Characterizing the Associations and Roles of DDK and Mcm2-7 DNA Replication Proteins in *Saccharomyces Cerevisiae*

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

The essential cell cycle kinase Dbf4/Cdc7 (DDK) triggers DNA replication through phosphorylation of the Mcm2-7 helicase at replication origins. Prior work has implicated various Mcm2-7 subunits as targets of DDK, however it is not well understood which specific subunits mediate the docking of the DDK complex. Through yeast two-hybrid and co-immunoprecipitation analyses, we found that Dbf4 and Cdc7 interact with distinct subunits of the Mcm2-7 helicase complex. Dbf4 showed the strongest interaction with Mcm2 while Cdc7 associated with Mcm4 and Mcm5. Dissection of the N-terminal region of Mcm2 revealed two regions that mediate the interaction with Dbf4, whereas in Mcm4, a region near the N-terminus has been previously identified by another group as the DDK docking domain. Mutant forms of Mcm2 (Mcm2ΔDDD) or Mcm4 (Mcm4ΔDDD) lacking the DDK docking domain were expressed in cells and resulted in modest growth and replication defects. Combining the two mutations resulted in synthetic lethality, suggesting a redundant mechanism of Mcm2 and Mcm4 in targeting the DDK complex to Mcm rings. Furthermore, growth inhibition could be induced in a Mcm4ΔDDD background by overexpressing Mcm2 to titrate Dbf4 from Mcm rings. These growth defects were exacerbated in the presence of genotoxic agents such as hydroxyurea and methyl methanesulfonate, suggesting that DDK-Mcm interactions may play a role in stabilizing replication forks under S-phase checkpoint conditions. Regions of Cdc7 were examined for their interaction with Mcm4 and Dbf4. Results have shown that the N-terminal amino acid region 55-124 and the C-terminal region 453-507 of Cdc7 are likely target regions for Dbf4-binding. Several conserved residues were identified within the N-terminal 55-124 Cdc7 region that interface with conserved residues

within motif-C of Dbf4. Conserved residues were identified within the DDD domain of Mcm2 and mutating these residues resulted in a decreased interaction with Dbf4. Lastly, bioinformatics analysis has revealed potential conserved residues within the Mcm4DDD region, which may play a role in binding to Cdc7. This research is significant because these factors, which are conserved in all eukaryotes studied to date, should give further insight as to how DNA replication is triggered and how it is affected when cells are exposed to DNA damaging or replication compromising agents. This research also has implications in cancer genetics, as prior studies have shown elevated DDK and Mcm protein levels in tumour cell lines and melanomas, with Cdc7 showing great promise as a cancer therapeutic target. Such knowledge will further enhance our understanding of the DNA replication process and the roles of cell cycle proteins involved, under both normal and checkpoint conditions.

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List of Abbreviations and Acronyms

APC/C:Anaphase promoting complex/cyclosome

ARS: Autonomously replicating sequence

Cdc: Cell division cycle

CDK: Cyclin dependent kinase

CMG: Cdc45, Mcm2-7, GINS

Co-IP: Co-immunoprecipitation

DDK: Dbf4-dependent kinase

DNA: Deoxyribonucleic acid

dNTP: deoxynucleotide phosphate

DTT: Dithiothreitol

EDTA: Ethylenediaminetetraacetic acid

FACS: Fluorescence activated cell sorting

FL: Full Length

5'FOA: 5-Fluoroorotic Acid

GAL/RAFF: Galactose/Raffinose

GINS: Go-Ichi-Ni-San

HA: Hemagglutinin

HU: Hydroxyurea

Kb: Kilobase

LEU: Leucine

MCM: Minichromosome maintenance

MMS: Methyl methanesulfonate

MYC: Myelocytamatosis

NLS: Nuclear Localization Signal

ONPG: 2-Nitrophenyl-β-D-galactopyranoside

ORC: Origin recognition complex

PCNA: Proliferating cell nuclear antigen

PCR: Polymerase chain reaction

PMSF: Phenylmethylsulphonyl fluoride

Pre-IC: Pre-initiation complex

Pre-LC: Pre-loading complex

Pre-RC: Pre-replicative complex

RPA: Replication protein A

SC: Synthetic complete

SDS: Sodium dodecyl sulfate

SV40: Simian Vacuolating Virus 40

TRP: Tryptophan

URA: Uracil

WCE: Whole cell extract

WT: Wild-type

YPD: Yeast extract, peptone, dextrose

Chapter 1: Introduction

1.1 Background

1.1.1 Features of Yeast

Yeasts are unicellular eukaryotes that have historically been used as a key component in fermentation processes like brewing, bread making and wine making dating as far back as 6000 B.C. Yeasts are members of the Fungi kingdom with more than 1500 yeast species identified to date. Yeasts are non-photosynthetic chemoorganotrophs meaning they require fixed, organic compounds as their source of energy. These can include sugars like glucose and fructose, alcohols, organic acids, fatty acids, and hydrocarbons (reviewed in Walker, 1998). The ability to utilize a variety of carbon sources allows their adaptation to a diverse range of natural habitats like plant surfaces, ripened fruits, guts of animals and insects, soils, freshwater, seawater, and even some extreme habitats like low temperature and high salt environments (reviewed in Walker, 1998; Turk *et al.*, 2010). Yeasts can also survive in aerobic or anaerobic environments. In the presence of oxygen, they utilize aerobic respiration to convert sugars to carbon dioxide and ATP, and in the absence of oxygen, energy is obtained from glycolysis and sugars are converted to ethanol (reviewed in Kruckeberg and Dickinson, 2004). In the 1930s, yeast was identified as a useful experimental organism and has since been used to study fundamental cellular processes, mechanisms and structures (reviewed in Feldmann, 2012).

Saccharomyces cerevisiae, also known as budding yeast, is commonly used as a model organism for genetic analysis because of its short generation time, easy cultivation methods and simple DNA transformation system for the manipulation of genes. The term "budding" comes from its asexual division process, where a small bud grows off the mother

cell and eventually pinches off to form a daughter cell (reviewed in Herskowitz, 1988). Wild type yeast has a doubling time of 90-100 minutes when grown at 30°C. It can exist in either haploid or diploid states, undergoing the simple life cycle of mitosis and growth (reviewed in Herskowitz, 1988). Typically, haploid yeast cells have a spherical shape measuring 2-4 μm in diameter whereas the larger ellipsoid-shaped diploid cells measure 5-6 μm (reviewed in Walker, 1998; reviewed in Feldmann, 2012). Haploids exist as either mating type-a or type-α (reviewed in Herskowitz, 1988). These mating types are determined by the transcriptional repression or activation of the mating type (*MAT)* locus, which confers sexual differentiation into one of the two types (reviewed in Herskowitz, 1988). A haploid cell can trigger the mating response pathway in a cell with the opposite mating type. For instance, a MATα type cell secretes the α-factor pheromone, which binds to the receptors on a nearby MATa cell, thereby activating its mating response (reviewed in Kruckeberg and Dickinson, 2004). This response involves cell projections that change the cell's morphology to form a pear-shape, a process known as "schmooing", which enables it to extend, conjugate, and form diploids (reviewed in Herskowitz, 1988). Subsequently, diploids undergo meiosis and sporulation under starvation conditions to form four haploids enclosed in a sac (ascus) (reviewed in Kruckeberg and Dickinson, 2004). The α-factor pheromone arrests cells in late G1-phase by inactivating the G1 cyclin/Cdc28 kinase complex (involved in the transition from G1 to S phase). In the lab, α-factor can be used to arrest cells and study relative cell cycle progression (Herskowitz and Matthias, 1994).

1.1.2 Yeast Genetics

A haploid *S. cerevisiae* cell contains 16 linear chromosomes ranging in size from 230 kilobases (kb) to 1532 kb (*Saccharomyces* Genome Database, 2014; Engel *et al.*, 2014). The budding yeast genome was the first eukaryotic organism to be fully sequenced in 1996 (Gofeau *et al.*, 1996). Currently 6607 open reading frames (ORFs) have been identified, of which 5094 ORFs (77.1%) have been confirmed as protein-encoding genes, 786 ORFs (11.9%) are "dubious" meaning they may not be true ORFs, and the remaining 727 ORFs (11%) have yet to be characterized (*Saccharomyces* Genome Database, 2014; Costanzo *et al.*, 2010). Their highly compact genome consists of 12, 157 kb of DNA, with introns making up only 4% of the genome (*Saccharomyces* Genome Database, 2014; Lin *et al.*, 2013). This relatively small genome size allows for easy manipulation, especially when creating mutants. Simple transformation methods can be used to introduce circular plasmids into yeast, which can then be maintained in the cell through selective measures. Lab strains commonly have deletions in metabolic genes involved in the production of essential organic compounds necessary for growth. For example, *URA3* is a gene that encodes orotidine 5-phosphate decarboxylase, an enzyme that is involved in the biosynthesis of uracil. Strains with a mutation or deletion of this gene cannot synthesize uracil *de novo*, therefore, must be grown in media supplemented with uracil in order to survive (reviewed in Funk *et al.*, 2002). Exogenous DNA containing the *URA3* gene can be transformed into *ura3*- mutants and selected for growth on media with no uracil added. The *URA3* gene can also be used in a negative selection technique when grown on 5-fluoroorotic acid (5'FOA) media. Strains possessing a functional *URA3* gene, will convert 5'FOA compound to its toxic form 5-

flurouracil, which causes cell death. This method can be used to select for the loss of a *URA3*-containing plasmid or select for *ura3*- mutants, which would be resistant.

Homologous recombination is another method for introducing exogenous DNA via integration into the genome. DNA (linear or circular form) containing altered or disrupted alleles along with flanking homologous chromosomal segments, can be transformed into yeast cells and directed to specific locations in the genome for replacement (reviewed in Funk *et al.*, 2002). Replacing or deleting normal wild-type genes can produce phenotypes that may shed light on gene and protein function. Another type of genetic manipulation is replacing native gene promoters with constitutive and regulatable promoters. A commonly used promoter is the *GAL1* promoter, which is activated in the presence of galactose and repressed in its absence (Mumberg *et al.*, 1994). This allows gene expression to be controlled and proteins to be expressed at 1,000-fold in the presence of galactose. Many commercially available yeast strains have other convenient modifications in the genome for research purposes such as epitope-tagged fusion proteins, temperature sensitive mutations, and single gene deletions. Examples of epitope tags used in this thesis work are Hemagglutinin (HA), LexA, and myelocytomatsis (Myc) tags. Antibodies such as anti-Myc , anti-LexA, and anti-HA can be used to detect these fusion proteins on a Western blot, proving especially useful when proteins are present in low levels. The yeast strain background used in this thesis is S288c, derived from a progenitor strain EM93 (Mortimer and Johnston, 1986).

1.1.3 Yeast Life Cycle

The mitotic cell cycle of budding yeast *Saccharomyces cerevisiae* consists of the stages: Gap 1 (G1), Synthesis (S), Gap 2 (G2), and Mitosis (M) as well as cytokinesis (Figure 1.1) (reviewed in Herskowitz, 1988). In the G1 phase, cells grow and mainly carry out RNA synthesis and protein production. An important point in control of the cell cycle is the START checkpoint in late G1 phase, which confirms that the proper order of events have occurred before progressing into S phase (reviewed in Morgan, 2007). A number of factors can hinder a cell from progressing into S phase. Under nutrient starvation conditions, cells may enter a quiescent G0 phase where they can remain until nutrients become available again. Growth of the cell to a critical threshold size is important and depends on external factors like availability of nutrients, exogenous compounds and temperature (reviewed in Hartwell, 1974). DNA damage can also be a factor that triggers arrest and inhibits progression into S phase. Lastly, presence of mating pheromones can signal cells to undergo sexual reproduction with the opposite mating cell type instead of continuing on with the mitotic cycle. In budding yeast, Cdc28, a cyclin-dependent kinase (CDK), is considered the master regulator of cell cycle events like DNA replication, bud initiation, mitotic commitment, spindle assembly, and chromosome segregation (reviewed in Mendenhall and Hodge, 1998). Cdc28 binds to G1-phase cyclins like Cln1, Cln2, and Cln3 to form G1/S-CDKs (reviewed in Mendenhall and Hodge, 1998). S-phase cyclins Clb5 and Clb6 bind to Cdc28 to form inactive S phase cyclin-CDKs (S-CDKs) inhibited by the Stoichiometric Inhibitor of CDK-Clb (Sic1) protein (Schwob and Nasmyth, 1993; Verma *et al.*, 2001). As the cell approaches S-phase, G1/S-CDKs phosphorylate and target Sic1 for destruction,

thereby activating S-CDKs (reviewed in Morgan, 2007). The G1 phase is also when the pre-Replicative Complex (pre-RC) forms at specific initiation sites in the genome known as origins of DNA replication (more details on pre-RC formation are discussed in the next section). CDK levels are low in G1 phase when pre-RC assembly occurs, but rise sharply in the late G1 phase in order to activate the pre-RC and initiate DNA replication. Subsequently, active CDK levels are maintained throughout S, G2 and M phases to prevent further pre-RC formation and re-replication (Chen *et al.*, 2011; Nguyen *et al.*, 2001). CDK-mediated phosphorylation of the pre-RC components ORC, Mcm proteins and Cdc6 is the minimal requirement to prevent re-replication in the G2 and M phase (Nguyen *et al.*, 2001).

DNA replication occurs in S phase and is highly regulated to ensure proper duplication of chromosomes. Replication proceeds in a semi-conservative fashion with both parental strands serving as a template for new DNA synthesis (reviewed in Morgan, 2007). The eukaryotic replicative helicase Mcm2-7 (Minichromosome maintenance) breaks the hydrogen bonds between bases to unwind the DNA double helix and allow replication forks to progress bi-directionally from the origins forming a replication bubble (reviewed in Sclafani and Holzen, 2007). Topoisomerase II nicks the DNA downstream of the replication fork to uncoil the tightly packed DNA and relieve the tension caused by DNA unwinding. Single-stranded binding proteins (called RPAs in yeast) bind to the unwound DNA to prevent the re-annealing of the strands. A number of DNA polymerases then come in to carry out the duplication of the DNA sequence. First DNA polymerase α-primase binds the exposed DNA and synthesizes a short complementary RNA primer to act as a starting point for elongation

(Reviewed in Garg and Burgers, 2005). DNA polymerase can only synthesize in the 5' - 3' direction, therefore the synthesis process of the leading and lagging strand is different. The leading strand, which opens in the 3' - 5' direction, is duplicated by DNA polymerase ε, which adds the dNTPs continually with the movement of the fork. The lagging strand requires the addition of multiple RNA primers in order for DNA polymerase to synthesize in the 5' – 3' direction (reviewed in Garg and Burgers, 2005). Once the fork has progressed far enough, DNA polymerase α-primase synthesizes a short RNA primer. The RNA primers provide 3' hydroxyl groups at regular intervals along the lagging strand for DNA polymerase. Next, Proliferating Cell Nuclear Antigen (PCNA) clamps the DNA triggering the polymerase α-primase to dissociate and recruits polymerase δ to synthesize new DNA using the RNA primer. Since the lagging strand elongates in the opposite direction (towards the origin) of the replication fork movement, the addition of nucleotides is discontinuous and forms short 150 bp fragments called Okazaki fragments (reviewed in MacNeill, 2001). The RNA primers are then removed by endonuclease 1 (FEN1) and polymerase δ fills in the gaps with corresponding nucleotides. Finally, DNA ligase I joins the fragments by forming covalent phosphodiester bonds between the 3' hydroxyl end of one nucleotide and the 5' phosphate end of the adjacent nucleotide, resulting in the formation of one continuous DNA strand (reviewed in Morgan, 2007).

Replicating eukaryotic linear DNA can pose a problem with respect to the ends of the chromosomes. When the RNA primer at the very end of the lagging strand is removed, a required 3' hydroxyl group is no longer present for DNA polymerase to fill in the region

where the primer was located. Consequently, the remaining unreplicated sequence would be degraded and result in progressively shorter chromosomes with each cycle. The cell however has mechanisms in place to prevent this continual loss of genetic material. The ends of the chromosomes contain repeated nucleotide sequences called telomeres. Telomerase, a ribonucleoprotein reverse transcriptase, binds to the 3' flanking end of the telomeres and adds bases using its own RNA molecule as a template. The fidelity of telomerase activity is thought to decrease over time and contribute to aging and cancer development. By the end of S phase, the duplicated DNA consists of a parental DNA strand and a newly synthesized strand (reviewed in Morgan, 2007).

In the G2 phase, cells prepare for mitosis by synthesizing the necessary proteins and increasing in size. The chromosomes start to condense and proteins involved in mitotic spindle formation are synthesized. The G2/M checkpoint is an important DNA damage detection step to ensure that damaged DNA is not passed onto the daughter cells. The Cdc28/Clb2 protein complex facilitates the entry into mitosis (Surana *et al.*, 1993). Mitosis is composed of five stages: prophase, prometaphase, metaphase, anaphase, and telophase (reviewed in Morgan, 2007). In prophase, chromosomes continue to condense while spindle pole bodies organize microtubules. The microtubules extend and attach to a region on the chromosomes called kinetochores to facilitate its movement. In metaphase, a spindle pole body positions at the bud neck and spindles align along the mother/bud axis (Yeh *et al.*, 2000). In yeast, chromosomes do not condense enough to show a distinct alignment at a conventional metaphase plate (Straight *et al.*, 1997; reviewed in Winey and O'Toole, 2001).

The Anaphase Promoting Complex/Cyclosome (APC/C) facilitates the entry into anaphase and marks cell cycle proteins like cyclins for ubiquitin-mediated proteolysis (reviewed in Peters, 2002; Manchado *et al.*, 2010). Sister chromatids are held together by the protein complex cohesin, which is cleaved by the separase enzyme. Separase activation occurs through APC/C-dependent ubiquitination and degradation of its inhibitory chaperone protein, securin (reviewed in Peters, 2002; reviewed in Nasmyth, 2005). Cleavage of cohesins enables the separation of sister chromatids to opposite ends of the cell in anaphase. Yeast differs from multicellular eukaryotes in that it undergoes a closed mitosis, whereby the disassembly and reassembly of the nuclear envelope does not occur (reviewed in Taddei *et al.*, 2010). Bud enlargement continues until the telophase stage when the protein machinery is redirected to prepare for cytokinesis (reviewed in Bi and Park, 2012; reviewed in Morgan, 2007). Cytokinesis is the process involving the separation of the mother and daughter cell. The physical division is accomplished through the formation of a contractile actomyosin ring and septum at the bud neck of the cell (reviewed in Wloka and Bi, 2012). Budding yeast undergoes asymmetric cell division with buds initially being smaller than the mother cell. After separation, a bud scar is left on the cell surface of the mother cell. Bud scars are an important determinant of cellular age (reviewed in Kruckeberg and Dickinson, 2004; reviewed in Walker, 1998; reviewed in Morgan, 2007).

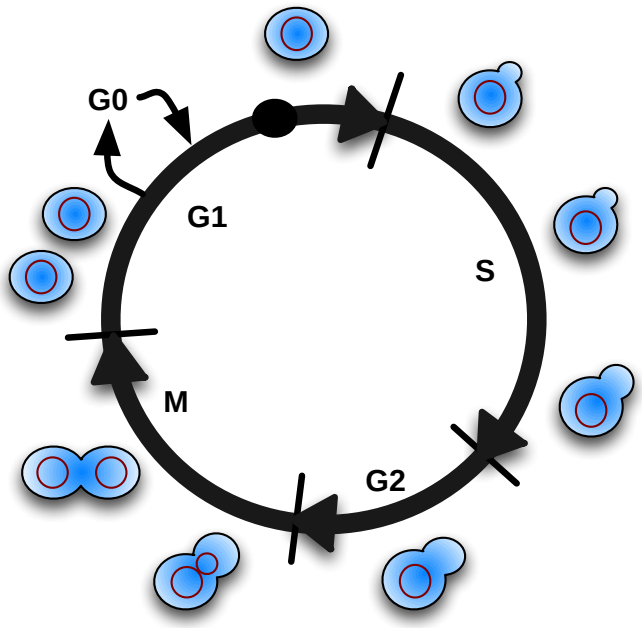

Figure 1.1: Cell Cycle of *Saccharomyces cerevisiae*. The life cycle encompasses the main stages of growth including G1, S, G2 and M phase followed by asymmetric cell division to form a mother and daughter cells. Entry into and exit from the G0 phase occurs before START (represented by the black circle in the G1 phase).

1.2 Initiation of DNA Replication

1.2.1 Formation of the pre-RC and Activation at Origins

The initiation of DNA replication in budding yeast begins at specific origin sites in the genome called Autonomously Replicating Sequences (ARS). These origins were first identified using plasmid-based assays in which fragments of the yeast genome were incorporated into bacterial plasmids lacking a eukaryotic origin (Brewer and Fangman, 1987). Any resulting plasmids that were able to replicate in yeast cells would have contained these origin sequences, and were subsequently identified as ARS origins (Brewer and Fangman, 1987). It is estimated that there are roughly 300 origins of replication found

throughout the yeast genome and activated origins fire only once per cell cycle (Raghuraman *et al.*, 2001; Nieduszynski *et al.*, 2006). Yeast origins fire in a temporal fashion activating in early, middle or late in S phase (Barberis *et al.*, 2010; Mantiero *et al.*, 2011). Cells potentially employ this temporal firing mechanism to preserve a number of unfired origins in the event when replication forks stall due to the DNA damage checkpoint response (Duch *et al.*, 2011). Preserving late origins also serves a role in restarting replication during checkpoint recovery (Santocanale and Diffley, 1998).

In the initiation of eukaryotic DNA replication, there are two main stages that ensure faithful replication of the genome. The first is the formation of the pre-RC, which is a preparatory process that involves the sequential binding of protein factors at replication origins (reviewed in Sclafani and Holzen, 2007) (Figure 1.2). The formation of the pre-RC occurs in the G1 phase and 'licenses' the origins to prepare them to fire. ORC (Origin Recognition Complex), composed of six subunits, Orc1-6, is the first protein complex to bind to the origin, acting as a scaffold for the binding of subsequent protein factors (reviewed in Labib, 2010). Next, Cdc6 (Cell Division Cycle 6) AAA+ ATPase protein binds to the ORC complex and acts as a clamp loader to enable the Mcm2-7 complex to bind via ATP hydrolysis (Randell *et al.*, 2006) (Figure 1.2). Mcm2-7 is a heterohexameric helicase complex that unwinds the DNA during the replication process (reviewed in Li and Araki, 2013). Cdt1 (Chromatin licensing and DNA replication factor-1) protein targets the inactive Mcm2-7 complex to origins, followed by ORC-dependent ATP hydrolysis and activation by Cdc6 (Figure 1.2) (reviewed in Takeda and Dutta, 2005; Tanaka and Diffley, 2002; reviewed

in Diffley, 2011). Orc6 specifically has been associated with Cdt1-binding and helicase recruitment, but not origin DNA association (Chen *et al.*, 2007). This final stable loading of the Mcm complex forms a 'licensed' origin, which is subsequently activated by kinases like CDK and DDK.

In the second stage of DNA replication initiation, Cyclin Dependent Kinase (CDK) and Dbf4 Dependent Kinase (DDK) complexes activate the licensed origins by phosphorylating the replication machinery components (reviewed in Diffley, 2011; reviewed in Li and Araki, 2013; see Figure 1.2). Many Sld (Synthetic lethal with Dpb11) proteins play important roles in replication initiation. DDK phosphorylates the Mcm2-7, allowing Sld3-Sld7 complex and Cdc45 to associate with the pre-RC (Heller *et al.*, 2011; see Figure 1.2). Sld7 association with Sld3 and Cdc45 ensures proper Sld3 functioning during initiation (Tanaka *et al.*, 2011). Sld2 protein is phosphorylated by CDK allowing it to bind to the N-terminus of the Dpb11 (DNA polymerase B 11) protein whereas CDK-dependent phosphorylation of Sld3 enables binding to the C-terminus of Dpb11 (Figure 1.2). This phosphorylation facilitates the recruitment of other replication proteins to form the pre-Loading Complex (pre-LC) consisting of phosphorylated Sld2, Dpb11, Pol ε, and GINS (Go-Ichi-Ni-San) (Muramatsu *et al.*, 2010; reviewed in Li and Araki, 2013; see Figure 1.2). The recruitment of GINS to the origins facilitates the formation of the CMG (Cdc45-Mcm2-7-GINS) complex, which activates the helicase (Yaabuchi *et al.*, 2006; Zegerman and Diffley, 2007; reviewed in Yekezare *et al.*, 2013). Cdc45 plays an essential role in replication initiation and elongation, and functions in the recruitment of polymerase α and polymerase δ

to origins. More recently Cdc45 has been shown to bind directly to ssDNA, an interaction that is important in helicase stalling during replication stress (Bruck and Kaplan, 2013). Another study has shown that DDK promotes the association of Cdc45 and Sld3 before CDK recruits Sld2, Dpb11 and GINS suggesting that DDK acts sequentially before CDK in replication initiation (Heller *et al.*, 2011). Mcm10 has been shown to be required for origin unwinding after the recruitment of the CMG components, possibly stimulating helicase activity and aiding in the Pol α and Pol δ association with replication forks (Kanke *et al.*, 2012; van Deursen *et al.*, 2012). Sld2, Sld3 and Dpb11 are released to form the replisome, a complex that unwinds the DNA and carries out replication by opening the fork bi-directionally (reviewed in Labib, 2010; see Figure 1.2).

