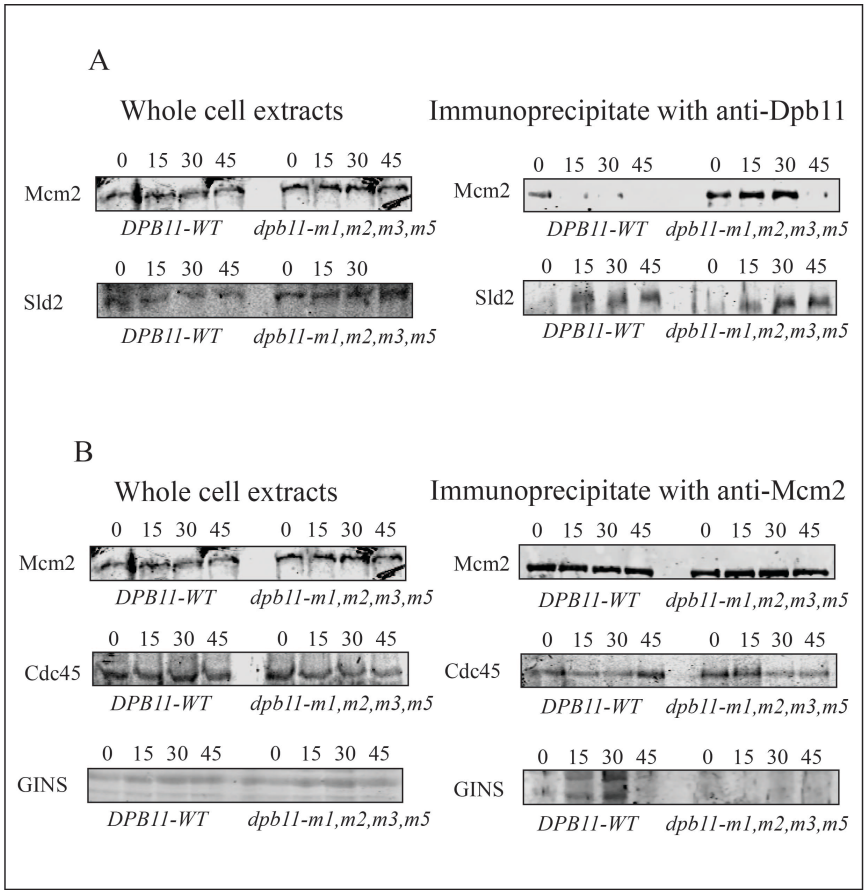


**Figure 4.6: Cells expressing *dpb11-m1,m2,m3,m5* exhibit no GINS-Mcm2-7 interaction.**

The *dpb11-td* cells expressing *DPB11-WT* or *dpb11-m1,m2,m3,m5* at the restrictive conditions were arrested with α-factor and then released into medium lacking α-factor for the indicated times. (A and B, left panel) Whole cell extracts were analyzed by Western blot for expression of the indicated proteins. (A, right panel) Cell extracts were immunoprecipitated with antibodies directed against Dpb11, followed by western analysis for Mcm2 or Sld2. (B, right panel) Cell

extracts were prepared to isolate loaded Mcm2-7 complex as described. Antibodies directed against Mcm2 were used for immunoprecipitation, followed by Western analysis of Mcm2, Cdc45, or GINS.

## Conclusion

### Summary of results

I found a mutant of Dpb11, *dpb11-m1,m2,m3,m5* that binds partially to ssDNA but it remains bound to Mcm2-7 even in the presence of ssDNA. I also found that when this mutant *dpb11-m1,m2,m3,m5* (BRCT4 motif mutant of Dpb11 that remains bound to Mcm2-7 in presence of ssDNA) is expressed in yeast cells, the cells suffer severe growth and DNA replication defects in the absence of wild type *DPB11*. The region aa430-615 of Dpb11 that encompasses Dpb11 BRCT4 is poorly conserved from yeast to humans, making it difficult to determine if the residues involved in ssDNA binding are conserved from yeast to human. Expression of *dpb11-m1,m2,m3,m5* results in a decreased RPA interaction with origin DNA compared to *DPB11-WT*. In addition, the *dpb11-m1,m2,m3,m5* mutant exhibits a prolonged interaction between Dpb11 and Mcm2-7 after release from $G_1$. GINS also fails to associate with Mcm2-7 during S phase in the mutant cells. These data suggest that release of Dpb11 from Mcm2-7 is important for GINS-Mcm2-7 interaction in S phase.

### Sld2 and Dpb11 block the premature interaction between GINS and Mcm2-7 in G1

The Mcm2-7 ring cracks open in late M and $G_1$ phase to encircle double-stranded DNA (37). However, the helicase is not active at this point because it is not bound to GINS in $G_1$. Sld3 binding to Mcm3/Mcm5 blocks GINS interaction with Mcm2-7 *in vitro* (58), Sld2 binding to Mcm3/Mcm5 blocks GINS interaction with Mcm2-7 *in vitro* and *in vivo* (60, 61), and Dpb11

interaction with Mcm3/Mcm5 blocks the interaction between GINS and Mcm2-7 *in vitro* (Figures 2.6) and *in vivo* (Figure 4.6 B). These data suggest that Sld3, Sld2, or Dpb11 can block the interaction between GINS and Mcm2-7 in $G_1$. Blocking premature CMG assembly may be important for two reasons. First, if GINS binds to Cdc45 prematurely, the helicase may be prematurely activated. It is critical that helicase activation is precisely coordinated with polymerase DNA synthesis, in order to prevent the accumulation of excess ssDNA and genome instability. Second, the CMG complex forms a sealed ring, and it cannot open to allow for the extrusion of ssDNA (83, 130). Thus, if the CMG complex were to form prior to ssDNA extrusion, the sealed CMG complex may block ssDNA extrusion from the central channel of Mcm2-7. Previous published work has demonstrated a pre- loading complex, composed of Sld2, Dpb11, GINS, and Pol ε, that is important for the recruitment of GINS to replication origins (55). Furthermore, Dpb11 has been show to bind directly to GINS through an interacting region between BRCT motifs 2 and 3 (131). I am pointing out here, that although the pre-loading complex may be important for recruiting GINS to origins, the direct interaction between GINS and Mcm2-7 is blocked by Dpb11, Sld2, and possibly Sld3 (60, 62).

**The Sld3-Sld2-Dpb11 ternary complex in S phase binds to ssDNA, allowing GINS to bind Mcm2-7**

In S phase, S-CDK phosphorylates Sld2 and Sld3 (52, 54). These phosphorylation events stimulate the interactions between Dpb11 and Sld2 and Sld3 to form the Dpb11-Sld3-Sld2 ternary complex (52, 54). The formation of the Sld3-Sld2- Dpb11 complex has been shown to be essential, and in fact the formation of this complex can bypass the requirement for CDK in the cell (52, 54). During S phase, the T-rich strand of origin DNA is extruded from the central

channel of Mcm2-7 (123). Recent work demonstrates that the Dbf4-dependent kinase phosphorylates Mcm2, opening the Mcm2-Mcm5 gate of the Mcm2-7 complex to allow for the extrusion of T-rich ssDNA from the central channel of Mcm2-7 (48). The Mcm2-Mcm5 gate, which may function to open and close the Mcm2-7 ring, appears to be conserved from yeast to *Drosophila* (37, 83, 132).