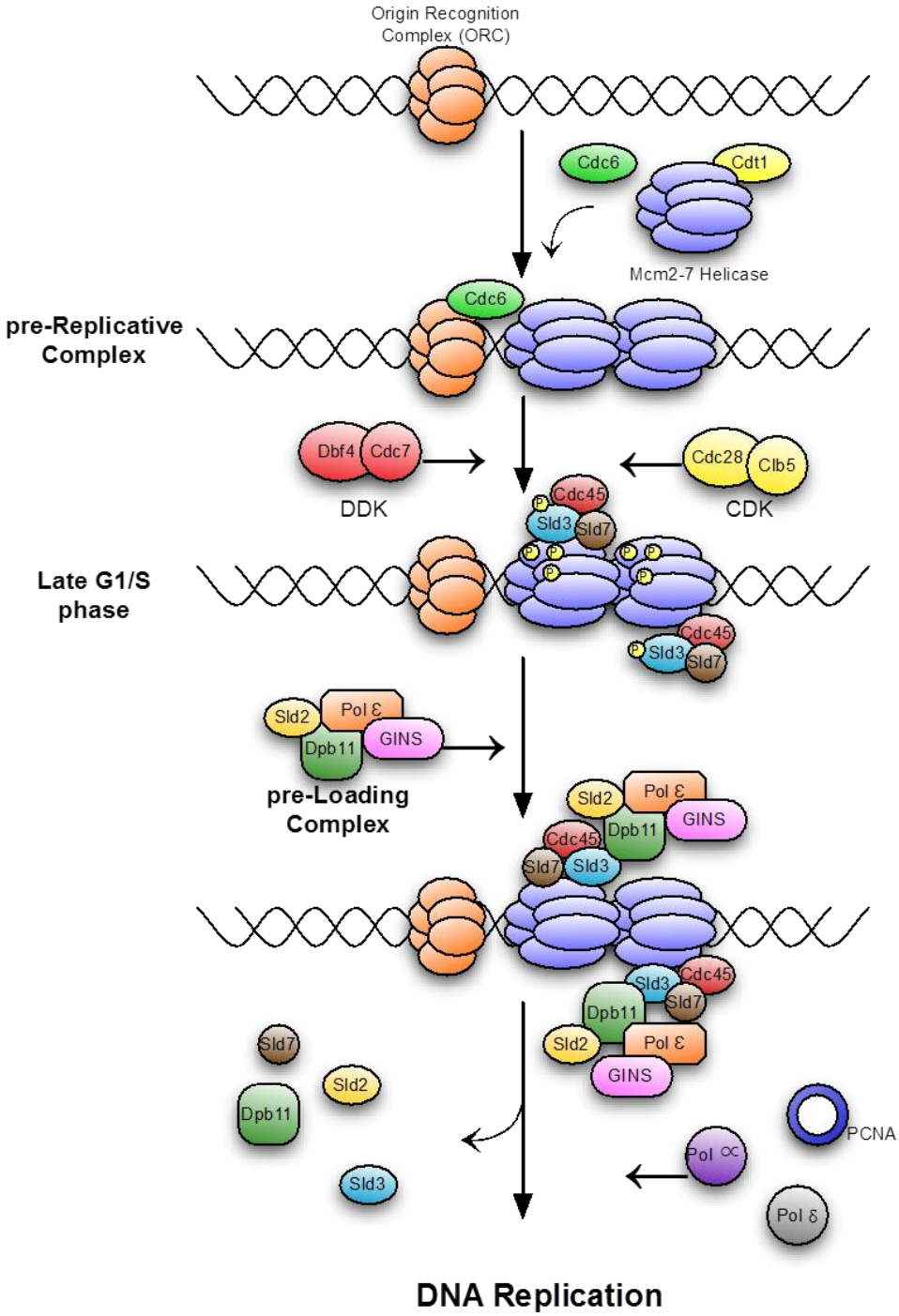

Figure 1.2: Formation of the pre-Replicative Complex and pre-Loading Complex. The formation of the pre-RC occurs in G1 phase. DDK and CDK phosphorylate components of the pre-RC to enable the recruitment of other replication factors like the pre-LC to eventually form the replisome, which carries out local DNA unwinding and new DNA synthesis in S phase (adapted from Li and Araki, 2013 and Labib, 2010).

1.2.2 Mcm2-7 Helicase Complex

The Mcm2-7 helicase is a heterohexameric AAA+ ATPase complex that utilizes ATP binding and hydrolysis to unwind double-stranded DNA (reviewed in Vijayraghavan and Schwacha, 2012). The complex contains each Mcm subunit in a 1:1:1:1:1:1 stoichiometry with features similar to the homohexameric archael Mcm ring (reviewed in Bochman and Schwacha, 2009). The complex loads onto DNA as a double hexamer in a head-to-head conformation. Each subunit contains a similar P-loop structure consisting of ATP-binding motifs (Walker A and Walker B) and ATP-interface motifs (arginine finger and sensor 2) (reviewed in Bochman and Schwacha, 2009). The presence of these two ATPase active sites in each Mcm subunit enabled the isolation of stable dimers to determine the structural arrangement of Mcm2-7. The dimeric subunit pairs are as follows: Mcm5/3, Mcm3/7, Mcm7/4, Mcm4/6, Mcm6/2 and Mcm2/5. The pairings reveal the ring order to be Mcm2-6-4-7-3-5. Biochemical studies have shown that Mcm2-7 lacked helicase activity *in vitro*, however a dimeric heterotrimer consisting of Mcm4-6-7 exhibited ATP-dependent helicase activity (Ma *et al.,* 2010). Mcm4 and Mcm7 were shown to oligomerize into a hexamer without Mcm6, and introducing mutations in Mcm4, Mcm6 or Mcm7 resulted in decreased ATP hydrolysis (Ma *et al.*, 2010). Each of the six Mcm genes has orthologs in all known eukaryotic genomes, signifying the importance of functionally distinct Mcm subunits. Biochemical analysis has revealed that the individual ATPase active sites are non-equivalent, playing specific roles in helicase activity or regulation. The Mcm7/4 active site was found to be essential for helicase activation while the addition of Mcm2 or Mcm5/3 dimer had an inhibitory effect on the Mcm4-6-7 helicase (reviewed in Vijayraghavan and Schwacha,

2012). Therefore, it was proposed that in *S. cerevisiae*, Mcm4, Mcm7 and possibly Mcm6 are required for helicase activation while Mcm2, Mcm3 and Mcm5 act as negative regulators (reviewed in Forsburg, 2004). The Mcm2/5 active site has been implicated in acting as a gate to open and close the Mcm2-7 ring, however that model has recently evolved to include the involvement Cdc45 and GINS in the gate-closing mechanism (Costa *et al.*, 2011). The exact mechanism of DNA helix unwinding has yet to be fully characterized though many models have been proposed (reviewed in Takahashi *et al.*, 2005; reviewed in Bochman and Schwacha, 2009). Pump models suggest that helicases rotate DNA. The rotary-pump model, for instance, proposes that multiple loaded helicases translocate along dsDNA, eventually anchoring and rotating in opposite directions to unwind the double helix by relieving torsional strain (reviewed in Takahashi *et al.*, 2005). Steric models suggest that the helicase translocates along one DNA strand while displacing the other strand. A variant of this idea is the 'ploughshare' model whereby Mcm2-7 translocates along double-stranded DNA as a single hexamer pushing ssDNA through the central channel (reviewed in Takahashi *et al.*, 2005). Studies have shown that the Mcms have positively charged β-hairpin fingers that protrude into the central channel, two of which are found within the AAA+ domain and are likely involved in ATP hydrolysis to facilitate DNA unwinding (reviewed in Forsburg, 2004). With respect to pre-RC formation, the C-terminal domains of Mcm2-7 mediate the interactions with ORC, Cdc6 and Cdt1 (reviewed in Vijayraghavan and Schwacha, 2012). The Mcm2 and Mcm3 subunits contain partial Nuclear Localization Signal (NLS) sequences whereas the Nuclear Export Signal (NES) is found only on Mcm3 (Young *et al.*, 1997; Braun and Breeden, 2007). Therefore, Mcm2-7 enters the nucleus either as intact heterohexamers or

at least as subcomplexes containing Mcm2 and Mcm3. Regulation of the Mcm2-7 levels occurs through phosphorylation by CDK and is important to ensure that inappropriate DNA re-replication does not occur (Liku *et al.*, 2005). In early G1 phase, a combination of low CDK activity and translation of new Mcm proteins drives the transport of Mcm2-7 into the nucleus, whereas export of Mcm proteins from the nucleus occurs in S phase when CDK activity is high (Nguyen *et al.*, 2000; Nguyen *et al.*, 2001). The regulation of Mcm proteins in the context of replication initiation is discussed in more detail in the next section.

1.2.3 The DDK Complex and its regulation of Mcm2-7

The conserved protein kinase complex DDK triggers DNA replication by primarily phosphorylating subunits of the Mcm2-7 complex. DDK is composed of serine threonine kinase subunit, Cell division cycle 7 (Cdc7), and a regulatory subunit, Dumbbell forming unit 4 (Dbf4). Cdc7 is a 58-kDa (kiloDalton) protein present at relatively constant levels throughout the cell cycle (reviewed in Bell and Dutta, 2002). Dbf4 is an 80-kDa protein that peaks from late G1-phase until late M-phase (reviewed in Bell and Dutta, 2002). The Anaphase Promoting Complex (APC/C) rapidly degrades Dbf4 at the start of anaphase in order to prevent further replication initiation (Cheng *et al.*, 1999; reviewed in Matthews and Guarné, 2013). Since Dbf4 binds and activates the inactive Cdc7 kinase subunit, regulation of kinase activity occurs through cell cycle regulation of Dbf4 levels. Orthologs of Dbf4 have been identified in all eukaryotes examined to date and sequence comparisons have revealed three conserved regions, motifs N, M, and C, each mediating interactions with other replication factors (reviewed in Masai and Arai, 2000). Motif-N has been shown to mediate

interactions with Orc2 and the Rad53 checkpoint kinase (Matthews *et al*., 2012; Varrin *et al*., 2005). Motifs-M and -C interact with Cdc7 and Mcm2, and deletion of either motif results in loss of cell viability (Sato *et al.*, 2003; Yamashita *et al.*, 2005; Jones *et al.*, 2010; Harkins *et al.*, 2009). Recent structural analysis revealed that the N-terminal region of Dbf4 contains an alpha helix projecting from a canonical BRCT domain that is necessary for mediating the interaction with Rad53 (Matthews *et al.*, 2012).

The interaction between Cdc7 and other replication factors is another interesting avenue of investigation. Cdc7 contains 11 conserved kinase domains and 3 kinase-insert domains, which mediate protein-protein interactions (reviewed in Masai and Arai, 2002) (Figure 1.3). Previous findings in budding yeast have shown that the C-terminal region of Cdc7 mediates binding with Dbf4, and specific residues within the kinase domains are essential for kinase activation and function (Jackson *et al,* 1993; Ohtoshi *et al.*, 1997). Similar findings in human cell lines have shown that the C-terminal region of Cdc7 mediates the interaction with Dbf4 motifs M and C (Kitamura *et al.*, 2011). Substitution of the yeast C-terminal 55 amino acids in place of equivalent human residues supported growth in a *cdc7Δ* yeast strain but only when paired with yeast Dbf4, suggesting a species-specific interaction in this region (Davey *et al.*, 2011). A recent study that has solved the crystal structure of human Dbf4-Cdc7 complex revealed that Dbf4 motif-C binds to the N-terminal lobe of Cdc7 and is sufficient for kinase activity, whereas Dbf4 motif-M binds to the C-terminal lobe acting as a tethering domain (Hughes *et al.*, 2012). Lack of Cdc7 function has

been linked to faster replication fork progression due to reduced origin firing and checkpoint signaling deficiency (Zhong *et al.*, 2013).

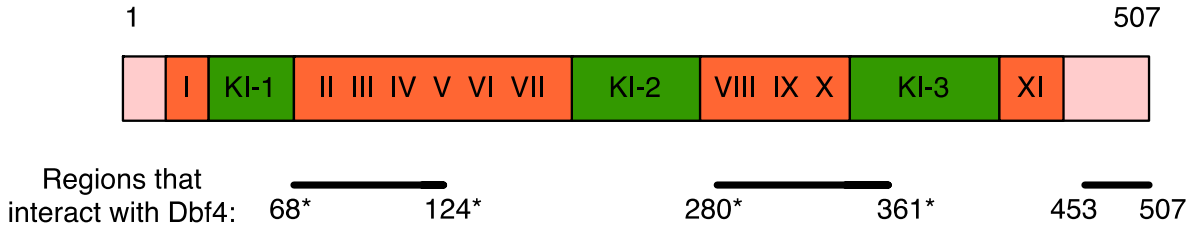

Figure 1.3: Schematic of the Cdc7 Structure. The above figure outlines the main domains and regions of Cdc7. The orange regions denote the 11 conserved, kinase domains (indicated by the roman numerals). The green regions denote the 3 Kinase Insert (KI) domains that mediate protein-protein interactions. The numbers above and below refer to approximate amino acid positions. The black bars outline regions that interact with Dbf4. The regions marked with an asterisk are based on human Dbf4-Cdc7 structural work, but the indicated amino acids are the equivalent *S. cerevisiae* regions (Hughes *et al.*, 2012). The region not marked with an asterisk is based on *S. cerevisiae* work (Jackson *et al.*, 1993; Ohtoshi *et al.*, 1997).

Under normal growth conditions, multiple subunits of the heterohexameric Mcm2-7 helicase complex are phosphorylated by DDK (Cho *et al.*, 2006). Mcm2, Mcm4 and Mcm6 are the preferred substrates of DDK (Francis *et al.*, 2009; Randell *et al.*, 2010; Sheu and Stillman, 2006). Additionally a region known as the NSD (N-terminal Serine/Threonine rich domain) contains the phosphorylation target sites for DDK and other kinases like CDK and Mec1 (Sheu and Stillman, 2006). A region within the NSD (amino acids 74-174) contains sites that are phosphorylated by DDK to relieve an inhibitory effect. Removal of this N-terminal region was shown to bypass the requirement for DDK activity, confirming that the phosphorylation of the Mcm4 NSD region is the essential DDK function (Sheu and Stillman, 2010). In Mcm4, a region near the N-terminus has been shown to contain a DDK-docking domain (amino acids 175-333) (Sheu and Stillman, 2010). DDK has been shown to

phosphorylate the N-terminal tails of Mcm2 and Mcm6 (Randell *et al.*, 2010). Phosphorylation of Mcm2 by DDK is not essential for viability but instead plays a role in suppressing sensitivity to DNA damaging agents (Stead *et al.*, 2011). It has been proposed that Mec1 kinase phosphorylates Mcm4 and Mcm6 as a 'priming' mechanism for subsequent DDK phosphorylation (Randell *et al.*, 2010). Mcm4 and Mcm6 are two phosphorylation and pre-RC-dependent targets of budding yeast DDK, but Mcm4 is the critical DDK target *in vivo* (Francis *et al.*, 2009; Randell *et al.*, 2010). *In vitro* analysis has revealed that DDK phosphorylates every Mcm subunit except for Mcm5. (Lei *et al.*, 1997; Weinrich and Stillman, 1999). Interestingly the *mcm5-bob1* allele, which contains a single amino acid change, P83L, in Mcm5, bypasses the requirement for DDK much like the Mcm4 NSD deletion (Hardy *et al.*, 1997; Fletcher *et al.*, 2003). The mutation is thought to cause a conformational change in the Mcm2-7 complex that mimics the effect of phosphorylation (Hardy *et al.*, 1997; Fletcher *et al.*, 2003). Recently, the telomeric chromatin component Rif1 (Rap-1-interacting factor) has been shown to associate with Protein Phosphatase 1 (PP1) to facilitate Mcm4 dephosphorylation as a means of counteracting DDK phosphorylation and controlling premature Mcm4 phosphorylation in G1 phase (Hiraga *et al.*, 2014). Rif1-PP1 interaction is also regulated by DDK phosphorylation, making it an attractive new pathway to further study Mcm4 phosphorylation (Hiraga *et al.*, 2014). Currently, the precise region of DDK that mediates the interaction with Mcm4 in budding yeast remains unknown. Deducing this region will improve our understanding of how DDK is targeted to the Mcm2-7 complex.

1.3 The Checkpoint Response and Relevance in Cancer

1.3.1 Role of DDK and Mcm2-7 in the Checkpoint Response

When DNA is compromised due to genotoxic agents that damage DNA or reduce nucleotide pools, checkpoint mechanisms are activated to correct the damage and allow for the cell cycle to progress. Hydroxyurea (HU) is a ribonucleotide reductase inhibitor that prevents the formation of dNTPs, thereby limiting their pools at the replication forks and inhibiting further replication (Stead *et al.*, 2012). Methyl methanesulfonate (MMS) is a DNA alkylating agent that induces DNA lesions, causing single-stranded and double-stranded breaks in the genome (Stead *et al.*, 2011). Other genotoxic agents include caffeine, which inhibits checkpoint proteins leading to accumulation of damaged DNA, and phleomycin, a radiomimetic drug that causes double stranded DNA breaks (Moore, 1989; Karunda *et al.*, 2006). DNA damage activates the checkpoint response, which subsequently leads to the replication fork stalling and the inhibition of replication initiation by blocking further origin firing. In the presence of DNA damage, the replicative helicase can become uncoupled from the DNA polymerases, resulting in exposed ssDNA. Single stranded DNA-binding Replication Protein A (RPA) proteins bind to the ssDNA and recruits Mec1 kinase to the stalled forks via Ddc2 protein (Enomoto *et al.*, 2002; Paciotti *et al.*, 2001) and activates the Rad53 checkpoint kinase (Zegerman and Diffley, 2010; Alcasabas *et al.*, 2001). When activated, Rad53 phosphorylates Dbf4 causing its dissociation from Cdc7, thereby reducing active DDK concentrations (reviewed in Duncker & Brown, 2003). Cells potentially employ this mechanism to prevent further replication initiation and preserve the number of unfired origins until the damage has been repaired. Exposure to hydroxyurea creates severe fork

impediments leading to attenuated DDK activity and subsequent delay in origin activation and S phase progression (Alvino *et al.*, 2007). A study found that a Dbf4 mutant containing altered Rad53 phosphorylation sites exhibited greater initiation of late origin firing and faster replication in the presence of genotoxic agents (Duch *et al.*, 2011). Non-phosphorylatable mutations in Sld3 and Dbf4 exhibited increased late origin firing in the presence of hydroxyurea (Zegerman and Diffley, 2010). Following checkpoint activation, further DDK phosphorylation of Mcm2-7 is thought to contribute to maintaining fork integrity so that DNA replication can resume efficiently once the DNA damage has been repaired (Jones *et al.*, 2010; Stead *et al.*, 2012). DDK phosphorylation of two Mcm2 N-terminal residues Ser-164 and Ser-170 was shown to be important for suppressing sensitivity to certain genotoxic agents (Stead *et al.*, 2012). Mutating these residues to non-phosphorylatable forms resulted in a greater number of RPA foci (representing the generation of single stranded DNA). This suggests that phosphorylation of Mcm2 by DDK normally slows down the helicase reducing the rate at which it separates from the replisome (Stead *et al.*, 2012) (Figure 4). In fission yeast, the DDK homolog Hsk1 has been shown to phosphorylate Rad9, a 9-1-1 checkpoint clamp protein that is recruited to aberrant DNA structures, and failure to phosphorylate it results in abnormal DNA repair (Furuya *et al.*, 2010). More recently, Cdc7 has been linked to a pro-survival signaling pathway through its interaction with and stabilization of Tob, a protein involved in inhibiting the pro-apoptotic signaling pathway in response to mild DNA damage (Suzuki *et al.*, 2012). Therefore, investigating the interactions between DDK and essential cell cycle proteins is important in understanding how those associations may regulate the rate of replication and maintain of fork stability under checkpoint conditions.

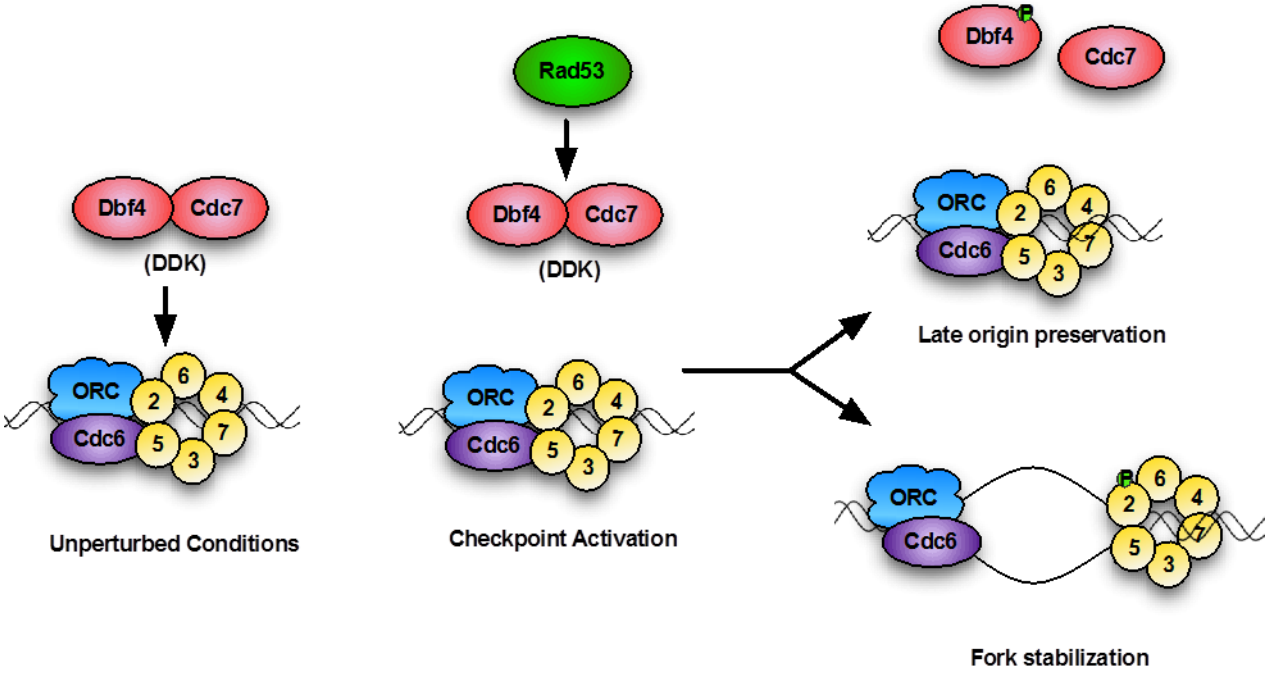

Figure 1.4: Checkpoint response involving Rad53, DDK and Mcms. When checkpoint mechanisms are activated, Rad53 phosphorylates Dbf4 causing its dissociation from Cdc7. This leads to a reduction in active DDK concentrations, thereby preserving late origins from firing. The helicase can uncouple from the replisome during checkpoint conditions therefore DDK phosphorylation of Mcm2 is thought to contribute to slowing down the helicase and stalling the forks as a result.

1.3.2 Relevance in Cancer Studies

Prior studies have shown elevated Dbf4 and Cdc7 levels in tumour cell lines and melanomas (Nambiar *et al.*, 2007; Bonte *et al.*, 2008). Given that DDK plays a vital role in the replication initiation process, it seems logical to investigate one of its subunits as a potential target for cancer therapeutics. Studies have shown Cdc7 to be a promising target using selective inhibitors. Typical chemotherapeutics inhibit DNA replication and activate the checkpoint response as a result (reviewed in Montagnoli *et al.*, 2010). Depletion of Cdc7 using siRNAs in tumour cells demonstrated a defective S phase and eventually cell death, without significant activation of the DNA checkpoint response (Montagnoli *et al.*, 2004). A

Cdc7 inhibitor, PHA-767491, has been shown to have an additive effect on tumour cell death when combined with currently used cancer therapeutics (Natoni *et al.*, 2013). Uncovering small molecule inhibitors of Cdc7 is a promising area of research in developing novel cancer therapeutics. Overall, acquiring such knowledge on the interactions between cell cycle proteins will enhance our understanding of the roles they play in the DNA replication process in normal and abnormal cells.

1.4 Research Objectives

The main objective of this thesis is to further characterize the interaction between the DDK and Mcm2-7 complexes in the budding yeast *Saccharomyces cerevisiae*. Prior work from the Duncker Lab has shown that Dbf4 exhibits a strong interaction with Mcm2, whereas Cdc7 interacts with Mcm4 and Mcm5. A region of Mcm4 near the N-terminus has been shown to contain a DDK-docking domain (amino acids 175-333) (Sheu and Stillman, 2006; Sheu and Stillman, 2010). The Duncker lab has found that in the Mcm2 subunit, two N-terminal regions (amino acids 2-4 and 10- 63) mediate the interaction with Dbf4 and deletion of these amino acids results in growth defects. Mutant Mcm2 and Mcm4 with deleted DDK-docking domains will hereafter be referred to as Mcm2ΔDDD and Mcm4ΔDDD. I hypothesized that Dbf4-Mcm2 and Cdc7-Mcm4 interactions represent redundant mechanisms for targeting of DDK to the Mcm2-7 complex. To investigate this, the simultaneous disruption of Dbf4-Mcm2 and Cdc7-Mcm4 interactions was compared to single disruptions. The growth effects were also examined in the presence of genotoxic agents such as hydroxyurea (HU) and methyl methanesulfonate (MMS). Investigating the interactions between DDK and the MCM complex is important in understanding the role that these

proteins play in regulating the rate of replication and maintaining fork stability under checkpoint conditions.

DDK Docking Domains have been identified within the Mcm2 and Mcm4 proteins, and precise regions within Dbf4 have been characterized for its interaction with Mcm2 and Cdc7. Previous findings in budding yeast have shown that the C-terminal region of Cdc7 mediates binding with Dbf4 (Jackson *et al.,* 1993). Currently the precise region that mediates the interaction with Mcm4 in budding yeast remains unknown. Therefore, my second objective aimed to further understand the interaction between DDK and the Mcm proteins by determining the minimal region(s) of Cdc7 responsible for its separate interactions with Dbf4 and Mcm4. Bioinformatics analysis was also conducted to determine conserved interface residues within Cdc7 and Dbf4. Determining the minimal region of Cdc7 that interacts with Dbf4 lays the groundwork for possible structural studies in the future. The third objective of this thesis was to identify conserved residues within the DDK Docking Domain of Mcm2 and Mcm4 that are essential for maintaining interactions with Dbf4 and Cdc7, respectively. Residues were mutated to determine their importance in mediating protein-protein interactions. These assays will allow for future experiments where mutant strains of the same kind can be created and examined for growth. It can also have implications in cancer therapeutic research whereby potential small molecule inhibitors can be designed to target specific residues within a protein. Therefore, knowing the important residues that mediate these essential interactions will be useful for designing therapeutic targets that could inhibit essential protein function and subsequent cell growth.

Chapter 2: Materials and Methods

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This research was originally published in The Journal of Biological Chemistry. Ramer, M.D., Suman, E.S., Richter, H., Stanger, K., Spranger, M., Bieberstein, N., Duncker, B.P. Dbf4 and Cdc7 Proteins Promote DNA Replication through Interactions with Distinct Mcm2-7 Protein Subunits. *J. Biol. Chem.* 2013; *288*(21): 14926-35.

Section 2.7 contributed by Dr. Matthew Ramer

2.1 Yeast Strains

DY-1 (*MATa, ade2-1, can1-100, trp1-1, his3-11,-15, ura3-1, leu2-3,-112, pep4:LEU2*) was used for the two-hybrid assays. DY-273 (*MATα, leu2Δ0, met15Δ, ura3Δ0, lys2Δ0, mcm2::his3*) and DY-274 (*MATα, his3Δ1, leu2Δ0, met15Δ, ura3Δ0, mcm4::KanMX*) supported for growth with wild-type *MCM2* or *MCM4* on a *URA3*, CEN/ARS vectors were used for the plasmid shuffle experiments (Stead *et al.*, 2009). Mcm2 and Mcm4 plasmid shuffle strains were transformed with YCplac111-Mcm2WT (wild-type), -Mcm2Δ2-4, 10-63, -Mcm4WT, or -Mcm4Δ175-333 and grown on Synthetic Complete (SC) media lacking uracil and leucine (SC-Ura-Leu). These were the 'shuffle in' strains. Colonies from these transformation plates were streaked out on SC-Leu +5'FOA (1mg/mL) plates to select for cells that had lost the *URA3*, CEN/ARS support plasmid. This results in the only copy of *MCM2* or *MCM4* being on the YCplac111 *LEU2*, CEN/ARS plasmid (See Table 2.1; DY-263, DY-264, DY-265, DY-266). These shuffle strains were then mated to DY-196 (*MATa his3Δ1, leu2Δ0, ura3Δ0*) and the resulting diploids were sporulated and dissected to generate the haploid *MATa* shuffle strains. DY-228 (MATa, *his3Δ200*, *met15Δ0*, *trp1Δ63*, *ura3Δ0*) was used as the parental strain to generate *GAL1-MCM2* (DY-215), *GAL1-MCM3* (DY-216), *GAL1- MCM4* (DY-230), *GAL1-MCM5* (DY-221), *GAL1-MCM6* (DY- 217), and *GAL1-MCM7* (DY-218) strains, using *TRP1* as a selectable marker. The DY-272 strain in which the genomic copy of *CDC7* was replaced with a *GAL1-CDC7-HA* allele induces Cdc7 expression in the presence of galactose and shuts off expression in the presence of glucose. This strain was used in the complementation assay in Chapter 4.

Table 2.1: Yeast Strains used in this study

Strain	Genotype
DY-1	*MATa, ade2-1, can1-100, trp1-1, his3-11,-15, ura3-1, leu2-3,-112, pep4:LEU2*
DY-26	*MATa, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0*
DY-215	*MATa, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, mcm2::PGal1-3HA-TRP1-MCM2*
DY-216	*MATa, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, mcm3::PGal1-3HA-TRP1-MCM3*
DY-217	*MATa, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, mcm6::PGal1-3HA-TRP-MCM6*
DY-218	*MATa, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, mcm7::PGal1-3HA-TRP1-MCM7*
DY-221	*MATa, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, mcm5::PGal1-3HA-TRP1-MCM5*
DY-228	*MATa, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0*
DY-230	*MATa, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, mcm4::PGal1-3HA-TRP1-MCM4*
DY-263	*MATa, leu2Δ0, met15Δ0, ura3Δ0, mcm2::his3* - supported for growth with YCplac111-Mcm2 WT
DY-264	*MATa, leu2Δ0, met15Δ0, ura3Δ0, mcm2::his3* - supported for growth with YCplac111-Mcm2Δ2-4, 10-63
DY-265	*MATa, leu2Δ0, met15Δ0, ura3Δ0, his3Δ0, mcm4::KanMX* - supported for growth with YCplac111-Mcm4 WT
DY-266	*MATa, leu2Δ0, met15Δ0, ura3Δ0, his3Δ0, mcm4::KanMX* - supported for growth with YCplac111-Mcm4 Δ175-333
DY-272	*MATa ura3::URA3[GPD-TK(7x)] ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 GAL psi+ cdc7*::*GAL-HA-CDC7-TRP1*

2.2 Plasmid Construction

pEG-Dbf4-FL and pJG4-6-Mcm2 FL have been previously described (Varrin *et al*., 2005). pJG4-6-Mcm3, pJG4-6-Mcm5, and pJG4-6-Mcm6 were generated by PCR amplification of genomic *MCM3, MCM5*, and *MCM6,* respectively, from DY-26 with the forward and reverse primers containing ApaI and XhoI restriction sites, respectively. pJG4-6-Mcm4 was generated by PCR amplification of genomic *MCM4* from DY-26 with the

forward and reverse primers containing NcoI and XhoI restriction sites, respectively. pJG4-6-Mcm7 was generated by PCR amplification of genomic *MCM7* from DY-26 with the forward and reverse primers containing Nco1 and EcoR1 sites, respectively. pJG4-6-MCM2Δ63 was generated by PCR amplification of genomic *MCM2* from DY-26 using forward and reverse primers corresponding to sequence encoding amino acids 64-868, containing NcoI and XhoI sites respectively. pJG4-6-Mcm2 1-63 was generated by PCR amplification of genomic *MCM2* from DY-26 with the forward and reverse primers corresponding to DNA sequence encoding amino acids 1-63, containing BglII and EcoRI sites, respectively. Both pJG4-6-Mcm2 505-868 and pJG4-6-Mcm2 1-504 were generated by PCR amplification of genomic *MCM2* from DY-1 with the forward and reverse primers corresponding to DNA encoding either amino acids 1-504 or 505-868, containing NcoI and XhoI, respectively. In all cases, the PCR products were kit-purified (GE Healthcare or Geneaid) and then ligated into the appropriately digested vector, followed by transformation of the entire ligation mix into DH5α competent bacterial cells. pEG-Cdc7-WT was generated by PCR amplification of the entire *CDC7* coding sequence from DY-26 genomic DNA with the forward and reverse primers containing EcoRI and BglII, respectively. pEG-202 was then cut with EcoRI and BamHI with the fragment and vector then ligated thus generating an in-frame fusion with the LexA coding sequence. pJG4-6-Mcm2Δ2-4,10-63 was generated by PCR amplification of genomic *MCM2* from DY-26 with a forward primer containing both NcoI and NdeI sites followed by the sequence encoding amino acids five through nine and 64-75. The reverse primer corresponded to the C-terminal coding sequence of *MCM2* containing BamHI and XhoI. pJG4-6-Mcm4Δ175-333 was generated by PCR amplification

of two fragments of *MCM4* from DY-26 genomic DNA (encoding amino acids 1-174 and 334-878) which were joined by an NcoI site that was engineered into the reverse primer of the first fragment and the forward primer of the second fragment. The two cut and purified fragments were cloned together into the pJG4-6 vector using the ApaI and XhoI sites in the multiple cloning site.

Specific residues within the *MCM2* gene were mutated using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). Sequence encoding Proline 22, proline 25, proline 39 and proline 43 were all mutated to sequence encoding alanines using mutagenic primers, which contained the desired mutation. QC1 denotes the mutation of proline 22 and 25 to alanines, QC2 denotes the mutation of proline 39 and 43, and QC3 denotes mutations to all four prolines to alanines. pJG-4-6-Mcm2 WT was used as the DNA template in the reaction. The resulting PCR product was mixed with *Dpn* I enzyme to digest the parental methylated and hemi-methylated DNA. After digestion, the resulting plasmids were transformed into XL-10-Gold Ultracompetent cells. A complete protocol can be found in the online QuikChange II XL Site-Directed Mutagenesis instruction manual: http://stanxterm.aecom.yu.edu/wiki/data/Product_manuals_attach/quikchange2xl.pdf.

The pCM190-Mcm2WT plasmid was generated by PCR amplification of *MCM2* from pJG4-6-Mcm2WT with forward and reverse primers corresponding to the gene coding sequence, containing NotI and BamHI, respectively. pCM190-Mcm4WT was generated by PCR amplification of MCM4 from pJG4-6-Mcm4WT with forward and reverse primers corresponding to the gene coding sequence, containing NotI and BglII sites, respectively.

pCM190-Mcm2Δ2-4, 10-63 was generated by PCR amplification of the gene coding sequence from pJG-4-6-Mcm2Δ2-4, 10-63 with forward and reverse primers containing NheI and BamHI sites, respectively. pCM190-Mcm4Δ175-333 was generated by PCR amplification of the gene encoding sequence from pJG4-6-Mcm4Δ175-333 with forward and reverse primers containing NheI and BglII sites, respectively. All the PCR products were cut with the respective enzymes and ligated into cut pCM190 at the equivalent sites. To add a Nuclear Localization Signal (NLS) sequence to the Mcm4 proteins, a pCM190-NLS construct was first made. The SV-40 (Simian Vacuolating Virus 40 Tag) NLS sequence was acquired from the pJG-4-6 vector. Synthetic oligonucleotides containing the NLS sequence were designed to anneal together and form sticky-end overhangs. The 5' and 3' overhangs were ligated into the pCM190 BamHI and NotI sites, respectively. pCM190-Mcm4-NLS and pCM190-McmΔ175-333-NLS were generated by PCR amplification of gene encoding region from pJG4-6-Mcm4 WT and pJG-4-6-Mcm4Δ175-333 with forward and reverse primers containing NotI and BglII sites, respectively. The PCR products were ligated to cut pCM190-NLS at the equivalent sites.

Bait plasmids expressing truncated forms of Cdc7 were all made using the same cloning strategy. The plasmids used in this study include: pEG-Cdc7 amino acids 453-507, pEG-Cdc7 1-452, pEG-Cdc7 276-507, pEG-Cdc7 1-275, pEG-Cdc7 125-507, pEG-Cdc7 1-124, pEG-Cdc7 55-507, pEG-Cdc7 1-54. These plasmids were generated by PCR amplification of sequence encoding the corresponding amino acid regions from pEG-Cdc7 WT using forward and reverse primers containing EcoRI and BglII sites, respectively. The

PCR products were ligated into cut pEG202 vector at the equivalent sites. pJG-4-6-Cdc7 1-452, pJG-4-6-Cdc7 55-507, pJG-4-6-Cdc7 276-507 and pJG-4-6-Cdc7 453-507 were all generated by PCR amplification of sequence encoding the corresponding amino acid regions from pEG-Cdc7 WT using forward and reverse primers containing EcoRI and BglII sites, respectively. The PCR products were ligated into cut pJG-4-6 vectors at the equivalent sites.

The plasmid shuffle vector (YCplac111) is a CEN/ARS vector with a *LEU2* selectable marker. The YCplac111-Mcm2WT and YCplac111-Mcm4WT plasmid shuffle vectors were obtained from Megan Davey (Stead *et al.,* 2009; unpublished). The YCplac111-Mcm2WT vector along with pJG4-6-Mcm2Δ2-4, 10-63 were cut with NdeI and BamHI and the resulting Mcm2Δ2-4, 10-63 fragment was cloned into YCplac111 vector at equivalent sites. YCplac111-Mcm2QC3 was generated by PCR amplification of the gene-encoding region from pJG4-6-Mcm2QC3 using forward and reverse primers containing NdeI and BamHI sites, respectively. Mcm4Δ175-333 was PCR amplified using pJG-4-6-Mcm4Δ175-333 as template using a forward primer corresponding to the *MCM4* N-terminal coding sequence and containing NdeI and a reverse primer corresponding to the C-terminal region of *MCM4*. This PCR product was then cut with NdeI and MluI (an internal site in Mcm4) and then cloned into the Mcm4FL plasmid shuffle vector, which was also cut with NdeI and MluI to generate the mutant plasmid shuffle vector. All plasmid constructs were sequenced in order to confirm that no additional mutations had been generated.

2.3 Yeast Transformation

Yeast cultures were first grown to an initial concentration of 1 x 10^7 cells/mL in 50 mL of the appropriate medium. Cultures were counted using a hemocytometer or by measuring the OD_{600} using a spectrophotometer. Cells were centrifuged for 5 minutes at 4,000 rpm, washed in sterile 1X TE (Tris-EDTA), spun down again, and resuspended in 2 mL of 100 mM lithium acetate (LiAc)/0.5X TE. The resuspended cultures were incubated for 10 minutes at room temperature. Plasmid DNA was added to sterile 1.5 mL tubes along with 100 μg of salmon sperm DNA and 100 μL of yeast suspension mix. Following this, 300 μL of 100 mM LiAc/40% PEG4000 (polyethylene glycol 4000)/1XTE solution was added to each of the tubes. The tubes were then incubated at 30°C for 30 minutes. After incubation, 40 μL of DMSO (dimethylsulfoxide) was added to each tube, followed by heat shock at 42°C for 7 minutes and cooling on ice immediately after for 2 minutes. The resulting transformants were plated on selective media and grown at 30°C for 2-3 days.

2.4 Yeast Two-hybrid Assay

The DY-1 strain was transformed with the *LacZ* reporter plasmid pSH18-34, pEG-202-derived bait, and pJG4-6-derived prey plasmids. Cultures were grown to an initial concentration of 1 x 10^7 cells/mL in 10 mL of SC medium (Amberg *et al*., 2005) lacking uracil, histidine, and tryptophan. Cells were then washed in sterile water and induced for 6 hours in 20 mL of 2% galactose-1% raffinose medium lacking uracil, histidine, and tryptophan. Cells were counted and ~5x10^6 cells were harvested and tested for the interactions between the fusion proteins and detected by the quantitative β-galactosidase (β-gal) assay. The cells were spun down and resuspended in 0.5mL of Z buffer (0.06 M

Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$, 0.05 M β-mercaptoethanol). Two drops of chloroform and one drop of 0.1% SDS were added to each tube and mixed by vortexing at the maximum speed for 10 seconds. Tubes were incubated at 28°C for 5 mins followed by simultaneous addition of 100 µL of ONPG (2-Nitophenyl-B-D-galatopyranoside) to each of the tubes. Adding 1 M Na_2CO_3 stops the reaction and the time elapsed between the addition of ONPG and addition of sodium carbonate was noted. The reporter plasmid pSH18-34 contains a *LacZ* gene, which encodes the β-galactosidase enzyme. β-galactosidase converts the added substrate ONPG to 2-Nitrophenol and galactose, resulting in a yellow colour change measurable via spectrophotometry at 420 nm. The β-gal activity was determined by the following formula: β-gal activity = 1,000 x A_{420} / (t x V x A_{600}), where t = time of the reaction (in minutes), and V = volume of culture used in the assay (in millitres). The strength of the protein-protein interaction is proportional to the amount of β-galactosidase enzyme produced. Therefore, a stronger yellow colour and increased absorbance represents stronger protein-protein interaction. Whole cell extracts (WCEs) were made from the remaining samples as described in the next section. Protein concentrations were determined by Bradford assays and protein expression was examined by western blot. The LexA-tagged bait proteins were detected using a rabbit polyclonal anti-LexA antibody (ABR or Cedarlane), while the HA-tagged prey proteins were detected using a mouse monoclonal anti-HA antibody (Sigma). Alexa Fluor 647 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse polyclonal secondary antibodies (Invitrogen) were used.

2.5 Whole Cell Extract Preparations And Western Blotting

Cultures were centrifuged at 4,000 rpm for 5 minutes and the pellets were resuspended in 400 μL of lysis buffer (10 mM Tris-HCl, pH 8; 140 mM NaCl; 1% Triton X-100; 1 mM EDTA; PMSF and 1X protease inhibitor cocktail from Fisher). The yeast suspension was transferred to 2 mL screw cap tubes containing 0.3 g of 0.5 mm glass beads. A bead beater (Biospec) was used to lyse samples for 8 cycles of alternating 30 seconds of beating and 30 seconds on ice. The tubes were centrifuged at 13,2000 rpm for 30 seconds and the supernatant was transferred to a new 1.5 mL tube. The protein concentration was determined in a Bradford Assay using BioRad reagent. Sample loading dye (60% 4X buffer [15% SDS; 40% glycerol, 166 mM tris-base]; 0.26 M DTT; 7% bromophenol blue) was then added to each of the samples at half the volume and boiled for 10 minutes. Samples were run on a 7.5% SDS polyacrylamide gel to separate the proteins based on size and then transferred to a nitrocellulose membrane by a wet transfer technique. A wet transfer involves sandwiching the gel and the membrane in between two pieces of Whatman paper and sponges in a cassette while submerged in transfer buffer (200 mM glycine; 25 mM tris-base; 20% methanol; 0.05% SDS). The cassette is placed in an OWL transfer apparatus, filled with transfer buffer and set at 50 volts to transfer for 2-12 hours at 4°C.

Following the transfer, the membranes were stained with 0.1% Ponceau S stain to check for proper transfer and even loading of proteins. The membranes were destained with 1XTEN+T (20 mM Tris-HCl; 1mM EDTA; 0.14 M NaCl; 0.05% Tween 20). The membrane was first incubated in a blocking agent (1XTEN + 5% skim milk powder) for 1 hour at room

temperature or overnight at 4°C. Following this, primary antibody (diluted in either 1XTEN with 5% skim milk solution or 3% bovine serum albumin solution) was added and incubated at room temperature for 1 hour. The membrane was then washed 3 times with 1X TEN+T for 10 minutes each. Following this, secondary antibody (diluted in 1XTEN with 5% skim milk solution) was added for incubation at room temperature for 1 hour in the dark (AlexaFlour secondary antibodies are light sensitive). Following a final 3 washes in 1X TEN+T at 10 minutes each, the membranes were imaged using a Pharos FX Plus (BioRad) and its corresponding software.

Table 2.2:Antibodies used in this study

Antibody	Source	Dilution
Anti-HA (mouse monoclonal)	Sigma	1:5000
Anti-LexA (rabbit polyclonal)	ABR/Cedarlane	1:5000
Anti-Myc (mouse monoclonal)	Sigma	1:5000
AlexaFlour 488 goat anti-mouse	Invitrogen	1:3000
AlexaFlour 647 goat anti-rabbit	Invitrogen	1:3000

2.6 Plasmid Shuffle Growth Assay

Spot plate growth assays were performed by growing cells to a concentration of $1x10^7$ cells/mL in the appropriate selective SC medium. Cultures were then serial diluted and 5 μL aliquots were spotted onto respective SC or Gal/Raff (2% galactose, 1% raffinose) selective media plates, which were then incubated at 30°C for 2-4 days. Spotting assays for genotoxic sensitivity were performed in the same manner, except that for the Mcm subunit over-expression assay the aliquots were plated on YPG/R (1% yeast extract, 2% peptone, 2% galactose and 1% raffinose) plates supplemented with 20-100 mM HU (Sigma) or 0.01%-0.02% MMS (Sigma), and for the plasmid shuffle genotoxic assay the aliquots were plated

on SC-Leu-Ura with or without doxycycline (DOX; 20 μg/mL) supplemented with 20-100 mM HU (Sigma), or 0.01%-0.02% MMS (Sigma). For the overexpression of Cdc7 truncated regions in a wild-type background, cultures were spotted on Gal/Raff-Trp plates. Growth curves were generated by growing cultures to a concentration of $5x10^6$ cells/mL in SC-Leu medium and then diluting the culture to a final starting concentration of $1.67x10^6$ cells/ml in SC-Leu medium. OD_{600} readings were taken at the indicated timepoints and converted to a cell concentration using the conversion factor of $0.36/1x10^7$ cells/ml. The final growth curve is an average of three independent experiments.

2.7 Mating Yeast Strains, Sporulation and Tetrad Dissection

Fresh cultures of cells of opposite mating types were grown to ~$5x10^6$ cells/ml. 5 μL of each culture was dropped adjacent to one another on a YPD plate and the drops were then mixed well using a sterile toothpick making a circle about 5 mm in diameter. Once the spot was dry, the plate was incubated at 30°C for 3 h. Following the incubation, a toothpick was used to streak a sample of the mating mixture across the edge of a fresh plate of selective medium (i.e. SC-Leu) such that it was visible for use in a tetrad- dissecting microscope. Zygotes were identified and plated in the matrix of the plate. Following zygote picking, the plate was incubated at 30°C until colonies formed from the original zygote. These were then transferred to sporulation media in order to begin the sporulation process.