Dpb11, Sld3, or Sld2 each bind individually to ssDNA *in vitro* (53, 62). Furthermore, ssDNA disrupts the interaction between Dpb11 and Mcm3/Mcm5 and allows GINS to bind Mcm3/Mcm5 *in vitro* and *in vivo* (Figures 2.7 and 4.6). Moreover, ssDNA also disrupts the interaction between Sld2 and Mcm3/Mcm5 *in vitro* and *in vivo,* allowing GINS to bind Mcm2-7 (60, 61). Finally, it has been shown using purified proteins that ssDNA disrupts the interaction between Sld3 and Mcm3/Mcm5, allowing GINS to bind Mcm2-7 (62). These data suggest that once ssDNA is extruded from the central channel of Mcm2-7 during S phase, the Dpb11-Sld3-Sld2 complex will bind to ssDNA instead of Mcm3/Mcm5. The Dpb11-Sld3- Sld2 complex has three different binding sites for ssDNA, which may all act together to form a very tight interaction with ssDNA. The disengagement of Dpb11, Sld3, and Sld2 from Mcm2-7, and the binding of the Dpb11-Sld3-Sld2 ternary complex to ssDNA, may allow for the subsequent binding of GINS to Cdc45-Mcm2-7. The binding of GINS to Cdc45-Mcm2-7 completes the assembly of the CMG complex, appropriately positioned to encircle ssDNA. The ability of ssDNA to displace Dpb11, Sld3, and Sld2 from Mcm2-7 thus fulfills an elegant regulatory mechanism to ensure that the CMG complex can only assemble once a single-strand is extruded from Mcm2-7.

The *in vivo* data presented in this chapter supports the *in vitro* data presented in chapter II, thus supporting our hypothesis that Dpb11 along with Sld3 and Sld2 plays an active role in

the formation of replication fork helicase. Dpb11 BRCT4 region is essential in the regulation of Dpb11-Mcm2-7 interaction and binding of Dpb11 BRCT4 region to ssDNA is essential for the displacement of Dpb11 from Mcm2-7 and for the formation of the CMG complex. Based on our results I describe a model of replication initiation in the following chapter.

**Acknowledgements**

I thank Dr. Susan Taylor for supplying purified PKA. I thank Dr. Mike O'Donnell for providing expression constructs for GINS. I thank Ruth Didier for help with FACS analysis and data collection. I also thank Dr. Yoichi Kato for the use of his sonicator. Thanks to Dr. Akash Gunjan and Yanchang Wang for helpful comments. This work was supported by the National Science Foundation Grant 1265431 to D.L.K.

# CHAPTER V

# DISCUSSION AND FUTURE DIRECTIONS

## Discussion

### A model for replication initiation

Based on the previous published data and the results presented in chapters II, III and IV, I suggest a model for replication initiation (Figure 5.1). According to this, model Dpb11, Sld3 and Sld2 independently bind Mcm2-7. These proteins bind the Mcm3/Mcm5 surface of the Mcm2-7 complex. Since Cdc45 interacts with the Mcm2 subunit, positioning of Dpb11 and Sld3 facilitates the recruitment of Cdc45 onto Mcm2-7. GINS also interacts with the Mcm3/Mcm5 subunits. Dpb11, Sld3, or Sld2 binding to Mcm2-7 thus prevents GINS from binding prematurely onto the Mcm2-7-Cdc45 complex (Figure 5.1 A). However, the action of Dbf4-dependent kinase (DDK) phosphorylates Mcm2, Mcm4 and Mcm6 subunits of the Mcm2-7 complex. DDK phosphorylation of Mcm2 weakens the interaction between Mcm2-Mcm5 subunits, thus opening the gate between Mcm2-Mcm5, which causes the extrusion of T-rich single-stranded origin DNA from the central channel of Mcm2-7. Since, Dpb11, Sld3 and Sld2 each bind ssDNA and ssDNA competes with their interaction with Mcm2-7, these proteins dissociate from the Mcm2-7-Cdc45 complex to associate with ssDNA. The action of cyclin-dependent kinase in the S-phase results in the phosphorylation of Sld2 and Sld3, which then bind to Dpb11. Together the Dpb11-Sld3-Sld2 ternary complex is associated with ssDNA (Figure 5.1 B). This allows for GINS to bind Mcm2-7-Cdc45 complex thus forming the Cdc45-Mcm2-7-

GINS complex (Figure 5.1 C). This mechanism ensures that GINS binds to the Mcm2-7-Cdc45 complex only after the extrusion of ssDNA. Single-stranded DNA thus acts as s trigger for the dissociation of Dpb11, Sld3, or Sld2 from Mcm2-7 and allows GINS to bind Mcm2-7. This also ensures that Dpb11, Sld3 and Sld2 do not travel with the replication fork.

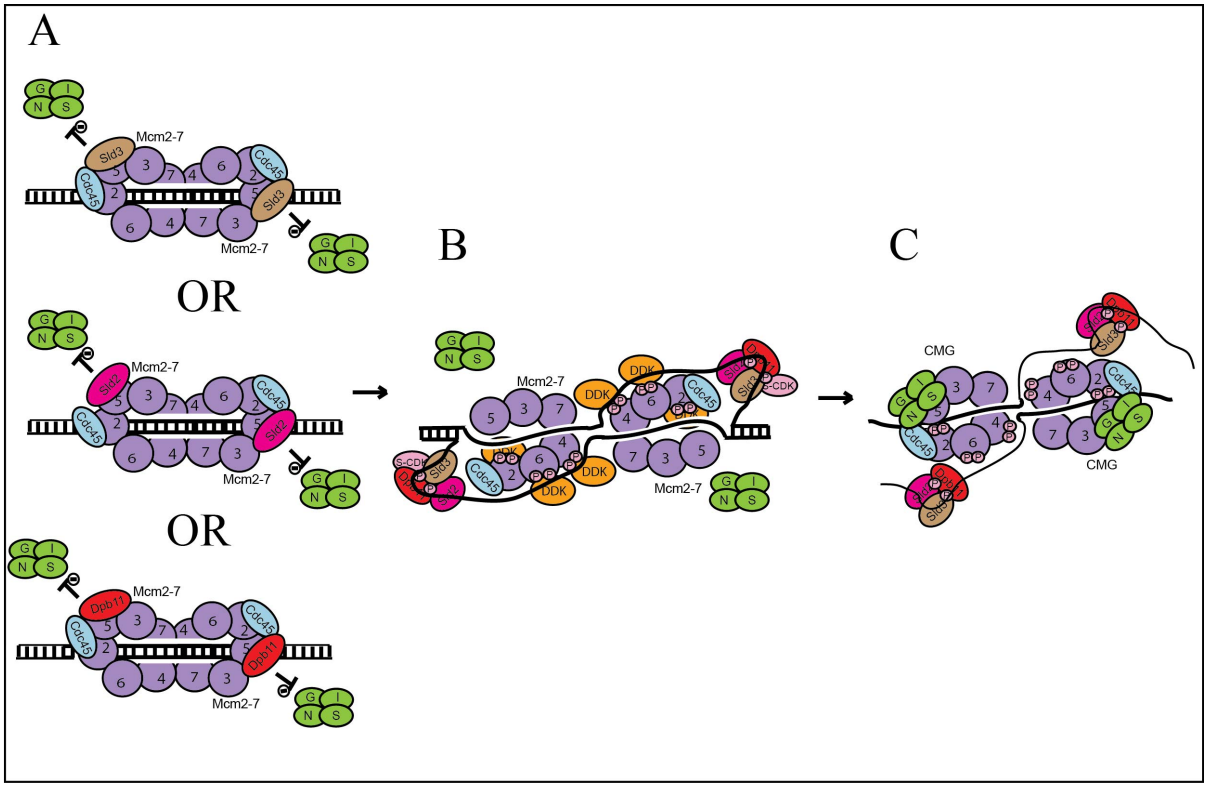


**Figure 5.1: Dpb11, Sld3, and Sld2 regulate the assembly of the CMG (Cdc45-Mcm2-7-GINS) helicase complex in budding yeast.**

(A) Mcm2-7 loads as a double hexamer to encircle double-stranded DNA during late M and G1 phase. Dpb11, Sld3 or Sld2 bind to Mcm2-7, and binding of Dpb11, Sld3, or Sld2 to Mcm2-7 blocks the premature interaction between GINS and Mcm2-7 during G1. Furthermore, Dpb11 or Sld3 may recruit Cdc45 to Mcm2-7, and this process may depend upon the Dbf4-dependent kinase (DDK). (B) DDK phosphorylates Mcm2, Mcm4 and Mcm6. DDK phosphorylation of Mcm2 opens the Mcm2-Mcm5 gate allowing single-stranded DNA to be extruded from the central channel of Mcm2-7 in S phase. In S phase, the S-phase cyclin dependent kinase phosphorylates Sld2 and Sld3, and these phosphorylated proteins each bind to Dpb11 to form a ternary complex of Dpb11-Sld3-Sld2. This Dpb11- Sld3-Sld2 ternary complex binds to single-stranded DNA. The Dpb11-Sld3-Sld2 ternary complex will disengage from Mcm2-7 upon

binding ssDNA. (C) The disengagement of Dpb11-Sld3-Sld2 from Mcm2- 7 will allow GINS to bind Cdc45-Mcm2-7. GINS binds to the Cdc45-Mcm2-7 complex, forming the closed Cdc45-Mcm2-7-GINS helicase complex that encircles single-stranded DNA.

**Dpb11**

Dpb11 is an essential yeast protein that functions during replication initiation and also in DNA damage response. During replication initiation, Dpb11 binds CDK-phosphorylated Sld3 and Sld2. It has 2 pairs of BRCT domains; BRCT 1 and 2 bind phosphorylated Sld3 while BRCT 3 and 4 bind phosphorylated Sld2. Formation of Dpb11-Sld3-Sld2 ternary complex is an essential step in replication initiation. Dpb11, Sld3 and Sld2 proteins do not travel with the replication fork. Thus, Dpb11 has been considered as a scaffold protein for the binding of phosphorylated Sld2 and Sld3. This study introduces a novel function of Dpb11 during replication initiation and its role in the formation of the replication fork helicase.

Based on this study, I suggest that Dpb11 binds replication origins via two mechanisms. It interacts with Mcm2-7 at the origins independent of DDK or S-CDK activity and also associates with the origin ssDNA after the action of DDK and S-CDK as a part of the Dpb11-Sld3-Sld2 ternary complex. Dpb11 is shown to interact directly with Mcm2-7 at the $G_1$/S phase boundary (Figure 4.6). Dpb11 interacts specifically with the Mcm3/Mcm5 subunits of the Mcm2-7 complex and helps in the recruitment of Cdc45 onto Mcm2. Dpb11 interaction with Mcm3/Mcm5 also prevents the premature binding of GINS to the Mcm2-7 complex. This interaction of Dpb11 with Mcm3/Mcm5 subunits is independent of kinase activity. A similar function has been shown for Sld2 and Sld3 and I suggest that Dpb11, Sld3, or Sld2 independently function in a redundant mechanism to prevent the association of GINS with Mcm2-7. GINS association with Mcm2-7 is the last known step in CMG formation in budding

yeast, so the cell employs this mechanism to ensure that GINS binds Mcm2-7 once ssDNA is extruded from Mcm2-7. However, ssDNA extrusion is dependent on DDK phosphorylation of Mcm2. Dpb11 binds ssDNA allowing GINS to bind Mcm2-7. Dpb11 might be present as a ternary complex on origin ssDNA and the formation of Dpb11-Sld3-Sld2 ternary complex is dependent on S-CDK phosphorylation of Sld2 and Sld3. This also ensures that Dpb11, Sld3 and Sld2 do not travel with replication fork. The interaction of Dpb11-Sld3-Sld2 ternary complex with origin ssDNA might also be important to stabilize the origin ssDNA that is extruded from the central channel of Mcm2-7 before the formation of active helicase. Binding of Dpb11-Sld3-Sld2 with origin ssDNA might prevent its re-annealing to its complementary strand.

## Future Directions

### To check whether Dpb11 function is conserved in higher eukaryotes

The functional homologs of Dpb11 have been identified in higher eukaryotes and include Rad4/Cut5 (*S. pombe*), Mus101 (*Xenopus* and *Drosophila*) and TopBP1 in humans (Figure 5.2). Even though yeast Dpb11 lacks any sequence similarity with Mus101 or TopBP1, the similarity is limited to the presence of BRCT domains. Similar to Dpb11, Mus101 and TopBp1 are also essential for replication initiation and bind other phosphorylated proteins (functional homologs of Sld2 and Sld3).

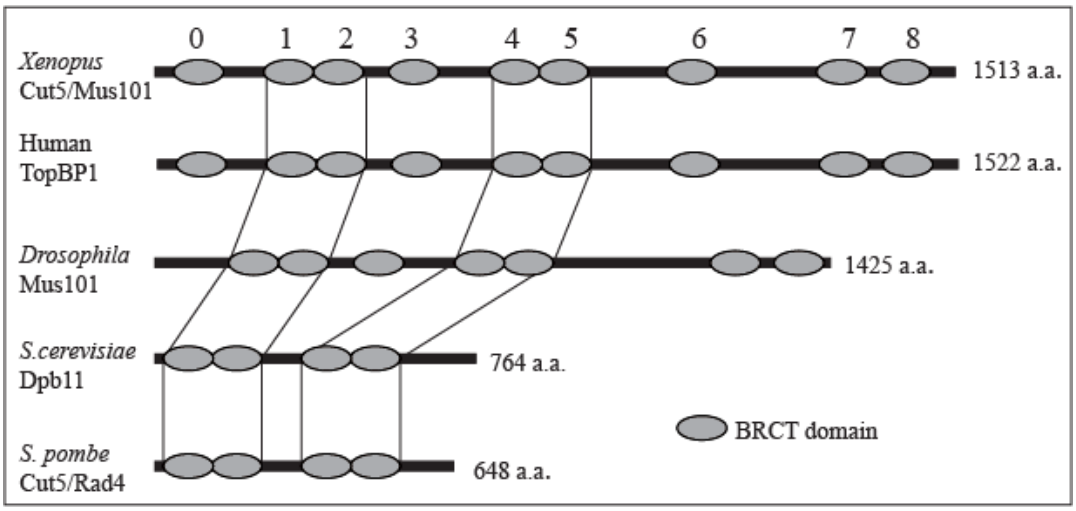


**Figure 5.2: Schematic comparison of Dpb11 homologs.**

Adapted from Hashimoto Y. and Takisawa H., 2003 (133).
Elliptical symbols represent BRCT domains and solid lines indicate highly conserved BRCT domains from yeast to human.