Colonies were picked from the zygote plates and used to inoculate sporulation media (1% potassium acetate, amino acids at 25% normal concentration) and incubated at 23°C for 2-3 days until asci were observed. 1 ml of the culture was centrifuged (13,000 rpm, 1 min),

washed in dH_2O and resuspended in 50 µL zymolyase solution (0.5 mg/ml in 1 M sorbitol) and incubated for 8-10 min at 30°C. Following incubation, 800 µL of sterile dH_2O was slowly added and the mixture allowed to rest on ice for 5 min. The supernatant was then slowly removed until the original volume was reached (~50-100 µL). The tip of a p1000 pipet tip was cut off creating a larger bore tip (thus reducing the stress on the fragile spores), which was used to pipet the culture into a drop on a selective medium plate. The plate was then tilted allowing the drop to run down the plate forming a streak suitable for tetrad dissection. Tetrads were then dissected as per the Singer MSM ascus dissection protocol (Singer Instruments, Sussex, England).

2.8 Synchronizing Yeast Cultures

The synthetic α-factor is a *S. cerevisiae* mating pheromone (New England Peptide), used to synchronize cultures in late G1-phase. The cells were grown to an initial concentration of ~$5x10^6$ cells/mL after which the α-factor was added and cultures were incubated for 1.5-3 hours at 30°C. The actual amount of α-factor used in each experiment was determined empirically by testing each strain, although typically 30 µg/ml was used for most strains. Following incubation, the cells were viewed under the microscope to look for the distinct G1-phase arrest morphology (i.e. few buds and the presence of shmoos) to ensure that an effective arrest has occurred. Cells were then washed with sterile dH_2O to remove the α-factor and released into either fresh medium lacking α-factor or the overnight growth medium (as it has a high concentration of Bar protein produced normally by growing cells which would help degrade the α-factor) containing 50 µg/mL Pronase E (Sigma), which also

helps to degrade any remaining α-factor. Aliquots of culture were taken at specific time points and prepared for FACS analysis.

2.9 Fluorescent Activated Cell Sorting (FACS) Analysis

Aliquots of 1 mL of culture were centrifuged (13,000 rpm, 30 sec) and resuspended in 1 mL of 70% ethanol and stored at 4°C until further processing. The fixed cells were treated with 500 uL RNase A (200 µg/mL in 50 mM Tris-HCl, pH 8) for 2-4 hours at 37°C. Following this, 500 µL Proteinase K (2 mg/ml in 50 mM Tris-HCl, pH7.5) was added and the tubes were incubated for 30-60 min at 50°C. Finally, the cells were centrifuged (13,000 rpm, 4 min) and resuspended in 50 µL FACS buffer (200 mM Tris-HCl, pH 7.5; 200 mM NaCl; 78 mM MgCl2) before being transferred to 500 µL Sytox solution (50 mM Tris-HCl, pH 7.5, 1:5000 dilution Sytox [Molecular Probes; 5mM Sytox in DMSO]) to stain the DNA. Cells were stored at 4°C in the dark and sonicated for 30 seconds just before analysis. The analysis was performed with a BD FACSVantage SE cell sorting system in the Molecular Core Facility in the Department of Biology at the University of Waterloo.

2.10 Bioinformatics Analysis

The protein sequences of the *S.cerevisiae* Mcm2 and Mcm4 DDK Docking domain regions were obtained in the FASTA format from NCBI. For Mcm2, the sequences were PSI-BLASTed (Position-Specific Iterated BLAST) against RefSeq (Reference Protein Sequences from the database) and run through three iterations/rounds of alignment. The resulting hits were aligned and transferred to the Seaview program. The Seaview-aligned sequences were transferred into weblogo (http://weblogo.berkeley.edu/logo.cgi) to generate a conservation logo for the residues. For the Mcm4 docking region, the sequences were BLASTed (protein-

protein BLAST) against RefSeq using the Standard Protein BLAST program on the NCBI website. The first 100 hits were selected and the sequences were transferred to the Seaview program and aligned with the built-in CLUSTALO algorithm. These alignments were transferred to weblogo to generate the conservation profile. The PyMol program was used to visualize the 3-D structure and identify the exposed residues.

For the Cdc7-Dbf4 regions, a python script was used to calculate the interface residues between Cdc7 and Dbf4 in PyMol. For the alignment, sequences from a number of organisms like chicken, fish, frog, yeast, and plant were chosen. Human Cdc7 protein sequence was BLASTed to find homologs in each of these species. A multiple sequence alignment (MSA) was generated in SeaView using the built-in MUSCLE algorithm with default parameters. The Jalview program was used to look at the MSA, reduce redundancy in sequences (if necessary) and check for conservation. The interface residues, which were previously identified using the python script, were checked for conservation among the species in the MSA. These Cdc7 residues were manually checked in a PyMol 3-D structure to see if they interact with some other residue in Dbf4. The resulting interface region was highlighted in the final PyMol structure.

Chapter 3: Dbf4 and Cdc7 Proteins Promote DNA Replication through Interactions with Distinct Mcm2-7 Protein Subunits

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This research was originally published in The Journal of Biological Chemistry. Ramer, M.D., Suman, E.S., Richter, H., Stanger, K., Spranger, M., Bieberstein, N., Duncker, B.P. Dbf4 and Cdc7 Proteins Promote DNA Replication through Interactions with Distinct Mcm2-7 Protein Subunits. *J. Biol. Chem.* 2013; *288*(21): 14926-35. © American Society for Biochemistry and Molecular Biology.

Figure 3.2A and 3.2B contributed by Dr. Matthew Ramer

3.1 Introduction

The Mcm2-7 helicase complex is composed of six distinct subunits that function together to facilitate DNA unwinding during replication in S phase of the cell cycle. The subunits are assembled into a heterohexamer in the cytoplasm and then co-imported into the nucleus with Cdt1 (Tanaka and Diffley, 2002). Cdt1 targets the Mcm2-7 helicase to the origin-bound ORC6 subunit, which is part of the heterhexameric ORC (Origin Recognition Complex) (Semple *et al.*, 2006; Chen *et al.*, 2007). Sequential ATP hydrolysis by Cdc6 and ORC enables the stable loading of two Mcm2-7 heterohexamers at individual origins (Randell *et al.*, 2006, Evrin *et al.*, 2009, Remus *et al.*, 2009). In late G1-phase, levels of Dbf4 rise, activating the Dbf4-dependent kinase Cdc7 (DDK), which then phosphorylates MCM subunits, thereby stimulating DNA replication. DDK has been previously shown to phosphorylate Mcm2, Mcm4 and Mcm6. The Mcm4 and Mcm6 subunits initially undergo priming phosphorylation by kinases, like Mec1, before being phosphorylated by DDK and stimulating DNA replication (Randell *et al.*, 2010).

Phosphorylation of the Mcm2-7 by DDK brings about a conformational change that activates the helicase complex. Mcm5 appears to be the only subunit that is not phosphorylated by DDK. The *mcm5-bob1* allele, which contains a single point mutation in Mcm5, bypasses the essential function of DDK (Weinreich and Stillman, 1999; Randell *et al.*, 2010). Structural analysis suggests that this Mcm5 mutant may impart a conformational change to the MCM ring rendering it competent for DNA replication (Hardy *et al.*, 1997; Hoang *et al.*, 2007). Regions of the Mcm4 subunit have been dissected to reveal an N-

terminal serine/threonine-rich domain (NSD) that is phosphorylated by DDK, and phosphomimetic mutations or removal of this domain can rescue cells lacking DDK activity (Sheu and Stillman, 2010). Therefore, this NSD region is suggested to play an inhibitory role that is relieved by processive phosphorylation by DDK (Sheu and Stillman, 2010). Although DDK-dependant conformational change in Mcm2-7 does not promote double hexamer separation, evidence suggests that it may stimulate association with two other firing factors required for recruiting DNA polymerases to origins, namely Sld3 and Cdc45 (Sheu and Stillman, 2006; Heller *et al.*, 2011; On *et al.*, 2014).

Given the essential role of Mcm2-7 helicase in replication, it makes sense that the deregulation of Mcm2-7 function has been linked to genomic instability and mammalian cancer phenotypes. Altered levels of MCM subunits have been associated with numerous human cancer types (reviewed in Alison *et al.*, 2002; Gonzalez *et al.*, 2005), and mice that are hypomorphic for MCM activity have demonstrated chromosomal abnormalities and a dramatic increase in cancer susceptibility (Shima *et al.*, 2006; Chuang *et al.*, 2010). DDK phosphorylation of the Mcm2 subunit is not required for normal growth but plays a role in the DNA damage response (Stead *et al.*, 2011). Mutation of two DDK target sites (Ser164 and Ser170) on Mcm2 to non-phosphorylatable alanines increased cell sensitivity to DNA damaging agents hydroxyurea (HU), methyl methanesulfonate (MMS), 5-fluorouracil (5-FU) and caffeine (Stead *et al.*, 2011; Stead *et al.*, 2012). This suggests a potential checkpoint role for DDK phosphorylation of Mcm2 via stabilization of replication forks.

Previous studies have identified precise amino acid residues within Mcm proteins that are phosphorylated by DDK (Bruck and Kaplan, 2009; Randell *et al.*, 2010; Stead *et al.*, 2011), however the targeting of the DDK to Mcm2-7 is poorly understood. It has been shown previously that two conserved regions of Dbf4 mediate interactions with the MCM complex (motifs-C and -M; Varrin *et al.*, 2005; Jones *et al.*, 2010). Mutation of these Dbf4 domains compromises cell growth, DNA replication, and MCM phosphorylation (Varrin *et al.*, 2005; Francis *et al.*, 2009; Harkins *et al.*, 2009; Jones *et al.*, 2010). A region on Mcm2 (amino acids 204-278) has been shown to bind to DDK *in vitro* (Bruck and Kaplan, 2009). Additionally, a region on Mcm4 (amino acids 175-333) has been identified as the docking domain of DDK and mutation of this region reduces levels of phosphorylated Mcm4 (Sheu and Stillman, 2006). Though these regions are known, determining which specific subunit interactions target the DDK complex to the Mcm2-7 ring still remained to be elucidated.

Prior work from the Duncker Lab had investigated the association of DDK with the Mcm2-7 helicase complex, by evaluating Dbf4 and Cdc7 separately for its interaction with each of the Mcm subunits. Using the yeast two-hybrid assay, Dbf4 exhibited a strong interaction with the Mcm2 subunit and a weaker, but reproducible, interaction with Mcm6 (Appendix A Figure 1A). Complementary co-immunoprecipitation (Co-IP) analysis was also conducted with Myc-tagged Dbf4 and HA-tagged Mcms (Appendix A Figure 1B). Similar to the yeast two-hybrid results, the Mcm2 and Mcm6 subunits were pulled down with Dbf4, while the extent of the Dbf4-Mcm6 seemed comparable to the Dbf4-Mcm2 interaction.

When combining both two-hybrid and Co-IP results, Cdc7 showed equal interactions with both Mcm4 and Mcm5 (Appendix A Figure 1C and 1D).

The robust association of Mcm2 and Dbf4 prompted further investigation into this interaction. A series of Mcm2 truncations were constructed to test for the interaction with Dbf4 in a two-hybrid assay (Appendix A Figure 2A). Either removal of the N-terminal end (1-504) or the C-terminal end (505-868) showed reduced interactions with Dbf4 (Appendix A Figure 2B). The removal of the N-terminal end however showed a greater reduction in interaction, suggesting that it may contain a region important for interacting with Dbf4. Further dissection of the N-terminal end revealed that removal of the first 63 amino acids abrogated the interaction with Dbf4 (Appendix A Figure 2C and 2D). As previously mentioned, Mcm2 contains the partial nuclear localization signal (NLS) sequence specifically at the N-terminal amino acids residues 5-9 (Liku *et al.*, 2005). The NLS was restored to examine the effect of native Mcm2 NLS on Dbf4-Mcm2 interactions. The resulting construct, Mcm2Δ2-4, 10-63, showed diminished interactions with Dbf4 (Appendix A Figure 2E and 2F). The Mcm2Δ2-4, 10-63 protein also did not significantly change the interaction with Mcm6 (Mcm2's neighbour in the Mcm ring complex) (data not shown).

These findings are congruent with the Stillman lab's identification of a region of Mcm4 DDK docking domain (amino acids 175-333), which mediates association with DDK (Sheu and Stillman, 2006). Taken together, the relative importance of the Dbf4-Mcm2 and Cdc7-Mcm4 interactions for cell proliferation and DNA replication was explored, using the

Mcm2Δ2-4, 10-63 and Mcm4Δ175-333 mutants. Hereafter, the Mcm4Δ175-333 and Mcm2Δ2-4, 10-63 will be referred to as Mcm4ΔDDD and Mcm2ΔDDD, respectively. Plasmid shuffle strains were constructed by deleting the genomic copy of either *MCM2* or *MCM4*, and supporting for growth with a CEN (1-2 copies per cell) vector expressing either wild type or mutant Mcm2 or Mcm4. When *mcm2ΔDDD* and *mcm4ΔDDD* were used to support growth in *mcm2* and *mcm4* deletion strains, respectively, modest growth impairment was observed relative to wild-type controls (Appendix A Figure 3A). Subsequently, DNA replication was assessed in these same strains. Log phase cultures were arrested in late G1 phase using the mating pheromone α-factor, followed by removal of the α-factor to allow for a synchronous release into the cell cycle (Appendix A Figure 3B). Both the Mcm2ΔDDD and Mcm4ΔDDD strains showed slight but reproducible defects in S-phase progression compared to their wild-type counterparts (compare FACS profiles at 35 min in Appendix A Figure 3B). Therefore disruption of either the Dbf4-Mcm2 or Cdc7-Mcm4 interaction had only minor consequences for DNA replication and cell cycle progression. This suggested that only one of these interactions is sufficient to target the DDK complex to Mcm2-7 and allow it to phosphorylate the critical MCM residues required to trigger DNA replication. To investigate whether the Dbf4-Mcm2 and Cdc7-Mcm4 interactions represented redundant targeting mechanisms, the Mcm2ΔDDD and Mcm4ΔDDD strains were crossed, sporulation was induced in the diploids, and the resultant tetrads were dissected. Of 55 spores analyzed, none were *mcm2Δ*, *mcm4Δ* supported by episomal Mcm2ΔDDD and Mcm4ΔDDD. Conversely, in a control cross of the Mcm2 and Mcm4 wild-type plasmid shuffle strains, 10 of 36 spores analyzed were *mcm2Δ*, *mcm4Δ* supported by episomal Mcm2WT and

Mcm4WT. These results suggested that the combination of Mcm2ΔDDD and Mcm4ΔDDD was synthetic lethal, consistent with a model whereby disruption of the redundant Mcm2-Dbf4 and Mcm4-Cdc7 interactions simultaneously, prevents targeting of the DDK complex to Mcm2-7.

Prior work has shown that mutations in Dbf4 motif C are compromised for their interaction with Mcm2 leading to hypersensitivity under replication stress conditions (Jones *et al.*, 2010). Similarly, DDK has been shown to phosphorylate Mcm2 to stabilize replication forks in the presence of genotoxic agents. The notion that the role of Dbf4/Cdc7 in stabilizing and/or restarting replication forks under checkpoint conditions may occur through association with Mcm2-7 was investigated by constitutively overexpressing MCM subunits that impact Dbf4/Cdc7 targeting. Yeast strains were generated in which the genomic promoters controlling expression of individual MCM genes were replaced with a strong *GAL1* promoter (Appendix A Figure 4). This resulted in the ability to overexpress each of the MCM subunits individually. When these strains were exposed to the DNA alkylating agent methyl methanesulfonate (MMS) or the ribonucleotide reductase inhibitor hydroxyurea (HU), both of which impede replication fork progression, those overexpressing Mcm2 and Mcm4 were highly sensitive, whereas those overexpressing other subunits were not (Appendix A Figure 4). Production of excess Mcm2 and Mcm4 can compete with the Mcm2-7 ring for interaction with Dbf4 and Cdc7, respectively. Under checkpoint conditions, this can lead to weaker associations between Dbf4/Cdc7 and the MCM helicase, thereby leading to more severe growth defects (Appendix A Figure 4).

This chapter provides further evidence to support the model that Dbf4 and Cdc7 interact with distinct subunits of Mcm2-7 helicase. It also aims to show that deletion of the DDK-interacting region on Mcm2 or Mcm4 compromises DNA replication, and that simultaneous impairment of both the Dbf4- and Cdc7-MCM interactions results in lethality. Overexpression of Mcm2 in a strain background compromised for the Cdc7-Mcm4 interaction results in a severe growth defects. Growth was also examined on genotoxic agents to evaluate the role of DDK association with Mcm2-7 under checkpoint conditions.

3.2 Results

3.2.1 Simultaneous disruption of Dbf4-Mcm2 and Cdc7-Mcm4 leads to growth defects

The Duncker lab had previously found that combining both Mcm2ΔDDD/Mcm4ΔDDD mutations results in synthetic lethality. Therefore, since there were no cells to work with due to his combined mutation, an induced disruption approach was used. This experimental strategy involves disrupting the Mcm2-Dbf4 interaction by displacing it via Mcm2 overexpression in a cell background where Mcm4-Cdc7 association is already compromised. Plasmid shuffle strains were first created by deleting the genomic copy of either *MCM2* or *MCM4*, and supporting for growth with a CEN (1-2 copies per cell) vector expressing either wild type or mutant Mcm2 or Mcm4 (Figure 3.1). The Mcm4ΔDDD and Mcm4WT background strains were then transformed with a doxycycline repressible vector expressing Mcm2, Mcm2ΔDDD, or an empty vector control (Figure 3.1). Similarly, Mcm2ΔDDD and Mcm2WT background strains were transformed with a doxycycline repressible vector expressing Mcm4, Mcm4ΔDDD, or an empty vector control (Figure 3.1). The Mcm2ΔDDD and Mcm4ΔDDD overexpression was used as additional controls to determine if the titration effect was due to the presence of the docking domains. Spot plate assays were conducted to assess the overall rate of growth in the presence and absence of doxycycline. In the presence of doxycycline, all transformants demonstrated comparable growth (Figure 3.2A). In the absence of doxycycline, the overexpression of Mcm2 in a Mcm4WT background resulted in mild growth defects, consistent the notion that surplus Mcm2 is able to partially titrate the DDK complex from the Mcm2-7 ring, through its interaction with Dbf4 (Figure 3.2A). Overexpression of Mcm2 in a Mcm4ΔDDD showed a

striking growth defect that was much more severe, lending support to a model whereby simultaneous disruption of the Dbf4-Mcm2 and Cdc7-Mcm4 interactions compromises the ability of the DDK complex to associate with Mcm2-7 (Figure 3.2A and 3.2B).

The overexpression of Mcm4 in a Mcm2ΔDDD background resulted in modest growth defects that varied from trial to trial (Figure 3.2A and 3.2C). This could be due to less effective sequestration of Cdc7 by free Mcm4 compared to the origin bound Mcm4. Another reason could be that unlike Mcm2 and Mcm3, Mcm4 does not contain a Nuclear Localization Signal (NLS) sequence, causing less efficient targeting of Mcm4 to the nucleus. This would reduce the titration effect of Mcm4 compared to Mcm2 overexpression. To test whether this was the case, an NLS sequence was cloned into the respective Dox-repressible vectors containing Mcm4, Mcm4ΔDDD and empty vector (Figure 3.3). The addition of the NLS showed negligible growth defects, suggesting that the titration effect of Mcm4 may not be strong enough to compromise DDK association with Mcm2-7 (Figure 3.3). The overexpression of Mcm2ΔDDD in a Mcm4ΔDDD background and vice versa resulted in very minor growth defects, most likely due to incorporation of the mutant Mcms into the Mcm2-7 rings (Figure 3.2C).

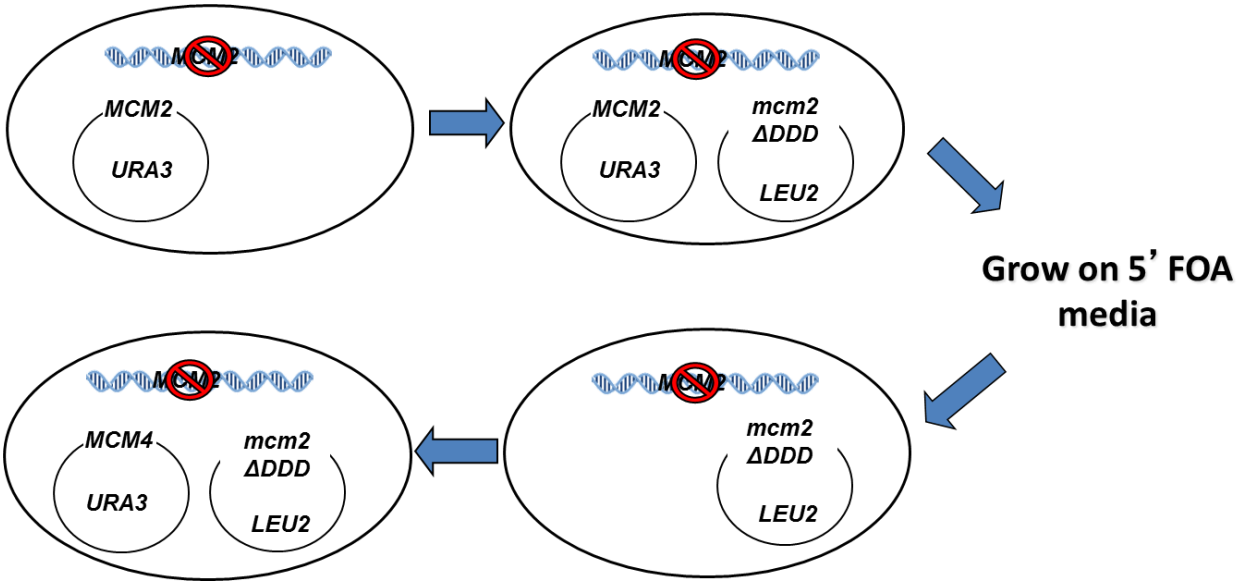

Figure 3.1: Construction of plasmid shuffle strains. Mcm2 is used as an example in this diagram, but the same strategy was applied to Mcm4 plasmid shuffle strains as well. Cells with the deleted genomic copy of *MCM2* were supported for growth with a CEN (single copy per cell) vector containing a wild-type *MCM2* gene and *URA3* selectable marker. A *LEU2*, CEN construct containing the mutant copy of the Mcm2 was then transformed into the strain. The resulting colonies from the transformation plates were streaked out on 5’FOA (5-flouroorotic acid) plates to select for cells that had lost the initial *URA3* support plasmid. Strains possessing a functional *URA3* gene, which encodes orotine-5-monophosphate decarboxylase (involved in the synthesis of uracil), will convert 5’FOA to its toxic form 5-flurouracil, thereby causing cell death. The only copy of the gene that should have remained in the cell was the mutant Mcm2 form on the *LEU2*, CEN plasmid. Other constructs like Mcm4 on a *URA3*, Dox-repressible vector were then transformed into those strains for overexpression/titration effects.