Studies using *Xenopus* Mus101 have already shown that this protein binds chromatin in a CDK independent manner in the $G_1$/S phase and helps in the recruitment of Cdc45. It would be of great significance to study if the Dpb11 interactions described in this document are conserved in higher eukaryotes and whether TopBP1 also functions in a way similar to Dpb11 to prevent the premature binding of GINS during CMG formation.

**Dpb11-Mcm2-7 interaction**

I show here that Dpb11 interacts directly with Mcm2-7. For this study I focused on the Mcm2, Mcm3 and Mcm5 region of the Mcm2-7 complex (as Cdc45 binds to Mcm2, and GINS binds to Mcm3 and Mcm5). Our results highlight the importance of Dpb11-BRCT4 region in Dpb11 interaction with Mcm3/Mcm5. Future studies should focus on determining the residues involved in this interaction. A Dpb11 mutant defective in Mcm3/Mcm5 binding will be a useful

tool to further establish the role of Dpb11 in GINS binding to Mcm2-7. This mutant might also effect Cdc45 recruitment onto Mcm2-7. According to the model described in Figure 5.1, Dpb11 defective in binding Mcm3/Mcm5 might result in the premature binding of GINS to Mcm2-7-Cdc45 complex. This would result in the formation of a closed CMG complex encircling double-stranded DNA. Due to the absence of ssDNA extrusion, a strain with mutant Dpb11 (defective in Mcm3/Mcm5 binding) might be defective in replication initiation. However, studies conducted by another group suggest the presence of a pre-loading complex (pre-LC) consisting of Dpb11, GINS, Sld2 and Pol e. The pre-LC formation is dependent on S-CDK activity (55). According to this model, Dpb11 binds GINS and helps in its recruitment onto replication origins. In this situation, a Dpb11 mutant defective for Mcm3/Mcm5 binding will be defective in the loading of GINS onto Mcm2-7.

While studying Dpb11 interaction with Mcm2-7 subunits, I observed that in addition to binding with Mcm3 and Mcm5, Dpb11 also interacts with Mcm7 and weakly with Mcm4. Dpb11 interaction with Mcm6 is similar to GST background (Figure 5.3). It will be useful to study the significance of Dpb11 interaction with Mcm7 and Mcm4. For example, Dpb11 may inhibit the helicase or ATPase activity of Mcm2-7 or Mcm4,6,7 by binding directly to Mcm7 or Mcm4. Alternatively, Dpb11 binding to Mcm4 or Mcm7 may help recruit Cdc45 to Mcm2-7. Finally, Dpb11 interaction with Mcm4 or Mcm7 may dissociate the Mcm2-7 double hexamers to single hexamers. Each of these ideas can be tested in the future with *in vitro* and *in vivo* experiments.

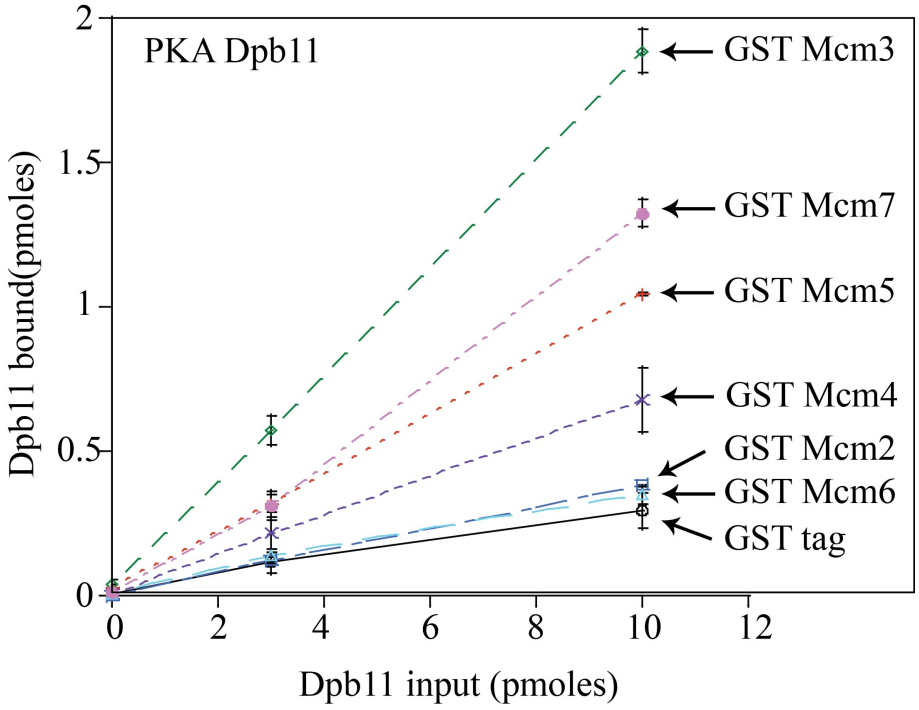


**Figure 5.3: Dpb11 interaction with different Mcm2-7 subunits.**

30 pmoles of GST-Mcm2, GST-Mcm3, GST-Mcm5, GST-Mcm6, GST-Mcm4, GST-Mcm7 or GST alone were incubated with glutathione sepharose and increasing concentrations of radiolabeled PKA-Dpb11 at 30°C for 10 minutes in a GST pulldown assay as described in the Experimental Procedures. The bound radioactive Dpb11 was analyzed by SDS/PAGE followed by phosphorimaging. The results from similar experiments were then quantified, averaged and plotted as pmoles of Dpb11 bound versus the pmoles of Dpb11 input. The figure shows that Dpb11 in addition to binding Mcm3 and Mcm5 also interacts with Mcm7 and Mcm4.

**Cdc45 recruitment onto replication origins**

Our studies have shown that both Sld3 and Dpb11 help in Cdc45 recruitment onto Mcm2-7. It would be interesting to see if Sld2 also performs a similar function. This could be another example of redundancy in a cell. The cell might employ such an efficient way of Cdc45 recruitment to ensure that the replication origins are marked for initiation. Since more number of Mcm2-7 double hexamers are loaded than the number of origins, it is hypothesized that the

recruitment of Cdc45 onto replication origins is a deciding factor for origin firing, as Cdc45 is a limiting factor (34).

The Dpb11-Cdc45 interaction described here has not been studied in detail and can be pursued further to study its significance in replication initiation. It is important to determine the region and the specific residues in Dpb11 that interact with Cdc45. Constructing a Dpb11 mutant defective in Cdc45 binding will be useful in answering the questions related to the role of Dpb11 in Cdc45 recruitment onto replication origins and formation of the CMG complex. An opposite study can also be performed, to determine the region of Cdc45 and the specific residues in Cdc45 that interact with Dpb11. These mutants can then be studied in both *in vitro* and *in vivo* experiments.