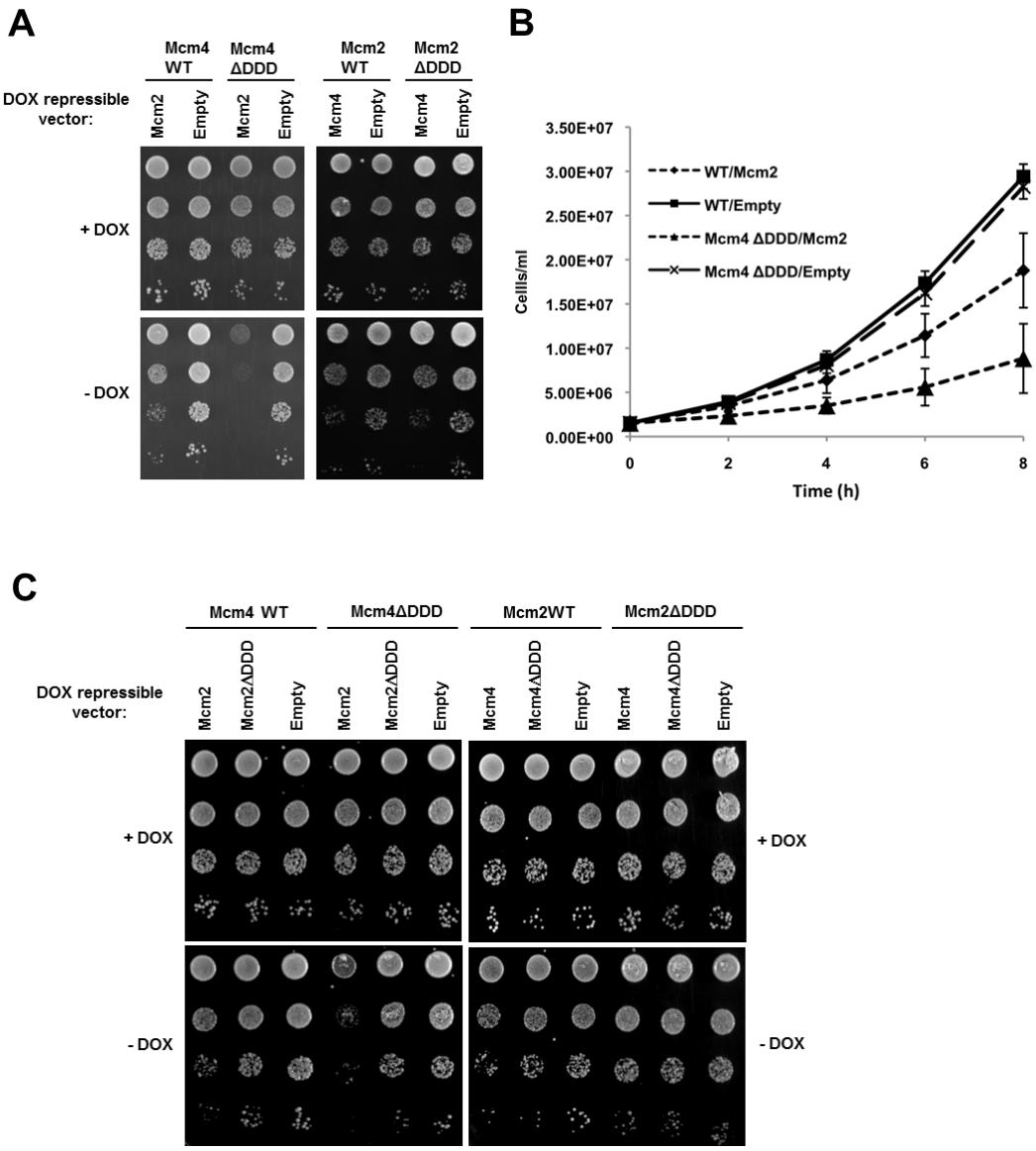

Figure 3.2: Mcm4ΔDDD cells are sensitive to Mcm2 overexpression. Mcm4WT, Mcm4ΔDDD, Mcm2WT, and Mcm2ΔDDD plasmid-supported strains were transformed with either empty pCM190, pCM190-Mcm2WT, or pCM190-Mcm4WT for which expression is under the control of a doxycycline (DOX)-repressible promoter. (A) 10-fold serial dilutions of each transformant were spotted on selective media with or without Dox at a starting concentration of $1x10^7$ cells/ml and grown for 2 days. (B) The same Mcm4WT and Mcm4ΔDDD transformants were grown in selective medium without Dox and the cell concentration determined at the indicated time points. The average of three replicates is shown $\pm$ S.D. (*error bars*). (C) Serial dilutions were carried as in A, with the addition of strains transformed with pCM190-Mcm2ΔDDD or pCM190-Mcm4ΔDDD, as indicated.

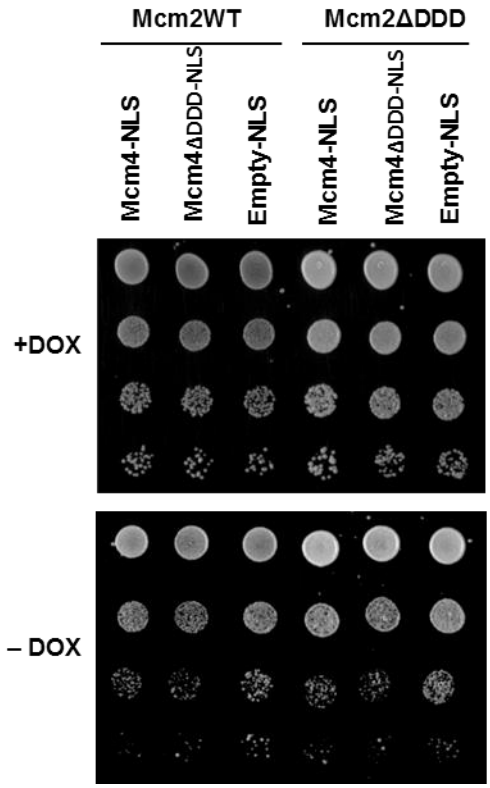

Figure 3.3: Overexpression of Mcm4-NLS shows negligible growth defects in Mcm2ΔDDD cells. Mcm2WT and Mcm2ΔDDD plasmid-supported strains transformed with pCM190-Mcm4WT-NLS, pCM190-Mcm4ΔDDD-NLS, or empty pCM190-NLS were tested for possible growth defects. Each of the pCM190 constructs contained a SV40 NLS added to the N-terminal end of the gene.10-fold serial dilutions of each transformed strain were plated on selective media with or without added Dox, at a starting concentration of $1x10^7$ cells/ml, and grown for 2 days at 30°C.

3.2.2 Exposure to genotoxic agents causes exacerbation of growth defects

Growth on genotoxic agents like hydroxyurea and methyl methanesulfonate resulted in exacerbation of growth inhibition in the Mcm4ΔDDD cells overexpressing Mcm2 (Figure 3.4). This is consistent with the previous results whereby growth defects were seen due to excess Mcm2 binding Dbf4 and titrating away the DDK complex from the Mcm2-7 ring. It was observed that Mcm4ΔDDD cells overexpressing Mcm2 were more sensitive than those overexpressing Mcm2 with wild-type Mcm4 present (Figure 3.4). The Mcm4 overexpression in the Mcm2ΔDDD showed slight growth inhibition in the presence of genotoxic agents (Figure 3.5; see the highest dilution). Ineffective sequestering of Cdc7 by Mcm4 could be a reason for the slight growth defect. The lack of an NLS sequence on Mcm4 could also be preventing the localization of overexpressed Mcm4 into the nucleus. Since genotoxic agents cause replication fork stalling, increased growth defects suggest that DDK-Mcm interactions may play a role in stabilizing replication forks under checkpoint conditions. Therefore, the ability to respond to replication stress seems to correlate with the degree of impairment in the association between Dbf4/Cdc7 and the Mcm2-7 complex.

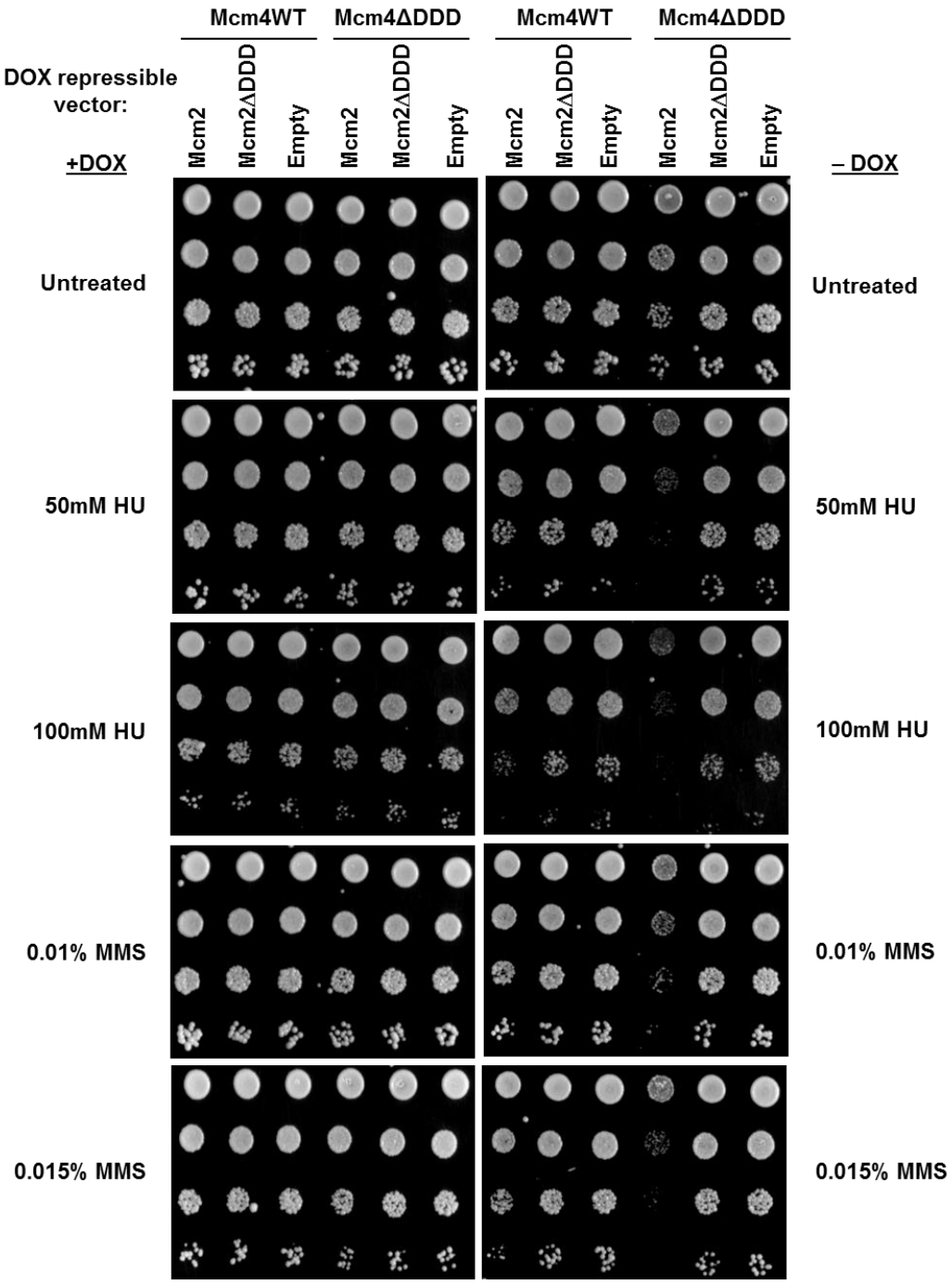

Figure 3.4: Exposure to genotoxic agents exacerbates growth defects in Mcm4ΔDDD cells overexpressing Mcm2. Mcm4WT and Mcm4ΔDDD plasmid-supported strains transformed with pCM190-Mcm2WT, pCM190-Mcm2ΔDDD, or empty pCM190 were tested for sensitivity to genotoxic agents. 10-fold serial dilutions of each transformed strain were plated on selective media containing the indicated concentrations of HU or MMS, with or without added Dox, at a starting concentration of $1x10^7$ cells/ml grown at 30°C for 3 days.

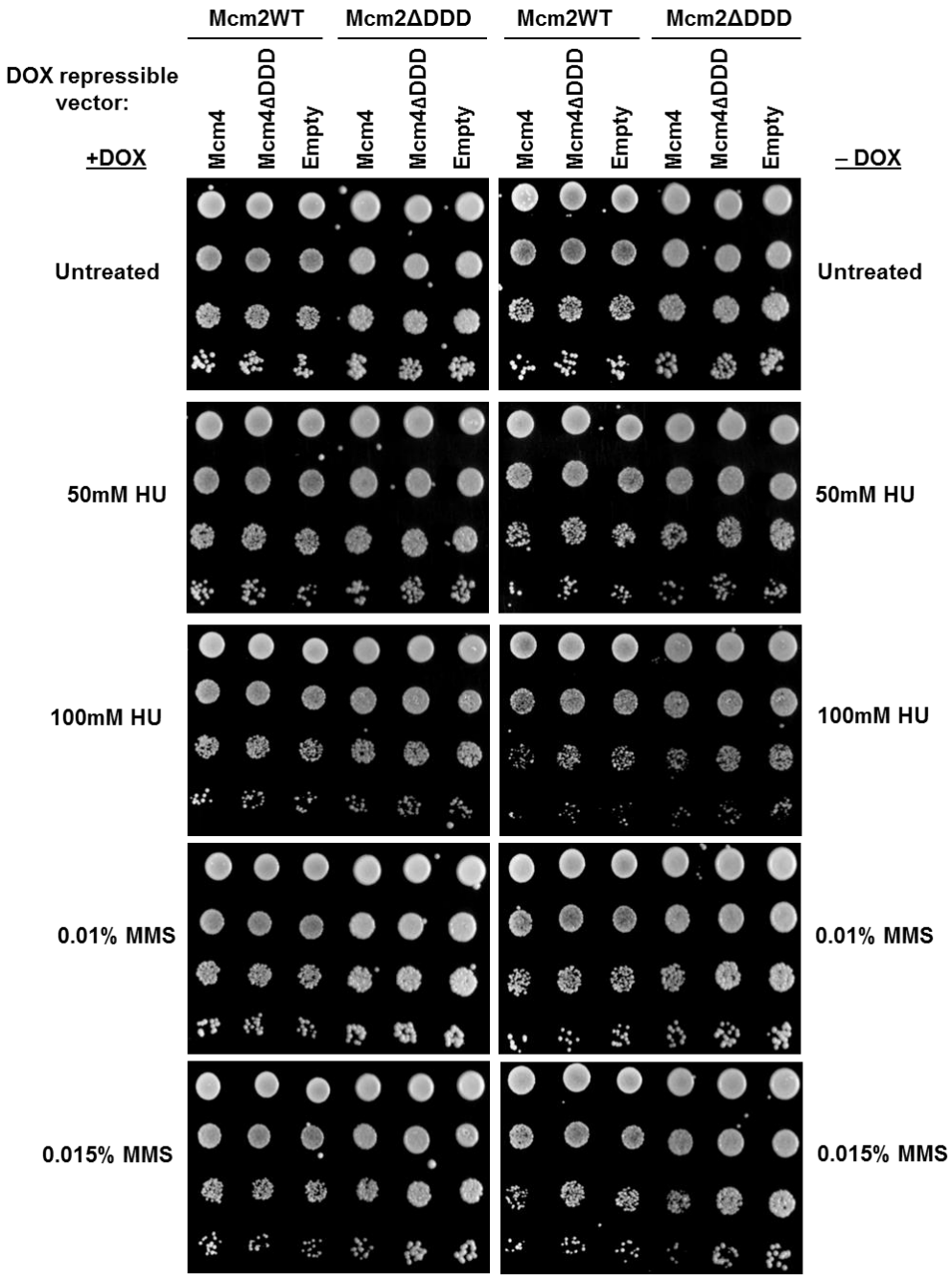

Figure 3.5: Exposure to genotoxic agents shows no significant exacerbation in growth defects in Mcm2ΔDDD cells overexpressing Mcm4. Mcm2WT and Mcm2ΔDDD plasmid-supported strains transformed with pCM190-Mcm4WT, pCM190-Mcm4ΔDDD, or empty pCM190 were tested for sensitivity to genotoxic agents. 10-fold serial dilutions of each transformed strain were plated on selective media containing the indicated concentrations of HU or MMS, with or without added Dox, at a starting concentration of $1x10^7$ cells/ml, and grown for 3 days at 30°C.

3.3 Discussion

The DDK complex triggers replication by phosphorylating subunits of the Mcm2-7 helicase complex at licensed origins. This phosphorylation leads to the recruitment of other replication protein factors to form the replisome that carries out local DNA unwinding. It is well known that the critical phosphorylation targets of DDK are Mcm2-7, however little is known about the way in which this essential replicative kinase is targeted to the MCM complex.

Previous findings from the Duncker lab have shown that Dbf4 and Cdc7 each contribute to the interaction with individual subunits of Mcm2-7. In the case of Dbf4, the results suggest that it interacts strongly with Mcm2, and exhibits a weaker association with Mcm6, a subunit that lies adjacent to Mcm2 in the MCM ring (Davey *et al*., 2003). These observations are consistent with previous work indicating that Dbf4, but not Cdc7, binds tightly to Mcm2 (Bruck and Kaplan, 2009). Combined two-hybrid and co-IP data indicate that Cdc7 interacts equally with Mcm4 and Mcm5, and displays no association with Mcm2 and Mcm6. Two-hybrid analysis of various Mcm2 domains reveal that residues in both the N- and C-terminal halves of the protein participate in the Dbf4 interaction, though the N-terminus appears to make the larger contribution. Removal of the N-terminal 63 amino acids of Mcm2 abrogated the interaction with Dbf4, suggesting that this region contains an essential uncharacterized functional domain. Restoration of the NLS in this N-terminal region showed the same diminished interaction with Dbf4. Another study has previously reported that Dbf4 binds to a second region on Mcm2 spanning amino acids 204-278, although the effect of removing this region was not evaluated (Bruck and Kaplan, 2009). The

overall results suggest that there are two possible mechanisms for targeting the DDK complex to the Mcm2-7 ring. In the first case, both Dbf4-Mcm2 and Cdc7-Mcm4 interactions are required for DDK targeting, ensuring that free Dbf4 or Cdc7 does not interfere with the association of the complex. A second possibility is that the separate interactions represent redundant mechanisms for targeting the DDK complex. This would ensure that the DDK-MCM complex forms more efficiently with minimal effects of mutations that may hinder individual subunit interactions. The results of this study support the second model of DDK targeting, where the interactions appear to the redundant. Using the identified N-terminal DDK docking domain of Mcm2 (amino acids 2-4, 10- 63) and the previously identified DDK docking domain of Mcm4 (amino acids 175-333; Sheu and Stillman, 2006) in an inducible fashion provided more evidence as to the targeting mechanism. The consequences of disrupting both Dbf4-Mcm2 and Cdc7-Mcm4 interactions displayed greater growth inhibition when compared to single disruptions (Figure 3.6). This was demonstrated through the overexpression of Mcm2 to titrate Dbf4 in a cell background where Cdc7-Mcm4 interaction was already hindered. Although Mcm4 overexpression did not exhibit the same severe growth defect in the Mcm2ΔDDD background, this could be due to ineffective sequestering of the Cdc7. Dbf4 exhibited a very robust interaction with Mcm2, but Cdc7 showed relatively similar strength in binding to Mcm4 and Mcm5. This could mean that Mcm4 alone is not sufficient to properly titrate Cdc7, even with the addition of an NLS. In this regard, examining the effect of the Mcm5-Cdc7 interaction might be worth exploring in future studies. However, since combining the Mcm2ΔDDD and Mcm4ΔDDD mutations results in synthetic lethality, the effect of Cdc7-Mcm5 and the weaker Dbf4-Mcm6

interactions is most likely not sufficient to target the DDK complex to Mcm2-7. The model in Figure 3.6 outlines the interactions between DDK and the Mcm2-7 complex.

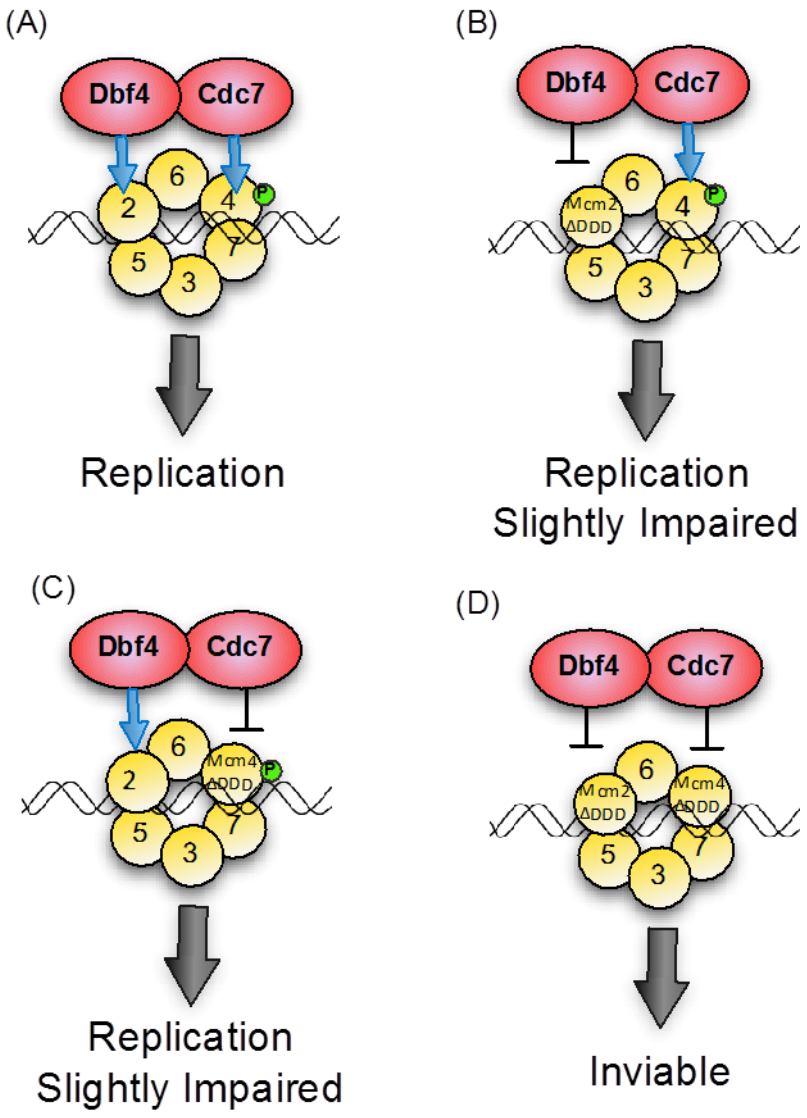

Figure 3.6: Model for DDK interaction with Mcm2-7 helicase complex. (A) Interaction between Dbf4-Mcm2 and Cdc7-Mcm4 targets the DDK complex to the Mcm2-7 ring to carry out phosphorylation of Mcm4, leading to normal DNA replication. (B) and (C) The disruption of one of these interactions will result in only slightly impaired replication. This is because the remaining interaction (either Dbf4-Mcm2 or Cdc7-Mcm4) is sufficient for DDK association with Mcm2-7. (D) Disruption of both interactions results in synthetic lethality due to abrogation of DDK association with Mcm2-7.

The simultaneous disruption of Dbf4-Mcm2 and Cdc7-Mcm4 interactions demonstrated exacerbated growth defects in the presence of genotoxic agents. This result complements findings in other studies where mutants of Dbf4 motif C (which interacts with Mcm2) displayed sensitivity to genotoxic agents (Jones *et al.*, 2010). Mutations of DDK phosphorylation sites on Mcm2 (Ser164 and Ser170) to non-phosphorylatable forms caused greater sensitivity to genotoxic agents (Stead *et al.*, 2011). These findings indicate that the targeting of DDK to the Mcm2-7 ring might be important under replication stress conditions. A possible reason for this targeting could be that DDK phosphorylation of one or more MCM subunits may help to stabilize and/or restart stalled or blocked replication forks. Recent evidence has shown an accumulation of Replication Protein A foci (representing generation of single stranded DNA) in Mcm2 mutants that have non-phosphorylatable DDK target sites, suggesting that the DDK acts to slow down the helicase in the presence of genotoxic agents (Stead *et al.*, 2012). Another likely scenario is that interaction with the MCM ring serves to direct DDK to other targets at or near the forks. Candidates include Cdc45 and the polα-primase complex, both of which are DDK substrates (Weinreich and Stillman, 1999; Nougarede *et al*., 2000; reviewed in Duncker and Brown, 2003), as well as histone H3, since its phosphorylation by DDK has been shown to play a role in maintaining genomic integrity (Baker *et al*., 2010). Aside from replication defects, agents that cause DNA damage can also induce apoptotic-like cell death in yeasts by activating caspase-like molecules (reviewed in Burhans *et al.*, 2003).

Interestingly, a number of the phenomena described here for budding yeast are similar to findings in more complex eukaryotes. For example, roles for both Mcm2-7 and Dbf4/Cdc7 during replication stress have been identified in *Xenopus* (Woodward *et al*., 2006; Tsuji *et al*., 2008). Several cancer and tumour derived cell lines have altered abundance of both MCM and DDK subunits (Bonte *et al*., 2008; Lau *et al*., 2010; reviewed in Mishra and Verma, 2010). Understanding the degree of conservation of Dbf4-Mcm2 and Cdc7-Mcm4 interactions in metazoan organisms and its influence on genome integrity maintenance would be an interesting avenue of exploration in further studies.