**Dpb11 interaction with single-stranded DNA**

I observed that Dpb11 binds tighter to a particular sequence from *ARS1* origin when compared to the other three sequences from the same origin. Similar results were observed with *ARS305* origin. Though I established that Dpb11 prefers ssDNA to dsDNA and that this interaction is mediated via pyrimidines (specifically by thymines), it is possible that Dpb11-ssDNA interaction at replication origins is governed by various other elements of the origin sequence. Future studies should investigate the nature of Dpb11-ssDNA interaction and its sequence dependence more extensively.

I also observed that in addition to Dpb11 BRCT4 region, the unstructured C-terminal region of Dpb11 binds weakly to ssDNA. This C-terminal region is however, dispensable for replication initiation but indispensable for $G_2$/M and S-phase checkpoint. It will be very interesting to see if Dpb11 functions during the checkpoint pathway by interacting with the

ssDNA present during replication fork stalling or during double strand breaks and if this interaction is mediated specifically via the unstructured C-terminal region of Dpb11.

**Conclusion**

The results described in this study are novel and elucidate the essential function of Dpb11 in DNA replication initiation. This document describes the mechanism of Dpb11 function at origin via its interaction with Mcm2-7, Cdc45 and single-stranded DNA. It also highlights the importance of the formation of Dpb11-Sld3-Sld2 ternary complex. By studying the biochemical function of Dpb11 during replication initiation, we get a new model for replication initiation (described earlier in this chapter), wherein Dpb11 functions similar to Sld2 and Sld3 in a redundant mechanism during replication initiation. This study contributes in deciphering the events that occur in S-phase during the transition of an inactive Mcm2-7 double hexamer to an active Cdc45-Mcm2-7-GINS complex. However the interactions of Dpb11 described here (especially Dpb11 interaction with Mcm4, Mcm7 and Cdc45) need to be studied in more detail to establish their role in replication initiation. This might help to elucidate more functions of Dpb11 and their contribution in the formation of an active helicase encircling ssDNA.

## REFERENCES


1. Newlon CS, Theis JF. The structure and function of yeast ARS elements. Curr Opin Gen Dev. 1993;3:752-8.

2. Huang R, Kowalski D. A DNA unwinding element and an ARS consensus comprise a replication origin within a yeast chromosome. EMBO J. 1993;12:4521-31.

3. Natale DA, Umek RM, Kowalski D. Ease of DNA unwinding is a conserved property of yeast replicatiion origins. Nucleic Acids Res. 1993;21:555-60.

4. Nieduszynski C, Knox Y, Donaldson A. Genome-wide identification of replication origins in yeast by comparative genomics. Genes Dev. 2006;20:1874-9.

5. Hyrien O. Peaks cloaked in the mist: The landscape of mammalian replication origins. J Cell Biol. 2015;208:147-60.

6. Rhind N, Gilbert D. DNA replication timing. Cold Spring Harb Perspect Biol 2013;5:a010132.

7. Ding Q, MacAlpine D. Defining the replication program through the chromatin landscape. Crit Rev Biochem Mol Biol. 2011;46:165-79.

8. MacAlpine D, Almouzni G. Chromatin and DNA replication. Cold Spring Harb Perspect Biol. 2013;5:a010207.

9. Ma E, Hyrien O, Goldar A. Do replication forks control late origin firing in Saccharomyces cerevisiae? Nucleic Acids Res. 2012;40:2010-9.

10. Aparicio O. Location, location, location: it's all in the timing for replication origins. Genes Dev. 2013;27:117-28.

11. Peace J, Ter-Zakarian A, Aparicio O. Rif1 regulates initiation timing of late replication origins throughout the S. cerevisiae genome. PLoS One. 2014;9:e98501.

12. Kamimura Y, Tak YS, Sugino A, Araki H. Sld3, which interacts with Cdc45 (Sld4), functions for chromosomal DNA replication in Saccharomyces cerevisiae. EMBO J. 2001;20(8):2097-107.

13. Mantiero D, Mackenzie A, Donaldson A, Zegerman P. Limiting replication initiation factors execute the temporal programme of origin firing in budding yeast. EMBO J. 2011;30:4805-14.

14. Pope B, Hiratani I, Gilbert D. Domain-wide regulation of DNA replication timing during mammalian development. Chromosome Res. 2010;18:127-36.

15. Pope B, Gilbert D. The replication domain model: regulating replicon firing in the context of large-scale chromosome architecture. J Mol Biol. 2013;425:4690-5.

16. Whitehouse I, Smith DJ. Chromatin dynamics at the replication fork: there's more to life than histones. Curr Opin Genet Dev. 2013;23(2):140-6.

17. Bell S, Stillman B. ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. Nature. 1992;357:128-34.

18. Costa A, Hood I, Berger J. Mechanisms for initiating cellular DNA replication. Annu Rev Biochem. 2013;82:25-54.

19. Hemerly A, Prasanth S, Siddiqui K, Stillman B. Orc1 controls centriole and centrosome copy number in human cells. Science. 2009;323:789-93.

20. Hoggard T, Shor E, Müller C, Nieduszynski C, Fox C. A Link between ORC-origin binding mechanisms and origin activation time revealed in budding yeast. PLoS Genet. 2013;9:e1003798.

21. McIntosh D, Blow J. Dormant origins, the licensing checkpoint, and the response to replicative stresses. Cold Spring Harb Perspect Biol 2012;4:a012955.

22. Hossain M, Stillman B. Meier-Gorlin syndrome mutations disrupt an Orc1 CDK inhibitory domain and cause centrosome reduplication. Genes Dev. 2012;26:1797-810.

23. Shen Z. The origin recognition complex in human diseases. Biosci Rep. 2013;33:e00044.

24. Bicknell L, Bongers E, Leitch A, Brown S, Schoots J, Harley M, et al. Mutations in the pre-replication complex cause Meier-Gorlin syndrome. Nat Genetics. 2011;43:356-9.

25. Guernsey D, Matsuoka M, Jiang H, Evans S, Macgillivray C, Nightingale M, et al. Mutations in origin recognition complex gene ORC4 cause Meier-Gorlin syndrome. Nat Genet. 2011;43:360-4.

26. Bleichert F, Balasov M, Chesnokov I, Nogales E, Botchan M, Berger J. A Meier-Gorlin syndrome mutation in a conserved C-terminal helix of Orc6 impedes origin recognition complex formation. Elife. 2013;2:e00882.

27. Maine G, Sinha P, Tye B. Mutants of S. cerevisiae defective in the maintenance of minichromosomes. Genetics. 1984;106:365-85.

28. Davey MJ, Indiani C, O'Donnell M. Reconstitution of the Mcm2-7p heterohexamer, subunit arrangement, and ATP site architecture. J Biol Chem. 2003;278:4491-9.

29. Bochman M, Schwacha A. The Mcm2-7 complex has in vitro helicase activity. Mol Cell. 2008;31:287-93.

30. Ilves I, Petojevic T, Pesavento JJ, Botchan MR. Activation of the MCM2-7 helicase by association with Cdc45 and GINS proteins. Mol Cell. 2010;37(2):247-58.

31. Coster G, Frigola J, Beuron F, Morris E, Diffley J. Origin Licensing Requires ATP Binding and Hydrolysis by the MCM Replicative Helicase. Mol Cell. 2014;55:666-77.

32. Remus D, Beuron F, Tolun G, Griffith J, Morris E, Diffley J. Concerted loading of Mcm2-7 double hexamers around DNA during DNA replication origin licensing. Cell. 2009;139:719-30.

33. Riera A, Tognetti S, Speck C. Helicase loading: how to build a MCM2-7 double-hexamer. Semin Cell Dev Biol. 2014;30:104-9.

34. Woodward AM, Göhler T, Luciani MG, Oehlmann M, Ge X, Gartner A, et al. Excess Mcm2-7 license dormant origins of replication that can be used under conditions of replicative stress. J Cell Biol. 2006;173(5):673-83.

35. Tognetti S RA, Speck C. Switch on the engine: how the eukaryotic replicative helicase MCM2-7 becomes activated. Chromosoma. 2014;Epub ahead of print:October 12.

36. Sun J, Fernandez-Cid A, Riera A, Tognetti S, Yuan Z, Stillman B, et al. Structural and mechanistic insights into Mcm2-7 double-hexamer assembly and function. Genes Dev. 2014;28.

37. Samel S, Fernández-Cid A, Sun J, Riera A, Tognetti S, Herrera M, et al. A unique DNA entry gate serves for regulated loading of the eukaryotic replicative helicase MCM2-7 onto DNA. Genes Dev. 2014;28:1653-66.

38. Tanaka S, Araki H. Helicase activation and establishment of replication forks at chromosomal origins of replication. Cold Spring Harb Perspect Biol. 2013;5:a010371.

39. Araki H. Cyclin-dependent kinase-dependent initiation of chromosomal DNA replication. Curr Opin Cell Biol. 2010;22:766-71.

40. Li Y, Araki H. Loading and activation of replication helicases: the key step of initiation of DNA replication. Genes Cells. 2013;18:266-77.

41. Calzada A, Hodgson B, Kanemaki M, Bueno A, Labib K. Molecular anatomy and regulation of a stable replisome at a paused eukaryotic DNA replication fork. Genes Dev. 2005;19(16):1905-19.

42. Hardy CFJ, Dryga O, Seematter S, Pahl PMB, Sclafani RA. *mcm5/cdc46-bob1* bypasses the requirement for the S phase activatorCdc7p. Proc Natl Acad Sci U S A. 1997;94:3151–5.

43. Sclafani R, Tecklenburg M, Pierce A. The mcm5-bob1 bypass of Cdc7p/Dbf4p in DNA replication depends on both Cdk-1 independent and Cdk-1-dependent steps in Saccharomyces cerevisiae. Genetics. 2002;161:47-57.

44. Sheu Y, Stillman B. The Dbf4-Cdc7 kinase promotes S phase by alleviating an inhibitory activity in Mcm4. Nature. 2010;463:113-7.

45. Sheu Y-J, Stillman B. Cdc7-Dbf4 phosphorylates MCM proteins via a docing site-mediated mechanism to promote S phase progression. Mol Cell. 2006;24:101-13.

46. Sclafani R, Holzen T. Cell Cycle Regulation of DNA Replication. Annu Rev Genet. 2007;41:237-80.

47. Hoang ML, Leon RP, Pessoa-Brandao L, Hunt S, Raghuraman MK, Fangman WL, et al. Structural changes in Mcm5 protein bypass Cdc7-Dbf4 function and reduce replication origin efficiency in S. cerevisiae. Mol Cell Biol. 2007;27:7594-602.

48. Bruck I, Kaplan DL. The Dbf4-Cdc7 kinase promotes Mcm2-7 ring opening to allow for single-stranded DNA extrusion and helicase assembly. J Biol Chem. 2015;290:1210-21.

49. Hiraga S, Alvino G, Chang F, Lian H, Sridhar A, Kubota T, et al. Rif1 controls DNA replication by directing Protein Phosphatase 1 to reverse Cdc7-mediated phosphorylation of the MCM complex. Genes Dev. 2014;28:372-83.

50. Davé A, Cooley C, Garg M, Bianchi A. Protein phosphatase 1 recruitment by Rif1 regulates DNA replication origin firing by counteracting DDK activity. Cell Rep. 2014;7:53-61.

51. Chen S, Bell S. CDK prevents Mcm2-7 helicase loading by inhibiting Cdt1 interaction with Orc6. Genes Dev. 2011;25:363-72.

52. Tanaka S, Umemori T, Hirai K, Muramatsu S, Kamimura Y, Araki H. CDK-dependent phosphorylation of Sld2 and Sld3 initiates DNA replication in budding yeast. Nature. 2007;445(7125):328-32.

53. Kanter D, Kaplan D. Sld2 binds to origin single-stranded DNA and stimulates DNA annealing. Nucleic Acids Res. 2011;39:2580-92.

54. Zegerman P, Diffley JF. Phosphorylation of Sld2 and Sld3 by cyclin-dependent kinases promotes DNA replication in budding yeast. Nature. 2007;445(7125):281-5.

55. Muramatsu S, Hirai K, Tak Y, Kamimura Y, Araki H. CDK-dependent complex formation between replication proteins Dpb11, Sld2, Pol (epsilon}, and GINS in budding yeast. Genes Dev. 2010;24:602-12.

56. Araki H, Leem S, Phongdara A, Sugino A. Dpb11, which interacts with DNA polymerase II(epsilon) in Saccharomyces cerevisiae, has a dual role in S-phase progression and at a cell cycle checkpoint. Proc Natl Acad Sci U S A. 1995;92:11791-5.

57. Masumoto H, Sugino A, Araki H. Dpb11 controls the association between DNA polymerases alpha and epsilon and the autonomously replicating sequence region of budding yeast. Mol Cell Biol. 2000;20:2809-17.

58. Bruck I, Kaplan D. GINS and Sld3 compete with one another for Mcm2-7 and Cdc45 binding. J Biol Chem. 2011;286:14157-67.

59. Dhingra N, Bruck I, Smith S, Ning B, Kaplan D. Dpb11 helps control assembly of the Cdc45-Mcm2-7-GINS replication fork helicase. J Biol Chem. 2015;Epub ahead of print:Feb 6.

60. Bruck I, Kaplan D. The replication initiation protein sld2 regulates helicase assembly. J Biol Chem. 2014;289:1948-59.

61. Bruck I, Kanter DM, Kaplan DL. Enabling association of the GINS tetramer with the Mcm2-7 complex by phosphorylated Sld2 protein and single-stranded origin DNA. J Biol Chem. 2011;286:36414-26.

62. Bruck I, Kaplan D. Origin Single-stranded DNA Releases Sld3 Protein from the Mcm2-7 Complex, Allowing the GINS Tetramer to Bind the Mcm2-7 Complex. J Biol Chem. 2011;286:18602-13.

63. Van Hatten RA, Tutter AV, Holway AH, Khederian AM, Walter JC, Michael WM. The Xenopus Xmus101 protein is required for the recruitment of Cdc45 to origins of DNA replication. J Cell Biol. 2002;159:541-7.

64. Schmidt U, Wollmann Y, Franke C, Grosse F, Saluz HP, Hänel F. Characterization of the interaction between the human DNA topoisomerase IIbeta binding protein 1 (TopBP1) and the cell division cycle protein 45 (Cdc45). Biochem J. 2007;Sept 21.