Chapter 4: Distinct Regions or Residues within Cdc7, Mcm2 and Mcm4 Potentially Facilitate DDK Association with Mcm2-7

Figure 4.4A and 4.6B contributed by Dr. Andrew Doxey

4.1 Introduction

The essential cell cycle kinase, DDK (Dbf4 dependent kinase), triggers the cell's transition from G1 to S phase. DDK is composed of the kinase subunit Cdc7 (cell division cycle 7) and its regulatory subunit Dbf4 (dumbbell forming unit 4). The key phosphorylation targets of DDK are multiple subunits of the heterohexameric Mcm2-7 helicase complex. As a result, phosphorylated Mcm2-7 unwinds the DNA double helix exposing single stranded DNA as a template for DNA polymerase. Mcm2-7 phosphorylation by Cdc7 facilitates the association of Cdc45 and Sld3, which subsequently recruit other replication factors to origins (Heller *et al.*, 2011). The Mcm2, Mcm4 and Mcm6 subunits have so far been implicated as direct targets of DDK (Sheu & Stillman, 2010; Randell *et al.*, 2010; Cho *et al.*, 2006). The previous chapter has shown that the Dbf4-Mcm2 and Cdc7-Mcm4 interactions represent redundant mechanisms in targeting DDK to the Mcm2-7 complex. Precise domains or regions that mediate these interactions have been characterized in Dbf4, Mcm2, and Mcm4, but not in Cdc7. The specific residues within the DDK docking domain of Mcm2 and Mcm4 that interact with Dbf4 and Cdc7, respectively, are also currently not known. Investigating these interactions will further enhance our understanding of the proposed targeting mechanism.

Cdc7 is an essential serine threonine kinase that is highly conserved among eukaryotes. The levels of Cdc7 remain constant throughout the cell cycle, and regulation of the kinase activity occurs through cell cycle regulation of its regulatory subunit, Dbf4. Dbf4 levels are high from G1 phase to late M phase when it is targeted by the APC/C (Anaphase Promoting Complex/Cyclosome) for degradation (Cheng *et al.*, 1999). Temperature sensitive

cdc7 mutants exhibit cell cycle arrest at G1 phase (Bousset and Diffley, 1998). The *mcm5-bob1* allele, which contains a single point mutation P83L in the Mcm5 subunit, bypasses the requirement for DDK most likely due to a conformational change conferred by the mutation (Hardy *et al.*, 1997; Hoang *et al.*, 2007). Several phosphorylation sites have been identified in the N-terminal regions of Mcm2, Mcm4 and Mcm6. Evidence has shown that kinases like Mec1 prime Mcm4 and Mcm6 to stimulate subsequent DDK phosphorylation (Randell *et al.*, 2010). Prior phosphorylation is also required for DDK to associate with Mcm2-7 in a Dbf4-dependent manner (Francis *et al.*, 2009). The N-terminal serine/threonine rich domain (NSD) of Mcm4 is processively phosphorylated by DDK to relieve an inhibitory effect and allow replication to proceed (Sheu and Stillman, 2010). Given the vital role of Cdc7, targeting it for inhibition has shown promise as a potential cancer therapeutic in many studies (Bonte *et al.*, 2008; reviewed in Montagnoli *et al.*, 2010; Natoni *et al.*, 2013). Therefore, understanding how Cdc7 interacts with Dbf4 and Mcm4 will further shed light on how DDK is targeted to the Mcm2-7 complex for replication initiation.

The DDK Docking Domains have been characterized in both Mcm4 (amino acids 175-333; Sheu and Stillman, 2006) and Mcm2 (amino acids 2-4, 10-63; see Chapter 2). Domains within Dbf4 have also been characterized. Dbf4 contains three conserved regions, identified as motifs-N, -M and -C (reviewed in Masai and Arai, 2000), each mediating interactions with replication factors. Motif-N has been shown to interact with Orc2 and the Rad53 checkpoint kinase, while motifs-M and -C exhibit some redundancy in interacting with Mcm2 and Cdc7 (Matthews *et al.*, 2012; Varrin *et al.*, 2005; Jones *et al.*, 2010; Harkins

et al., 2009). The Cdc7 structure, on the other hand, is composed of 11 conserved kinase domains and 3 Kinase-Insert domains, which mediate protein-protein interactions (reviewed in Masai and Arai, 2002; Masai *et al.*, 1995). Previous work in budding yeast has found that the C-terminal region of Cdc7 binds with Dbf4, and specific residues within the kinase domains are essential for kinase activation and function (Jackson *et al.*, 1993; Ohtoshi *et al.*, 1997). Consistently, findings in human cell lines have shown that the C-terminal region of Cdc7 mediates the interaction with Dbf4 motifs-M and -C (Kitamura *et al.*, 2011). The recently solved crystal structure of human Dbf4-Cdc7 complex revealed that Dbf4 motif-C binds to the N-terminal lobe of Cdc7 and is sufficient for kinase activity, whereas Dbf4 motif M binds to the C-terminal lobe acting as a tethering domain (Hughes *et al.*, 2012). These studies provide a good framework for deducing interacting regions in the equivalent budding yeast proteins.

4.2 Results

4.2.1 Regions of Cdc7 Show Overlap or Differences in Binding to Mcm4 and Dbf4

In order to determine which region of Cdc7 mediates the interaction with Mcm4, yeast two-hybrid assays were conducted. Several LexA-tagged Cdc7 truncations were cloned into bait vectors and tested for interaction with HA-tagged full-length Mcm4 expressed by prey vectors (Figure 4.1; see schematic of Cdc7 truncations). The Cdc7 regions were also examined for interaction with Dbf4 to determine whether certain regions exhibit overlap in interactions with both proteins. The two-hybrid results revealed that regions near the C-terminal end (Cdc7 amino acids 453-507) and N-terminal end (Cdc7 1-54) of Cdc7 show weak, but reproducible interactions with Mcm4 (Figure 4.1). Though these interactions are

weak, they appear to be important as removal of these regions resulted in abrogated interactions with Mcm4. The Cdc7 C-terminal end (Cdc7 453-507) showed a similar weak association with Dbf4 as well. Removal of the first 54 amino acids of Cdc7 maintained the binding to Dbf4 similar to the wild-type levels (Figure 4.1; see Cdc7 55-507 construct). This region seems to be sufficient for the interaction with Dbf4, but showed no interaction with Mcm4. In the previous chapter, it was proposed that Dbf4-Mcm2 and Cdc7-Mcm4 interactions represent redundant mechanisms for targeting DDK to the Mcm2-7 helicase. If the Cdc7-Mcm4 interaction was disrupted but the Dbf4-Mcm2 interaction was maintained, one would expect that Cdc7 55-507 would complement a *cdc7* mutant, assuming the kinase activity is still intact. To determine if Cdc7 55-507 could still promote DNA replication, the construct was tested for its ability to complement the lack of wild-type Cdc7 in a cell (Figure 4.2A). To achieve this, a strain in which the genomic copy of *CDC7* was replaced with a *GAL1-CDC7-HA* allele was used so that Cdc7 expression could be induced in the presence of galactose and shut off in the presence of glucose (Figure 4.2A). This strain was transformed with the two-hybrid bait vector containing Cdc7 55-507 and then grown on glucose and galactose media. The Cdc7 55-507 region did not complement growth when wild-type Cdc7 expression was turned off in the presence of galactose (Figure 4.2A). Though this region exhibited a disrupted interaction with Mcm4, DDK should still be targeted to the Mcm2-7 complex since the Dbf4-Mcm2 interaction is still maintained. This means that although the removal of the first 54 amino acids maintains binding to Dbf4, it may have affected other aspects like protein kinase activation or function.

Bioinformatics analysis was used to analyze the Dbf4-Cdc7 interaction and locate conserved interface residues. Appendix B Figure 1A shows a PyMol generated structure of human Dbf4-Cdc7 interacting regions where yellow/red represents Cdc7 amino acids 89-148 and blue/green represents Dbf4 amino acids 288-336. This region of Dbf4 encompasses conserved motif-C, which aligns with the *S.cerevisiae* Dbf4 motif-C region (amino acids 656-697) (Appendix B Figure 1B; see Dbf4 alignment; Hughes *et al.*, 2012; Harkins *et al.*, 2009). Motif-C has been previously shown to interact with Cdc7, however a precise Dbf4-binding domain has not yet been identified in *S.cerevisiae* Cdc7 (Kitamura *et al.*, 2011; Sato *et al.*, 2003; reviewed in Masai and Arai, 2000). Alignments of human and yeast Cdc7 sequences revealed 4-5 key Cdc7 residues that are both conserved and interact with residues in Dbf4 (see Section 2.10 for details on identification of residues). In Appendix B Figure 1B, Cdc7 residues highlighted in red indicate conserved residues that may interact with Dbf4. Similarly, in the Dbf4 alignment, residues highlighted in red may interact with Cdc7. Residues highlighted in colours other than red in Cdc7 are ones that show clear contact with residues of the same colour in the Dbf4 sequence (Appendix B Figure 1B; see both alignments). For example, the highlighted blue residue in Cdc7 shows a clear contact with the blue residue highlighted in Dbf4. The identified conserved interface residues within Cdc7 are Threonine 81 (number constitutes amino acid position), Serine 82, Proline 84, Arginine 111, and Aspartic Acid 114. Within Dbf4, the conserved interface residues are Glutamic acid 630, Histidine 648, Phenylalanine 658, and Aspartic Acid 672. The region of human Cdc7 that interacts with Dbf4 corresponds to amino acid region 75-113 in *S.cerevisiae*. This seems consistent with the two-hybrid data in Figure 4.1, as the removal of the first 124 amino acids

of Cdc7 abrogated the interaction with Dbf4, but the Cdc7 55-507 region maintained a robust association. Therefore, amino acid region 55-124 appears to be a key Dbf4 interacting motif in Cdc7.

To further test the binding between the Cdc7 truncations and Mcm4, several Cdc7 regions were overexpressed in a wild-type background and spotting assays were conducted. Overexpression of the C-terminal constructs (Cdc7 453-507 and Cdc7 276-507) resulted in minor growth inhibition (Figure 4.2B). The truncated proteins can weakly bind to either Dbf4 or Mcm4 (Figure 4.1), possibly sequestering them from normal replication functions. Since Cdc7 55-507 seems to be sufficient for interaction with Dbf4, overexpression of this region caused more significant growth defects, most likely due to sequestering of Dbf4 (Figure 4.2B). The Cdc7 1-452 region was overexpressed to see if a construct that showed no binding to Dbf4 or Mcm4 would show no growth defects due to the inability to sequester both proteins. Interestingly, overexpression of Cdc7 1-452 caused significant growth defects, despite showing no interaction with Dbf4 or Mcm4 in the two-hybrid data. One possibility could be that it titrates Mcm5 or Mcm7, which have also been shown to interact with Cdc7 (see Chapter 3). Two-hybrid analysis, however, revealed that Cdc7 1-452 does not interact with either Mcm5 or Mcm7 (Figure 4.3). Therefore, this Cdc7 truncation may be sequestering another as yet unidentified replication factor, thereby inhibiting subsequent DNA replication.

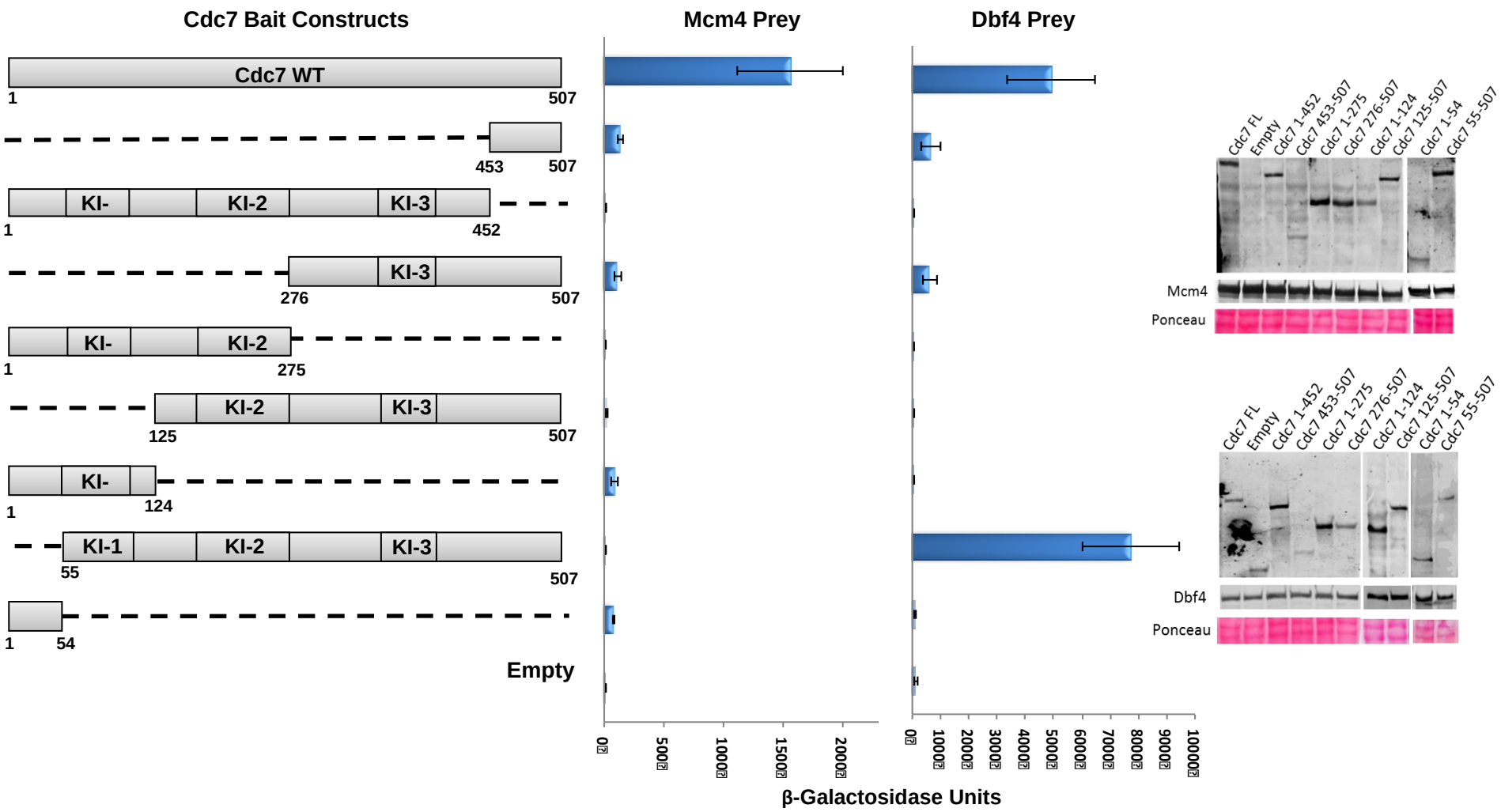

Figure 4.1: Distinct regions of Cdc7 exhibit binding to Mcm4 and Dbf4. Two-hybrid assays were carried out using HA-tagged prey plasmids pJG-Mcm4 or pJG-Dbf4. Several truncated forms of Cdc7 were cloned into LexA-tagged bait vectors. These include pEG-Cdc7 Full Length, pEG-Cdc7 453-507 (number denotes amino acids), pEG-Cdc7 1-452, pEG-Cdc7 276-507, pEG-Cdc7 1-275, pEG-Cdc7 125-507, pEG-Cdc7 1-124, pEG-Cdc7 55-507, pEG-Cdc7 1-54 and pEG-202 (empty). An average of three replicates is shown ± S.D. (*error bars*). To confirm that all baits and preys were properly expressed, aliquots of culture were removed prior to the measurement of β-galactosidase activity, and whole-cell extracts were prepared and subjected to immunoblot analysis. Bait proteins were detected with rabbit polyclonal anti-LexA antibody and prey proteins were detected with mouse monoclonal anti-HA antibody. Ponceau S staining of the membrane was carried out to check for equal loading of samples. The schematics outline the regions of Cdc7 that have been removed (indicated by the dotted lines) and the regions that have been retained (grey boxes). KI-1, KI-2, and KI-3 denote Kinase Insert-1, Kinase Insert-2, and Kinase Insert-3 domains, respectively.

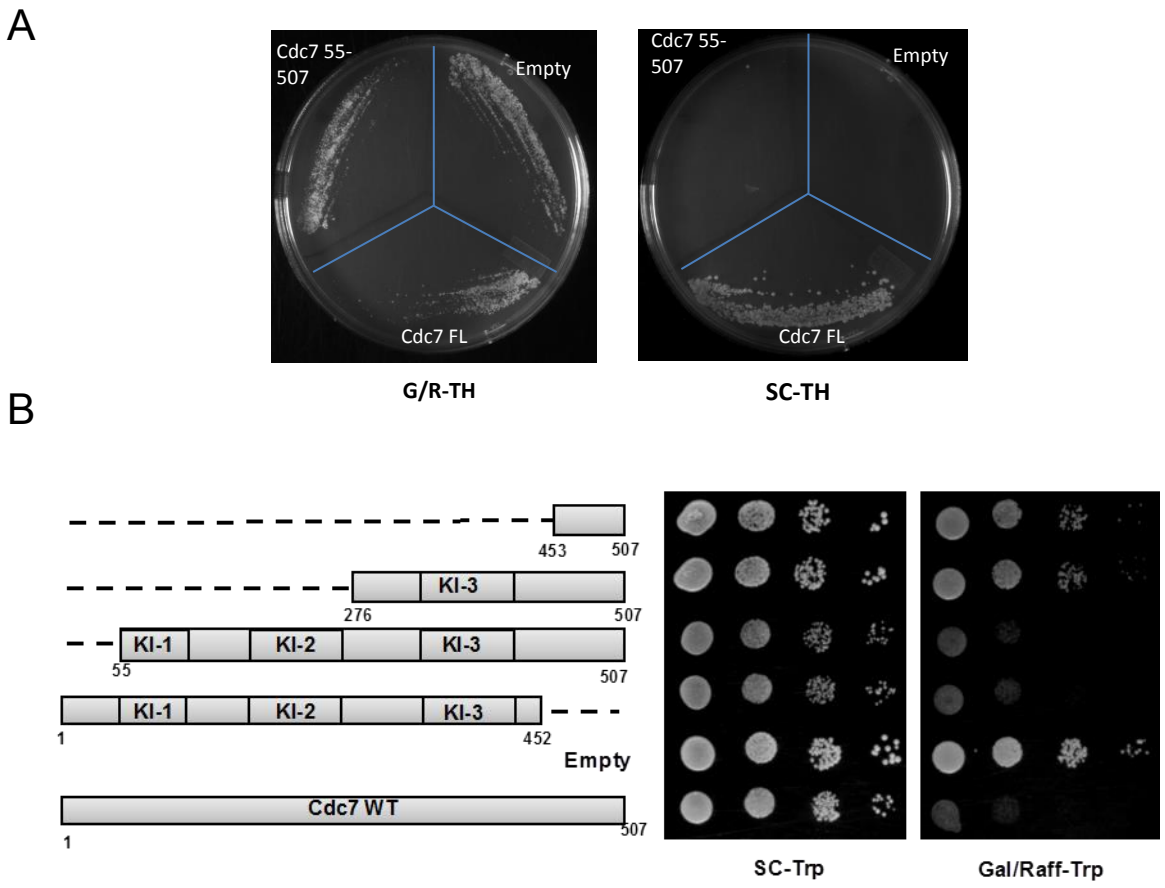

Figure 4.2: Overexpression of certain Cdc7 truncations results in growth defects. (A) The pEG-Cdc7 Full Length (FL), pEG-Cdc7 55-507, and pEG-202 (empty) vectors were transformed into a *GAL1-CDC7-HA* strain and streaked out onto glucose media without tryptophan and histidine (SC-TH) and galactose media without tryptophan and histidine (G/R-TH). Plates were grown for 2 days at 30°C. (B) Several Cdc7 constructs were cloned into prey pJG-4-6 vectors and transformed into the DY-1 wild type yeast strain. The constructs are pJG-Cdc7 Full Length (FL), pJG-4-6 (empty), pJG-Cdc7 1-452 (number denotes amino acids), pJG-Cdc7 55-507, pJG-Cdc7 276-507, and pJG-Cdc7 453-507. 10-fold serial dilutions of each construct were plated on glucose media with no tryptophan added (SC-Trp) or galactose media with no tryptophan (Gal/Raff-Trp) with a starting concentration of 1 x 10^7 cells/mL. Plates were grown for 2 days at 30°C.

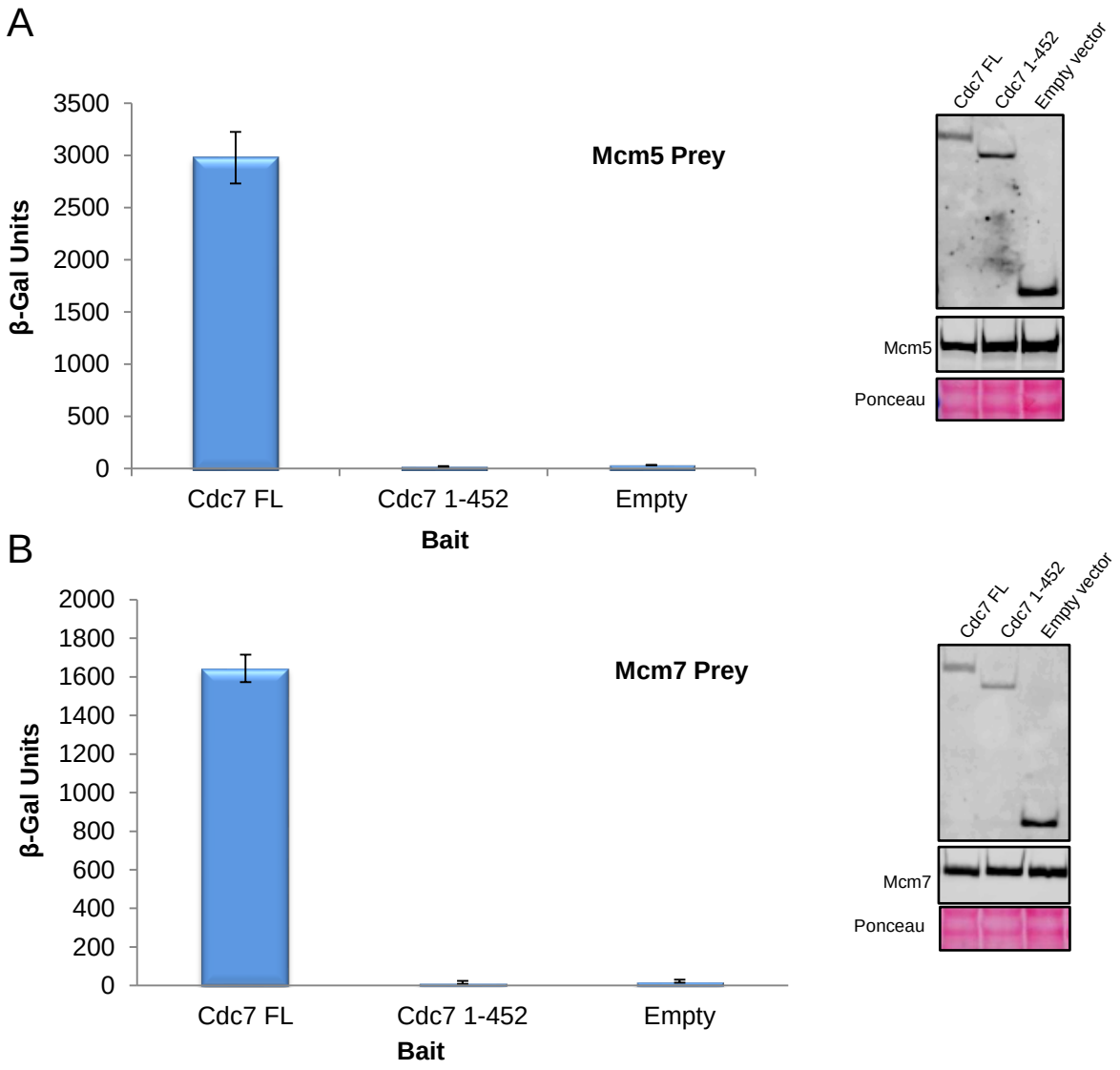

Figure 4.3: The Cdc7 1-452 construct exhibits no interaction with the Mcm5 or Mcm7 subunits. Two-hybrid assays were carried out using HA-tagged prey plasmids (A) pJG-Mcm5 or (B) pJG-Mcm7. The bait vectors expressed LexA-tagged Cdc7 constructs. These include pEG-Cdc7 Full Length, pEG-Cdc7 1-452 and pEG-202 (empty). An average of three replicates is shown ± S.D. (*error bars*). Immunoblot analysis to verify bait and prey expression was carried out as described in the legend for Figure 4.1.