65. Boos D, Sanchez-Pulido L, Rappas M, Pearl LH, Oliver AW, Ponting CP, et al. Regulation of DNA replication through Sld3-Dpb11 interaction is conserved from yeast to humans. Curr Biol. 2011;21(13):1152-7.

66. Ohlenschläger O, Kuhnert A, Schneider A, Haumann S, Bellstedt P, Keller H, et al. The N-terminus of the human RecQL4 helicase is a homeodomain-like DNA interaction motif. Nucleic Acids Res. 2012;40:8309-24.

67. Zegerman P. Evolutionary conservation of the CDK targets in eukaryotic DNA replication initiation. Chromosoma. 2015;Epub ahead of print:Jan 11.

68. Alver R, Zhang T, Josephrajan A, Fultz B, Hendrix C, Das-Bradoo S, et al. The N-terminus of Mcm10 is important for interaction with the 9-1-1 clamp and in resistance to DNA damage. Nucleic Acids Res. 2014;42:8389-404.

69. Thu YM, Bielinsky AK. Enigmatic roles of Mcm10 in DNA replication. Trends Biochem Sci. 2013;38(4):184-94.

70. van Deursen F, Sengupta S, De Piccoli G, Sanchez-Diaz A, Labib K. Mcm10 associates with the loaded DNA helicase at replication origins and defines a novel step in its activation. EMBO J. 2012;31(9):2195-206.

71. Watase G, Takisawa H, Kanemaki M. Mcm10 plays a role in functioning of the eukaryotic replicative DNA helicase, Cdc45-Mcm-GINS. Curr Biol. 2012;22:343-9.

72. Kanke M, Kodama Y, Takahashi TS, Nakagawa T, Masukata H. Mcm10 plays an essential role in origin DNA unwinding after loading of the CMG components. EMBO J. 2012;31(9):2182-94.

73. Thu YM, Bielinsky AK. MCM10: one tool for all-Integrity, maintenance and damage control. Semin Cell Dev Biol. 2014;30:121-30.

74. Onesti S, MacNeill SA. Structure and evolutionary origins of the CMG complex. Chromosoma. 2013;122(1-2):47-53.

75. Krastanova I, Sannino V, Amenitsch H, Gileadi O, Pisani FM, Onesti S. Structural and functional insights into the DNA replication factor Cdc45 reveal an evolutionary relationship to the DHH family of phosphoesterases. J Biol Chem. 2012;287(6):4121-8.

76. Bruck I, Kaplan DL. Cdc45 protein-single-stranded DNA interaction is important for stalling the helicase during replication stress. J Biol Chem. 2013;288(11):7550-63.

77. Szambowska A, Tessmer I, Kursula P, Usskilat C, Prus P, Pospiech H, et al. DNA binding properties of human Cdc45 suggest a function as molecular wedge for DNA unwinding. Nucleic Acids Res. 2014;42:2308-19.

78. Takayama Y, Kamimura Y, Okawa M, Muramatsu S, Sugino A, Araki H. GINS, a novel multiprotein complex required for chromosomal DNA replication in budding yeast. Genes Dev. 2003;17(9):1153-65.

79. Kubota Y, Takase Y, Komori Y, Hashimoto Y, Arata T, Kamimura Y, et al. A novel ring-like complex of Xenopus proteins essential for the initiation of DNA replication. Genes Dev. 2003;17(9):1141-52.

80. Boskovic J, Coloma J, Aparicio T, Zhou M, Robinson CV, Méndez J, et al. Molecular architecture of the human GINS complex. EMBO Rep. 2007;8(7):678-84.

81. Kamada K, Kubota Y, Arata T, Shindo Y, Hanaoka F. Structure of the human GINS complex and its assembly and functional interface in replication initiation. Nat Struct Mol Biol. 2007;14(5):388-96.

82. Chang YP, Wang G, Bermudez V, Hurwitz J, Chen XS. Crystal structure of the GINS complex and functional insights into its role in DNA replication. Proc Natl Acad Sci U S A. 2007;104(31):12685-90.

83. Costa A, Ilves I, Tamberg N, Petojevic T, Nogales E, Botchan MR, et al. The structural basis for MCM2-7 helicase activation by GINS and Cdc45. Nat Struct Mol Biol. 2011;18(4):471-7.

85. Lord CJ, Ashworth A. The DNA damage response and cancer therapy. Nature. 2012;481(7381):287-94.

86. Hustedt N, Gasser SM, Shimada K. Replication checkpoint: tuning and coordination of replication forks in s phase. Genes (Basel). 2013;4(3):388-434.

87. Zou L. Four pillars of the S-phase checkpoint. Genes Dev. 2013;27:227-33.

88. Navadgi-Patil V, Burgers P. Yeast DNA replication protein Dpb11 activates the Mec1/ATR checkpoint kinase. J Biol Chem. 2008;283:35853-9.

89. Navadgi-Patil VM, Burgers PM. The unstructured C-terminal tail of the 9-1-1 clamp subunit Ddc1 activates Mec1/ATR via two distinct mechanisms. Mol Cell. 2009;36(5):743-53.

90. Recolin B, van der Laan S, Tsanov N, Maiorano D. Molecular mechanisms of DNA replication checkpoint activation. Genes (Basel). 2014;5(1):147-75.

91. Sirbu BM, Cortez D. DNA damage response: three levels of DNA repair regulation. Cold Spring Harb Perspect Biol. 2013;5(8):a012724.

92. Navadgi-Patil VM, Burgers PM. Cell-cycle-specific activators of the Mec1/ATR checkpoint kinase. Biochem Soc Trans. 2011;39(2):600-5.

93. Navadgi-Patil VM, Kumar S, Burgers PM. The unstructured C-terminal tail of yeast Dpb11 (human TopBP1) protein is dispensable for DNA replication and the S phase checkpoint but required for the G2/M checkpoint. J Biol Chem. 2011;286(47):40999-1007.

94. Cremona CA, Sarangi P, Yang Y, Hang LE, Rahman S, Zhao X. Extensive DNA damage-induced sumoylation contributes to replication and repair and acts in addition to the mec1 checkpoint. Mol Cell. 2012;45(3):422-32.

95. Segurado M, Tercero JA. The S-phase checkpoint: targeting the replication fork. Biol Cell. 2009;101:617-27.

96. Kumar S, Burgers PM. Lagging strand maturation factor Dna2 is a component of the replication checkpoint initiation machinery. Genes Dev. 2013;27(3):313-21.

97. Labib K, Piccoli GD. Surviving chromosome replication: the many roles of the S-phase checkpoint pathway. Phil Trans R Soc B. 2011;366:3554-61.

98. Zegerman P, Diffley JF. Checkpoint-dependent inhibition of DNA replication initiation by Sld3 and Dbf4 phosphorylation. Nature. 2010;467(7314):474-8.

99. De Piccoli G, Katou Y, Itoh T, Nakato R, Shirahige K, Labib K. Replisome stability at defective DNA replication forks is independent of S phase checkpoint kinases. Mol Cell. 2012;45:699-704.

100. Anand RP, Lovett ST, Haber JE. Break-induced DNA replication. Cold Spring Harb Perspect Biol. 2013;5(12):a010397.

101. Lydeard JR, Lipkin-Moore Z, Sheu YJ, Stillman B, Burgers PM, Haber JE. Break-induced replication requires all essential DNA replication factors except those specific for pre-RC assembly. Genes Dev. 2010;24(11):1133-44.

102. Malkova A, Ira G. Break-induced replication: functions and molecular mechanism. Curr Opin Genet Dev. 2013;23(3):271-9.

103. Wang H, Elledge S. Genetic and physical interactions between DPB11 and DDC1 in the yeast DNA damage response pathway. Genetics. 2002;160:1295-304.

104. Mordes D, Nam E, Cortez D. Dpb11 activates the Mec1-Ddc2 complex. Proc Natl Acad Sci U S A. 2008;105:18730-4.

105. Qu M, Rappas M, Wardlaw CP, Garcia V, Ren JY, Day M, et al. Phosphorylation-dependent assembly and coordination of the DNA damage checkpoint apparatus by Rad4(TopBP[1]). Mol Cell. 2013;51(6):723-36.

106. Pfander B, Diffley JF. Dpb11 coordinates Mec1 kinase activation with cell cycle-regulated Rad9 recruitment. EMBO J. 2011;30(24):4897-907.

107. Wardlaw CP, Carr AM, Oliver AW. TopBP1: A BRCT-scaffold protein functioning in multiple cellular pathways. DNA Repair (Amst). 2014;22:165-74.

108. Gritenaite D, Princz LN, Szakal B, Bantele SC, Wendeler L, Schilbach S, et al. A cell cycle-regulated Slx4-Dpb11 complex promotes the resolution of DNA repair intermediates linked to stalled replication. Genes Dev. 2014;28(14):1604-19.

109. Germann S, Oestergaard V, Haas C, Salis P, Motegi A, Lisby M. Dpb11/TopBP1 play distinct roles in DNA replication, checkpoint response and homologus recombination. DNA Repair. 2011;10:210-24.

110. Tak Y, Tanaka Y, Endo S, Kamimura Y, Araki H. A CDK-catalysed regulatory phosphorylation for formation of the DNA replication complex Sld2-Dpb11. EMBO J. 2006;25:1987-96.

111. Bochman M, Bell S, Schwacha A. Subunit organization of Mcm2-7 and the unequal role of active sites in ATP hydrolysis and viability. Mol Cell Biol. 2008;28:5865-73.

112. Evrin C, Clarke P, Zech J, Lurz R, Sun J, Uhle S, et al. A double-hexameric MCM2-7 complex is loaded onto origin DNA during licensing of eukaryotic DNA replication. Proc Natl Acad Sci U S A. 2009;106:20240-5.

113. Masai H, Taniyama C, Ogino K, Matsui E, Kakusho N, Matsumoto S, et al. Phosphorylation of MCM4 by Cdc7 kinase facilitates its interaction with Cdc45 on the chromatin. J Biol Chem. 2006;281:39249-61.

114. Labib K. How do Cdc7 and cyclin-dependent kinases trigger the initiation of chromosome replication in eukaryotic cells? Genes Dev. 2010;24:1208-19.

115. Fu Y, Yardimci H, Long D, Ho T, Guainazzi A, Bermudez V, et al. Selective bypass of a lagging strand roadblock by the eukaryotic replicative DNA helicase. Cell. 2011;146:931-41.

116. Gambus A, Jones RC, Sanchez-Diaz A, Kanemaki M, Deursen Fv, Edmondson RD, et al. GINS maintains association of Cdc45 with MCM in replisome progression complexes at eukaryotic DNA replication forks. Nature Cell Biology. 2006;8:358 - 66.

117. Moyer S, Lewis P, Botchan M. Isolation of the Cdc45/Mcm2-7/GINS (CMG) complex, a candidate for the eukaryotic DNA replication fork helicase. Proc Natl Acad Sci U S A. 2006;103:10236-41.

118. Pacek M, Tutter A, Kubota Y, Takisawa H, Walter J. Localization of MCM2-7, Cdc45, and GINS to the site of DNA unwinding during eukaryotic DNA replication. Mol Cell. 2006;21:581-7.

119. Heller R, Kang S, Lam W, Chen S, Chan C, Bell S. Eukaryotic Origin-Dependent DNA Replication In Vitro Reveals Sequential Action of DDK and S-CDK Kinases. Cell. 2011;146:80-91.

120. Gros J, Devbhandari S, Remus D. Origin plasticity during budding yeast DNA replicaiton in vitro. EMBO J. 2014;33:621-36.

121. On K, Beuron F, Frith D, Snijders A, Morris E, Diffley J. Prereplicative complexes assembled in vitro support origin-dependent and independent DNA replication. EMBO J. 2014;18:605-20.

122. Kaplan DL, Davey MJ, O'Donnell M. Mcm4,6,7 uses a 'pump in ring' mechanism to unwind DNA by steric exclusion and actively translocate along a duplex. J Biol Chem. 2003;278:49171-82.

123. Geraghty D, Ding M, Heintz N, Pederson D. Premature structural changes at replication origins in a yeast minichromosome maintenance (MCM) mutant. J Biol Chem. 2000;275:18011-21.

124. Yeeles JT, Deegan TD, Janska A, Early A, Diffley JF. Regulated eukaryotic DNA replication origin firing with purified proteins. Nature. 2015;519(7544):431-5.

125. Kamimura Y, Masumoto H, Sugino A, Araki H. Sld2, which interacts with Dpb11 in Saccharomyces cerevisiae, is required for chromosomal DNA replication. Mol Cell Biol. 1998;18:6102-9.

126. Kanemaki M, Labib K. Distinct roles for Sld3 and GINS during establishment and progression of eukaryotic DNA replication forks. EMBO J. 2006;25(8):1753-63.

127. Tanaka S, Nakato R, Katou Y, Shirahige K, Araki H. Origin association of Sld3, Sld7, and Cdc45 proteins is a key step for determination of origin-firing timing. Curr Biol. 2011;21:2055-63.

128. Randell J, Fan A, Chan C, Francis L, Heller R, Galani K, et al. Mec1 is one of multiple kinases that prime the Mcm2-7 helicase for phosphorylation by Cdc7. Mol Cell. 2010;40:353-63.

129. Masumoto H, Muramatsu S, Kamimura Y, Araki H. S-Cdk-dependent phosphorylation of Sld2 essential for chromosomal DNA replication in budding yeast. Nature. 2002;415:651-5.

130. Costa A, Renault L, Swuec P, Petojevic T, Pesavento J, Ilves I, et al. DNA binding polarity, dimerization, and ATPase ring remodeling in the CMG helicase of the eukaryotic replisome. Elife. 2014;Aug 12:e03273.

131. Tanaka S, Komeda Y, Umemori T, Kubota Y, Takisawa H, Araki H. Efficient initiation of DNA replication in eukaryotes requires Dpb11/TopBP1-GINS interaction. Mol Cell Biol. 2013;33:2614-22.

132. Bochman M, Schwacha A. The Saccharomyces cerevisiae Mcm6/2 and Mcm5/3 ATPase active sites contribute to the function of the putative Mcm2-7 'gate'. Nucleic Acids Res. 2010;38:6078-88.

133. Hashimoto Y, Takisawa H. Xenopus Cut5 is essential for a CDK-dependent process in the initiation of DNA replication. EMBO J. 2003;22:2526-35.