4.2.2 Specific Residues within the Mcm2 N-terminal Region Mediate Binding to Dbf4

In the previous chapter, the DDK docking domain of Mcm2 was shown to be amino acids 2-4, 10-63. The next step involved identifying precise residues within this docking domain that may play a role in interacting with Dbf4. First, bioinformatics analysis was conducted to identify potential residues based on their conservation (Figure 4.4A). The first 63 amino acids were PSI-BLASTED (Position-Specific Iterative Basic Local Alignment Search Tool) against NCBI Reference Sequences (RefSeq) and run through three iterations/rounds. In the first iteration, a normal BLASTp run was conducted and generated a multiple alignment from which a Position Specific Scoring Matrix (PSSM) was constructed. The PSSM gives a position-specific score for each position in the alignment, with more conserved positions receiving high scores and less conserved positions receiving low scores. This profile was then used in a second BLAST search or iteration to detect sequences that match the initial conservation pattern. After the third iteration, a final conservation logo was generated and revealed two potential protein interaction motifs (Figure 4.4A). These are P-x-x-P and P-x-x-x-P, where P stands for proline and the x constitutes any other amino acid (reviewed in Kay *et al.*, 2000; Figure 4.4A). More specifically, the residues are Proline 22, Proline 25, Proline 39 and Proline 43. The proline residues were mutated to alanines using the QuikChange Site-Directed Mutagenesis kit (see Materials and Methods Section 2.2). Three different forms of the Mcm2 gene were constructed: one expressing protein with the first two prolines mutated (QC1), one expressing protein with the last two prolines mutated (QC2), and one expressing protein with all four prolines mutated (QC3). The resulting Mcm2 proteins were tested for interaction with Dbf4 (Figure 4.4B). Two-hybrid analysis showed that mutations to all four prolines results in a greater than 50% decrease in the interaction

with Dbf4, suggesting that both of these protein interaction motifs play a role in mediating binding to Dbf4 (Figure 4.4B).

To test the importance of the four prolines in mediating the interaction with Dbf4, an induced disruption approach (similar to the one used in Chapter 3). In this approach, Mcm4 would be overexpressed to bind and titrate Cdc7 in a background where Dbf4-Mcm2 interaction was already hindered. The experiment would be similar to the spot plate assay in Chapter 3 (Figure 3.6), except Mcm2QC3 would be used to disrupt the interaction with Dbf4 instead of Mcm2ΔDDD. The *mcm2QC3* mutant was cloned into a *URA3,* CEN (centromeric) vector and used to support the growth of a *Δmcm2* strain. This strain was then transformed with a pCM190 doxycycline repressible expression vector containing Mcm4-NLS (Mcm4 with nuclear localization signal sequence), Mcm4ΔDDD-NLS, or an empty vector control (Figure 4.5). In the presence of doxycycline, all three transformants demonstrated comparable growth (Figure 4.5; see +DOX panels). In the absence of doxycycline, the overexpression of Mcm4-NLS in the Mcm2QC3 background demonstrated modest growth defects compared to NLS overexpression alone (Figure 4.5; see highest dilution). Despite the addition of an NLS to Mcm4, the titration of Cdc7 by Mcm4 may not be strong enough to compromise DDK association with Mcm2-7. Interestingly, Mcm4ΔDDD-NLS overexpression in the Mcm2QC3 background also resulted in slight growth defects most likely due to the simultaneous incorporation of Mcm2QC3 and mutant Mcm4ΔDDD into the Mcm2-7 ring.

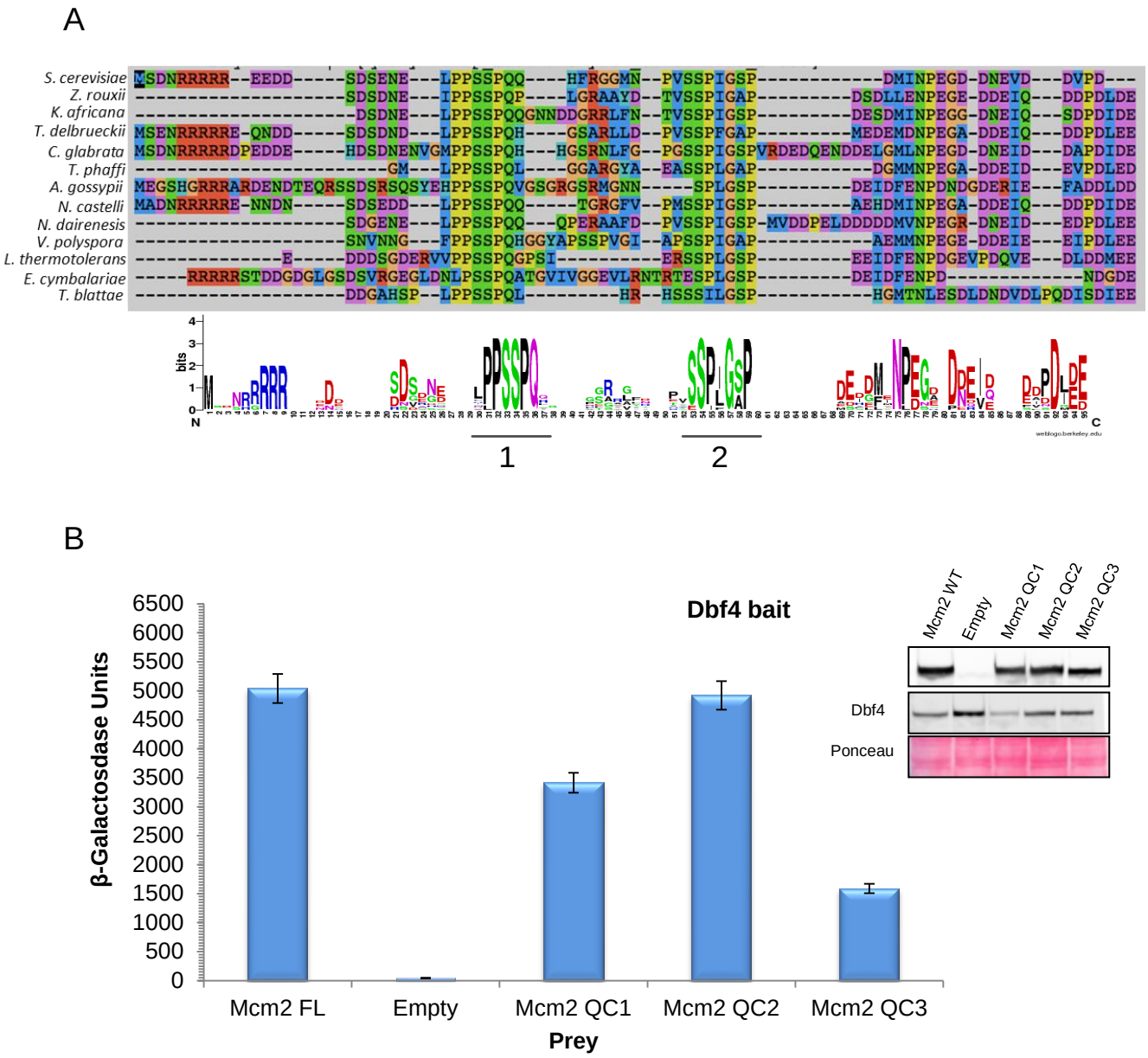

Figure 4.4: Mutating conserved prolines in Mcm2 N-terminal protein interaction motifs reduces the interaction with Dbf4. (A) Bioinformatics analysis was conducted on the Mcm2 DDK docking domain (amino acids 1-63). The region was PSI-BLASTED against reference sequences (RefSeq) and run through three rounds of alignments/iterations. A final conservation logo was generated and identified two conserved regions: PPSSPQ (region 1) and SSPxGxP (region 2). These resemble protein interaction motifs P-x-x-P and P-x-x-x-P. These prolines were then mutated to alanines using the QuikChange Mutagenesis kit. Mcm2 QC1 denotes mutations to the first two prolines to alanines in region 1, Mcm2 QC2 denotes mutations to the second two prolines in region 2, and Mcm2 QC3 denotes mutations to all four prolines. These HA-tagged wild type and mutant Mcm2 proteins were expressed off pJG-4-6 prey plasmids. The bait plasmids expressed LexA-tagged full-length Dbf4. An average of three replicates is shown ± S.D. (*error bars*). Immunoblot analysis to verify bait and prey expression was carried out as described in the legend for Figure 4.1.

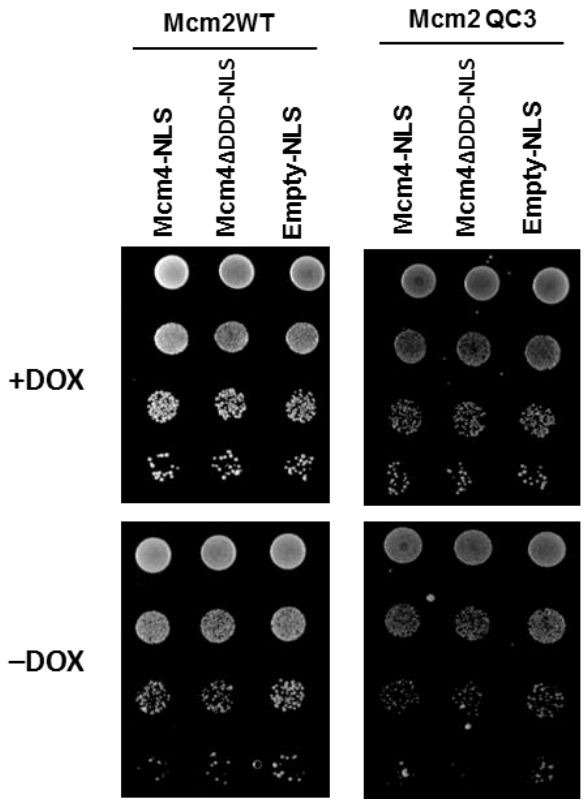

Figure 4.5: Overexpression of Mcm4-NLS shows modest growth defects in Mcm2 QC3 cells. Mcm2 WT and Mcm2QC3 plasmid-supported strains were transformed with pCM190-Mcm4WT-NLS, pCM190-Mcm4ΔDDD, or empty pCM190-NLS. The resulting transformants were test for possible growth defects. Each of the pCM190 constructs had a SV40 NLS added to the N-terminal end of the gene. 10-fold serial dilutions of each transformed strain were plated on selective media with or without added Dox (doxycycline), at a starting concentration of $1x10^7$ cells/mL, and grown for 2 days at 30°C.

4.2.3 Bioinformatics Analysis of the Mcm4 DDK Docking Domain reveal possible key interacting residues

The evolutionary conservation on the protein surface of the Mcm4 DDK docking region (amino acids 175-333) was mapped, similar to the analysis conducted for the Mcm2 docking region (Figure 4.6A). The results reveal a number of residues that appear to be conserved. When analyzed in 3D space, certain residues seem to cluster closely together and are also exposed along the same plane (Figure 4.6B; highlighted yellow residues). These include Tryptophan 181, Glutamine 266, Glutamic acid 267, Proline 319, Isoleucine 322, and Aspartic acid 323. These residues may represent possible interaction sites within the docking region of Mcm4 and serve as good candidate residues to mutate for protein interaction analysis. Mutating these residues could potentially reveal their importance in mediating the binding to Cdc7. Attempts at two-hybrid analysis with Mcm4 point mutants were unsuccessful as the background signals with full length Mcm4 prey and an empty bait vector were too high to draw any conclusions. Co-immunoprecipitation assays or GST pull-down assays may serve as an alternative approach to evaluate protein-protein interactions.

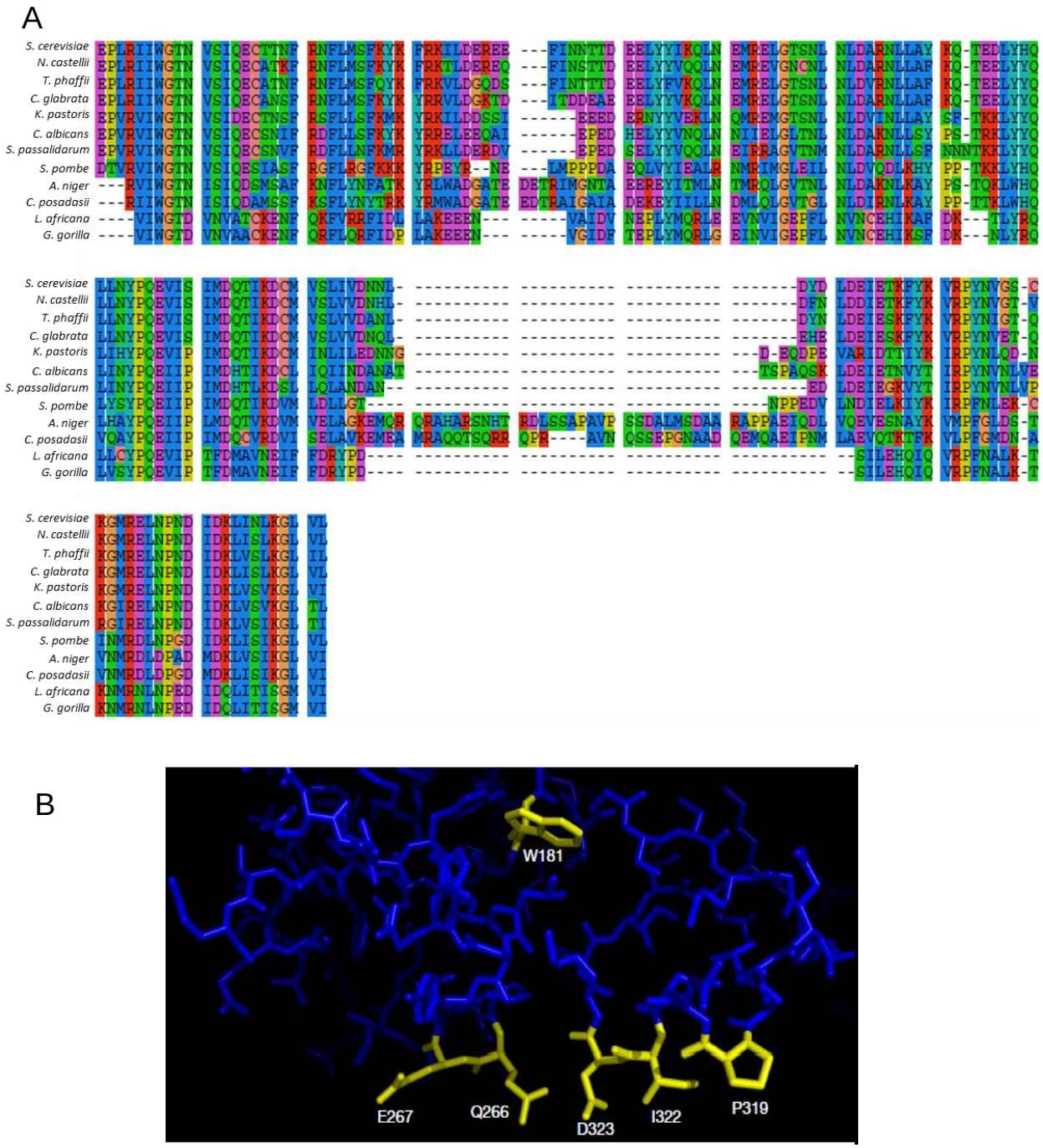

Figure 4.6: Bioinformatics reveals potential key interacting residues in Mcm4 (A) The Mcm4 DDK docking region (amino acids 175-333) was BLASTed against RefSeq and aligned using CUSTALO algorithm as implemented in the SeaView program. The alignment was transferred to weblogo to reveal conserved residues. (B) Structural visualization of the Mcm4 DDK docking region (amino acids 175-333) was generated using the PyMol program. The highlighted yellow residues are highly conserved residues that cluster closely together in 3D space. The residues are: Tryptophan 181 (W181; number denotes amino acid position), Glutamine 266 (Q266), Glutamic acid 267 (E267), Proline 319 (P319), Isoleucine 322 (I322), and Aspartic acid 323 (I322). These residues could represent possible interaction sites.

4.3 Discussion

4.3.1 Cdc7 and its interaction with Dbf4 and Mcm4 proteins

The Cdc7 kinase subunit of DDK (Dbf4-dependent kinase) shares significant similarity in its catalytic domains between yeasts and higher eukaryotes (reviewed in Masai and Arai, 2002). The recently solved crystal structure of human Dbf4-Cdc7 has revealed that Cdc7 has a characteristic bi-lobal structure (Hughes *et al.*, 2012). The active site is located in a cleft between the N- and C-terminal lobes, similar to other protein kinases (Hughes *et al.*, 2012; reviewed in Matthews and Guarné, 2013). In the human structure, the N- and C-lobes of Cdc7 interface with Dbf4 motif-N and motif-C, respectively (Hughes *et al.*, 2012). These results seem consistent with the yeast two-hybrid data, which show that the N- and C-terminal ends of Cdc7 interact with Dbf4. The C-terminal Cdc7 453-507 region showed weak, but reproducible interactions with Dbf4. This is also consistent with previous *S. cerevisiae* work, which showed that the C-terminal 55 amino acid residues interacts with Dbf4 and confers species-specific formation of functional DDK (Jackson *et al.*, 1993; Davey *et al.*, 2011). The Cdc7 55-507 construct exhibited a robust interaction with Dbf4, whereas the Cdc7 125-507 showed no interaction. The region spanning amino acids 55-124 in combination with the C-terminal residues 453-507 seem to be the key regions that bind to Dbf4. In human Cdc7, the first 36 residues were found to be exposed and subsequently subject to proteolysis during structural analysis (Hughes *et al.*, 2012). A truncated version of human Cdc7 with a deletion of this N-terminal region as well as large portions of Kinase Inserts 2 and 3 successfully crystallized with Dbf4 motifs-M and -C (Hughes *et al.*, 2012). Since the removal of the first 54 amino acids of yeast Cdc7 did not disrupt the interaction with Dbf4, this suggests that this region is also exposed like the N-terminal region in the

human Cdc7 structure. This information is useful for narrowing down regions that strongly bind to Dbf4 for crystallization studies in the future. The kinase insert domains do not seem to play a major role in mediating interactions on their own, but specific deletion mutants may need to be tested to confirm this. Overall, the amino acids 453-507 and 55-124 in *S. cerevisiae* Cdc7 seem to be the likely target regions for Dbf4 binding.

The N- and C-terminal regions of Cdc7 showed very weak, but reproducible interactions with Mcm4. The overlap in the binding of the Cdc7 C-terminal construct (amino acids 453-507) with Dbf4 and Mcm4 could explain the weak interaction, as the C-terminal end seems to be interacting with multiple targets. The first 54 amino acids of Cdc7 show weak binding with Mcm4 but not with Dbf4. This region could also potentially bind to other Mcm proteins like Mcm5 and Mcm7. Though binding to multiple targets seems like an interesting possibility, this would be difficult to determine without analyzing the enzyme in its active state. A serine/threonine kinase active site is controlled by the orientation of a region called the activation segment, found within the cleft of the N- and C-lobes (Nolen *et al.*, 2004; reviewed in Matthews and Guarné, 2013). This region facilitates the change from the inactive kinase to an active, stable form. Since Cdc7 undergoes this conformational change, most likely due to the binding with Dbf4, it would be difficult to conclude if the Cdc7 regions analyzed in the two-hybrid analysis represents the actual Cdc7 and Mcm4 interaction. The extreme N- and C-terminal ends seem to be required for binding to Mcm4 but this would be better analyzed through interactions with an active Cdc7-Dbf4 complex.

Previous work has shown that overexpression of kinase-negative Cdc7 mutants can dominantly inhibit the function of wild-type Cdc7 (Ohtoshi *et al.*, 1997). Interestingly, overexpressing wild-type Cdc7 caused severe growth inhibition (Figure 4.2B). This differs from previous findings, which have shown that a combination of Dbf4 and Cdc7 overexpression causes growth inhibition but individual subunit overexpression does not (Nougarede *et al.*, 2000; Devault *et al.*, 2008). One explanation could be that the HA fusion tag may be interfering with normal DDK function, thus preventing growth. Overexpression of the C-terminal constructs (Cdc7 453-507 and Cdc7 276-507) showed mild growth inhibition, while the overexpression of Cdc7 55-507 showed more severe growth inhibition. The Cdc7 truncations most likely form inactive kinase complexes with Dbf4 or sequester Mcm4 and Dbf4. One particularly interesting result is that the Cdc7 1-452 region, did not interact with either Mcm4 or Dbf4 in the two-hybrid data, yet showed severe growth inhibition. Two-hybrid analysis with Mcm5 and Mcm7 revealed no interaction with this construct, suggesting that it may be interacting with yet another protein factor essential for replication or has a general toxicity effect. The enhanced binding seen with the Cdc7 55-507 and Dbf4 did not retain DDK function, as it failed to complement the lack of wild-type Cdc7. The removal of the first 54 amino acids could have disrupted the essential kinase function of Cdc7, thereby resulting in a failure to initiate DNA replication.

4.3.2 Potential targeting residues within Mcm2, Mcm4, and Cdc7

The bioinformatics analysis of the Mcm2 DDK Docking domain revealed two regions with potential protein interaction motifs: P-x-x-P and P-x-x-x-P. These motifs are known to participate in binding to SH3 (Src-homology 3) domain-containing motifs (Chandra *et al.*,

2004; Cesarini *et al.*, 2002; Tong *et al.*, 2002). SH3 domains are commonly found in eukaryotic cytoskeletal and signal transduction proteins (reviewed in Kay *et al.*, 2000). It could be that Dbf4 contains an SH3-like fold that recognizes these motifs. The mutation of all four proline residues to alanines reduced the interaction by more than 50%, suggesting they are important for Dbf4 binding. To apply this finding to the Dbf4-Mcm2 and Cdc7-Mcm4 targeting model proposed earlier, an induced disruption approach was used (similar to Figure 3.6). Mcm4-NLS was overexpressed to titrate Cdc7 in a *mcm2QC3* mutant where the Dbf4-Mcm2 interaction is decreased. Mcm4-NLS overexpression in a Mcm2QC3 background resulted in slight growth defects. The Mcm4 alone may not be sufficient to properly titrate Cdc7, even with the addition of an NLS. Overexpressing the Mcm5 subunit in combination with Mcm4 overexpression could have a better titration effect, since both subunits have shown relatively equal strength in binding to Cdc7 (see Chapter 3).

The analysis of the DDK docking region (amino acids 175-333) of Mcm4 revealed a number of conserved residues that are exposed and clustered together. These residues vary in their chemical properties (hydrophobicity, chemical polarity, charge). Therefore, analyzing combinations of point mutants may be useful in understanding the precise roles of these residues in interacting with Cdc7. The conserved interface residues of Cdc7 are found in the region next to the Kinase-Insert 1 domain, but before Kinase-Insert 2. This region could be part of the potential yeast Cdc7 N-lobe that may interact with Dbf4 motif-C, similar to what was seen in the human DDK structure (Hughes *et al.*, 2012; reviewed in Matthews and Guarné, 2013). Mutating these residues would reveal their potential importance in binding to

Dbf4 and further narrow down essential Dbf4-Cdc7 interface regions for future structural studies.

Chapter 5: Discussion, Main Conclusions and Future Directions

5.1 Interactions between DDK and the MCM complex

The initiation of DNA replication involves a series of coordinated and regulated steps to ensure faithful replication of the genome once per cell cycle. Dbf4-Dependent Kinase (DDK) complex triggers replication initiation in late G1 phase by phosphorylating components of the Mcm2-7 helicase complex. The end goal of this phosphorylation is to recruit other replication factors and form an active replicative helicase to carry out local DNA unwinding for new DNA synthesis. The main physiological targets of DDK are Mcm2, Mcm4 and Mcm6. Many studies have investigated the DDK phosphorylation target sites on the Mcm subunits and its consequences on replication under normal and checkpoint conditions (Sheu and Stillman, 2010; Randell *et al.*, 2010; Stead *et al.*, 2011). Though much is known about DDK function, understanding how DDK is targeted to the MCM complex was unknown and was the focus of this thesis. Investigation of interactions between individual subunits of the MCM complex with DDK subunits revealed that Dbf4 and Cdc7 interact with mutually exclusive subunits of Mcm2-7 helicase. Dbf4 exhibited a strong interaction with Mcm2 and a weaker interaction with Mcm6, whereas Cdc7 interacted with Mcm4 and Mcm5 with relatively equal strength. Further dissection of Mcm2 revealed a DDK docking region within the N-terminal amino acids 2-4 and 10-63. Removal of the Mcm2 DDK docking region in combination with a previously identified Mcm4 DDK Docking domain (amino acids 175-333; Sheu and Stillman, 2006) resulted in synthetic lethality. These results show that disrupting Dbf4-Mcm2 and Cdc7-Mcm4 interactions simultaneously is sufficient to impair DDK targeting to the Mcm2-7 complex. This result was further supported by an induced disruption approach, where Mcm2 overexpression caused growth defects due to its titration of Dbf4 in a background where Cdc7-Mcm4 interactions were already

compromised. Though the overexpression of Mcm4 in a disrupted Dbf4-Mcm2 background did not achieve the same result, this may have been due to the ineffective sequestering of Cdc7 by Mcm4. The lack of a Nuclear Localization Signal (NLS) sequence on Mcm4 could have also been a reason for the lack of a titration effect, however the addition of an NLS to the constructs did not produce appreciably different results.

The lack of growth defects in the Mcm4 overexpression experiments could be due to a number of reasons. Despite the addition of NLS-encoding sequence to the Mcm4 constructs, we cannot be sure that the NLS-tagged proteins efficiently localized to the nucleus. The addition of green fluorescent protein (GFP) tags and detection via fluorescence microscopy could help further confirm the localization. Nuclear fractionation assays could also be used to look at the levels of NLS-tagged Mcm4 proteins in this organelle. Addition of two tandem copies of SV40 NLS sequence to Mcm4 could help to constitutively localize Mcm4 into the nucleus (Nguyen *et al.*, 2000). Given that Cdc7 interacts with both Mcm4 and Mcm5, it makes sense that overexpressing just the Mcm4 subunit may not effectively titrate Cdc7 in the induced disruption approach. Possibly overexpressing both Mcm4 and Mcm5 would improve the sequestering of Cdc7. Simultaneous disruption of Dbf4-Mcm2 and Cdc7-Mcm5 could also be sufficient to impair DDK targeting to the Mcm2-7 complex. Additionally, recent work in the Duncker Lab has shown that the Mcm2 region 1-278, which combines the N-terminal docking region identified in this study and a previously identified Mcm2-binding region (Bruck and Kaplan, 2009), is sufficient for the interaction with Dbf4 (Beaudoin and Duncker, unpublished). This region could be overexpressed in a wild-type

background to see if it would cause growth defects due to the titration of Dbf4. These possibilities will need to be further investigated with experiments.

5.2 Cdc7 and its interaction with Mcm4 and Dbf4

The 11 conserved kinase domains of budding yeast Cdc7 share significant similarity to the kinase domains of higher eukaryotes (reviewed in Masai and Arai, 2002). The kinase-insert domains (involved in protein-protein interactions) however show great variability (reviewed in Masai and Arai, 2002). The recently solved crystal structure of human DDK complex has identified specific domains and residues that participate in the Dbf4-Cdc7 interface regions (Hughes *et al.*, 2012). Currently, precise domains or regions of budding yeast Cdc7 that interact with Dbf4 or Mcm4 are unknown. In this study, several truncated forms of Cdc7 were evaluated for their interaction with Dbf4 and Mcm4. The C-terminal end of Cdc7 (amino acids 453-507) showed overlap in mediating weak binding to both Dbf4 and Mcm4. These interactions were further confirmed when overexpression of this region in a wild-type background caused growth defects, most like due to Mcm4 or Dbf4 sequestering or formation of inactive kinase complexes with Dbf4. The first 54 amino acids of Cdc7 exhibited weak binding to Mcm4, whereas when the region was removed the binding with Dbf4 was maintained. Taken together, Cdc7 N-terminal amino acids 55-124 and C-terminal amino acids 453-507 seem to be the likely target regions of Dbf4 binding. Bioinformatics analysis and alignments have also revealed conserved residues that interface with Dbf4 found within the 55-124 amino acid region of Cdc7. The first 54 amino acids of Cdc7 may be exposed when binding to Dbf4, much like the extreme N-terminal region of human Cdc7 when bound to Dbf4 in the crystal structure (Hughes *et al.*, 2012). Overexpression of Cdc7

55-507 showed severe growth defects most likely due to sequestering of Dbf4, but was unable to complement lack of Cdc7, possibly due to disruption of the kinase function.

The lack of Cdc7 complementation by the Cdc7 55-507 construct could be explored using *in vitro* kinase assays to detect for Mcm4 phosphorylation. Purified Mcm4 protein would be incubated with [γ-^{32}P]ATP and extracts containing Cdc7 55-507 and wild-type Dbf4. The incorporation of radiolabelled phosphate from the ATP into Mcm4 can be detected via separation of proteins on a SDS gel and exposure to X-ray film. If Mcm4 were phosphorylated, it would confirm that the kinase activity is retained in the shortened Cdc7 region. Another possibility is that the removal of the Cdc7 N-terminal region could be affecting the kinase activation instead of the kinase function (catalysis). The Cdc7 region involved in activating the kinase to its functional form may be different from the region that mediates the phosphotransfer for its substrate. Differentiating between these two functions may be difficult as some mutations/deletions may affect both aspects. *In vitro* kinase assays have shown that active Dbf4-Cdc7 complexes demonstrate a gel mobility shift due to autophosphorylation compared to catalytically inactive DDK kinases (Weinreich and Stillman, 1999). This approach could be applied to the Cdc7 55-507-Dbf4 complex. Determining a minimal region of Cdc7 that is still able to retain DDK function could help us to better analyze its interaction with Mcm subunits. In this approach, the complex is in its active form and would more closely represent the native interaction with Mcm4. Future studies could also look at mutating the conserved Dbf4-interface residues within Cdc7 like Threonine 81, Serine 82, Proline 84, Arginine 111, and Aspartic Acid 114 to verify their significance in mediating interactions with Dbf4. Dbf4 motif-C region in the alignment

encompasses a CCHH-type zinc finger which was previously examined by the Duncker Lab (Jones *et al.*, 2010). Mutations to the zinc finger residues in this region did not decrease the interaction with Cdc7 (Jones *et al.*, 2010). Other potential residues that could be mutated include Glutamic acid 630, Histidine 648, Phenylalanine 658, and Aspartic Acid 672. These mutations can be combined previously characterized C-motif mutations and tested for the interaction with Cdc7.

5.3 Evaluating Specific Residues within Mcm2 and Mcm4

Bioinformatics analysis has revealed conserved residues within the DDK docking domains of Mcm2 and Mcm4. Within Mcm2, two protein interaction domains P-x-x-P and P-x-x-x-P were identified, and mutation of all four conserved prolines in these motifs resulted in a more than 50% reduction in interaction with Dbf4. These protein interaction motifs are typically recognized by SH3 motifs found mostly in cytoskeletal and cell signaling molecules (Chandra *et al.*, 2004). The SH3 domain consists of 5 or 6 β-sheets, which fold to form a characteristic β-barrel structure (Wang *et al.*, 2007). The human Dbf4-Cdc7 structural work has shown 5 β-sheets within motif-M and -C to be involved in the interaction with Cdc7 (Hughes *et al.*, 2012). Yeast Dbf4 motif-M and -C could also contain β-sheets that resemble an SH3-like fold that interacts with Mcm2, but this would need to be confirmed with structural analysis. Mutations of more conserved residues within DDK docking region of Mcm2 could further decrease the interaction with Dbf4. Other conserved candidate residues include the Serine 23, Serine 24 or Glutamine 26 located next to the first two prolines. Additionally, Serine 38 and Glutamine 41 located near the second two prolines could also serve as good candidates. Conserved residues could be identified within the extended Mcm2

1-278 region and mutated to check for potential roles in facilitating binding to Dbf4. Bioinformatics analysis of the Mcm4 DDK docking domain (amino acids 175-333) revealed several conserved residues that are exposed and cluster together in 3D space. Though analysis of mutations in these residues using two-hybrid assays was unsuccessful, other protein-interaction methods can be used. Co-immunoprecipitation or GST pull-down assays could be used as alternative methods to examine protein-protein interactions.

5.4 Relevance to Cancer

Given that DDK and Mcm proteins play an essential role in DNA replication process, it would be reasonable to think that deregulation of these factors may lead to cancer phenotypes. Several studies have shown elevated levels of Cdc7 and Dbf4 in tumour cell lines and cancer tissues (Bonte *et al.*, 2008; Kulkarni *et al.*, 2009; Choschzick *et al.*, 2010; Cheng *et al.*, 2013). The critical role of Cdc7 in origin firing makes it an important target for novel cancer therapeutic studies (reviewed in Montagnoli *et al.*, 2010). For instance, PHA-767491 is a potent Cdc7 inhibitor that causes apoptotic cell death in multiple cancer cell types (Montagnoli *et al.*, 2008). Additionally, it has been shown to have an additive effect on myeloma cell death when combined with current chemotherapy drugs (Natoni *et al.*, 2013). An interesting avenue of research would be to investigate if truncated versions of proteins like Cdc7, Dbf4, Mcm2, or Mcm4 could be more selective inhibitors and serve as potential alternatives to chemical compounds. Though the presented data does not focus on cancer, it provides a greater insight into essential protein factors that govern replication initiation and also establishes the groundwork for possible structural studies in the future.

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Appendix A: Chapter 3 Supplementary Material

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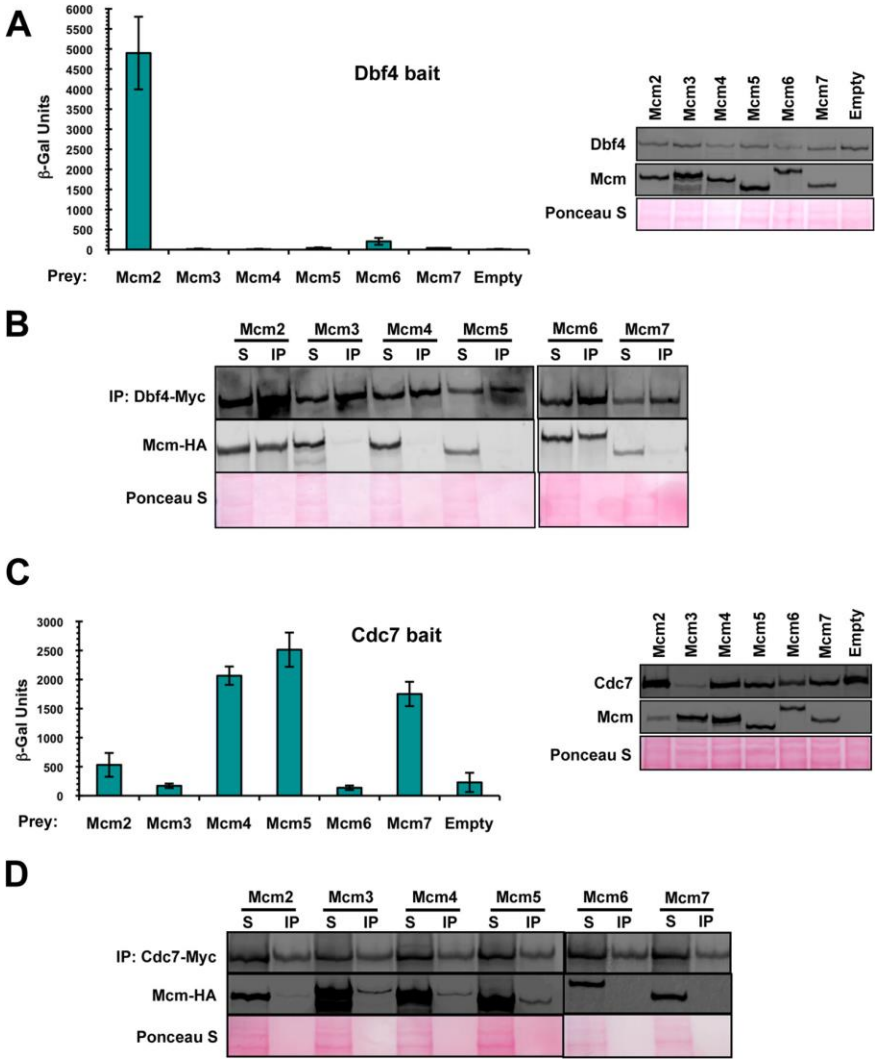
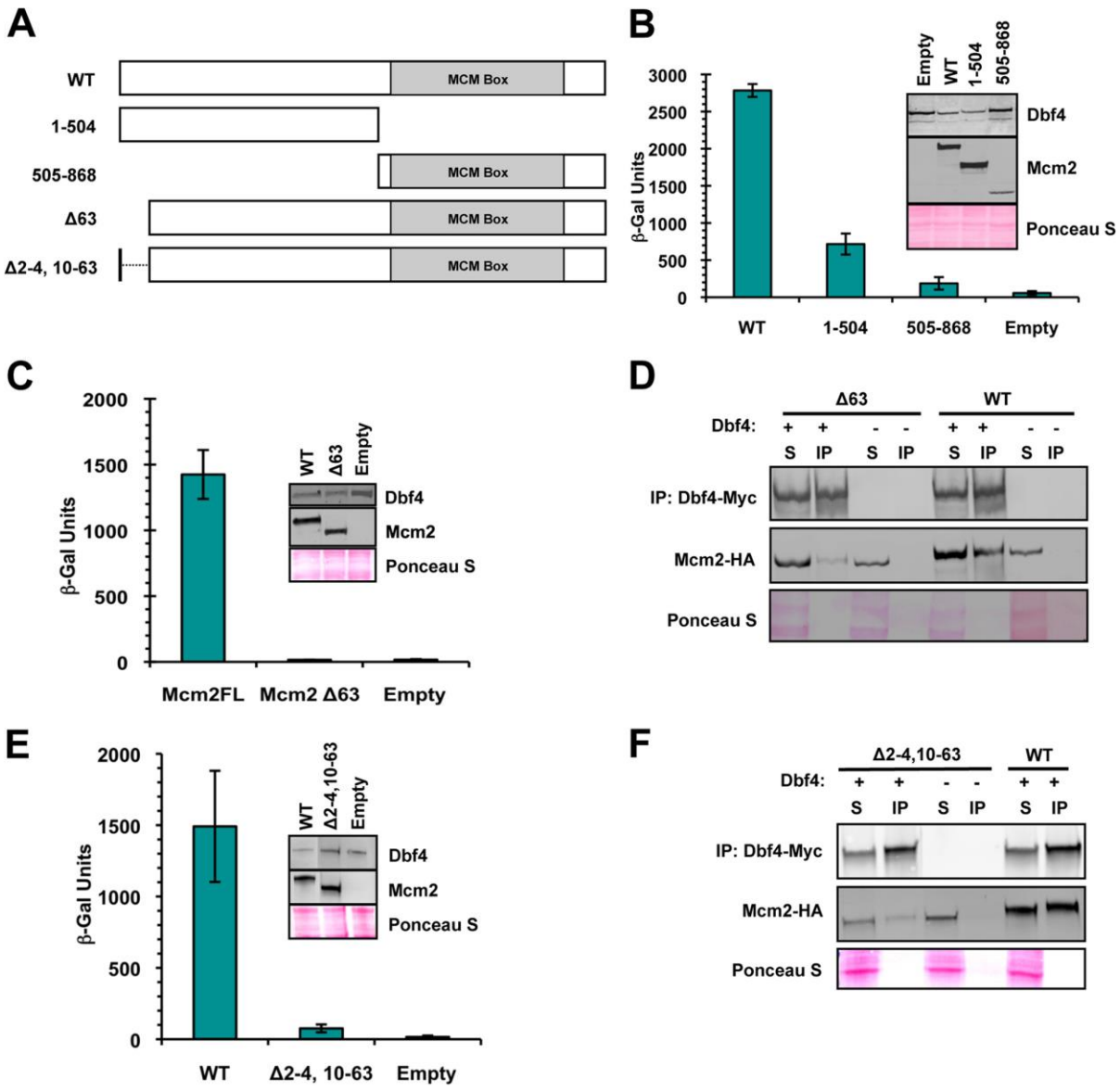
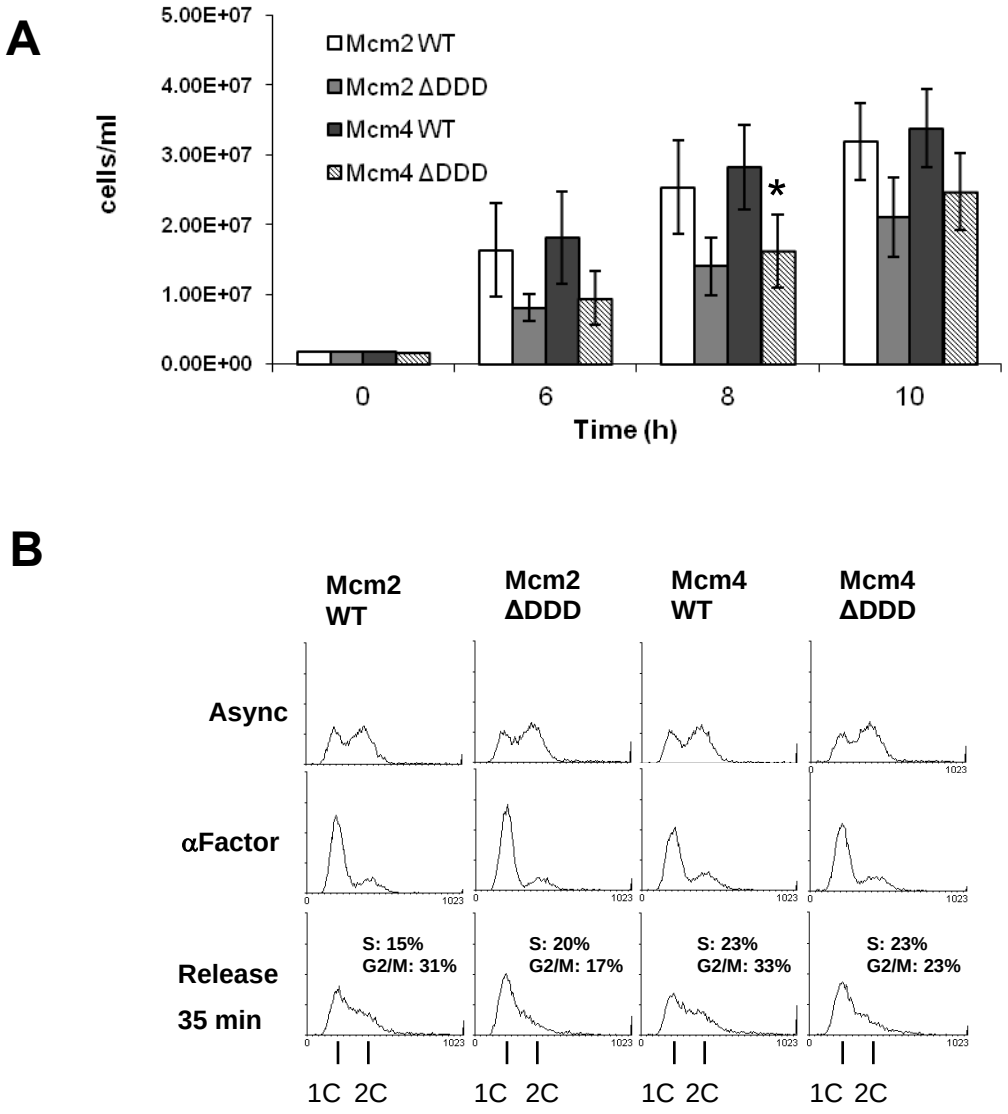
Figure 1, Figure 2A, Figure 2D-F, and Figure 3 contributed by Dr. Matthew Ramer
Figure 2B contributed by Martina Spranger
Figure 2C contributed by Nicole Bieberstein
Figure 4 contributed by Hagen Richter



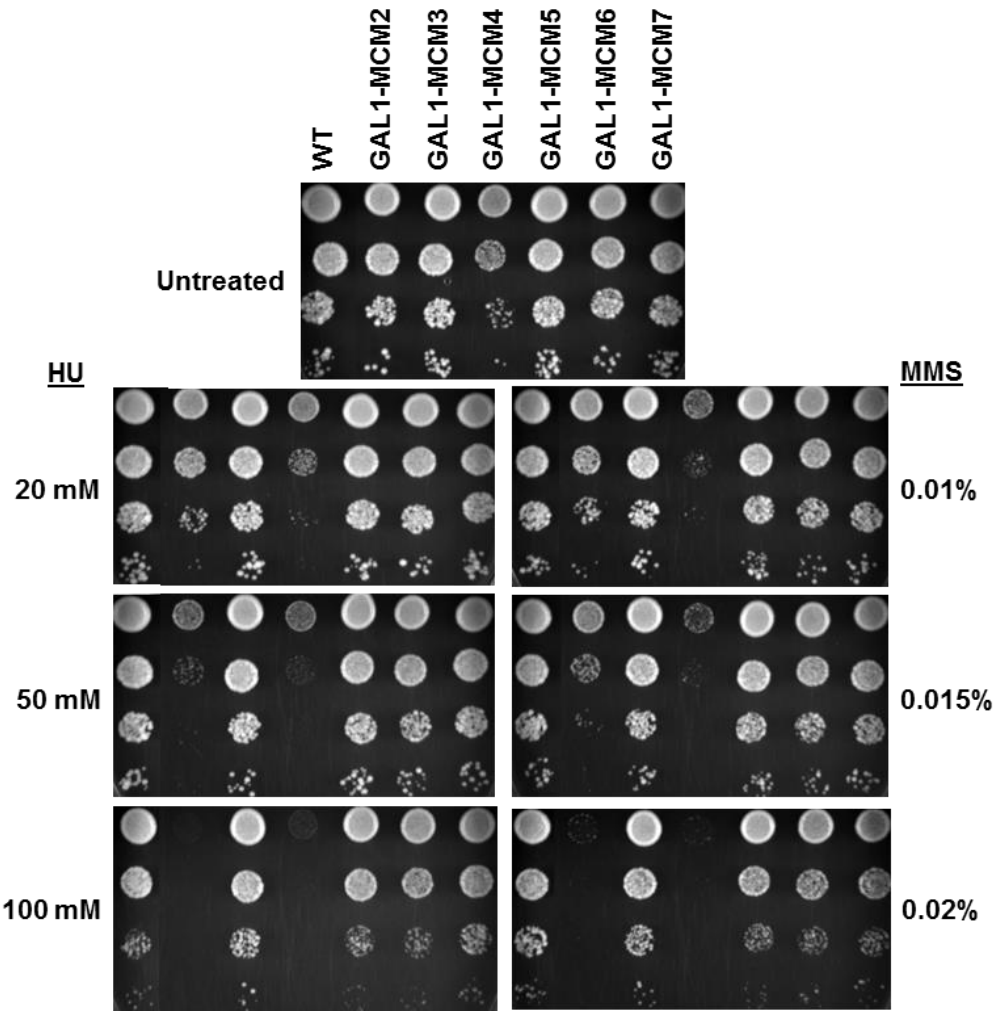
Appendix A Figure 1: Dbf4 and Cdc7 interact with mutually exclusive subunits of Mcm2–7. A and C, two-hybrid assays were carried out using either bait construct pEG-Dbf4 (A) or pEG-Cdc7 (C), whereas pJG-Mcm2, -Mcm3, -Mcm4, -Mcm5, -Mcm6, and -Mcm7 were used as prey constructs, along with pJG4–6 as an empty vector control. Culture aliquots were removed just prior to the measurement of β-galactosidase activity, and whole cell extracts were prepared and subjected to immunoblot analysis to confirm bait and prey expression. The average of three replicates is shown ± S.D. (error bars). Bait proteins were detected with anti-LexA antibodies, and prey proteins were detected with anti-HA antibodies. Ponceau S staining of the membrane was carried out to determine relative sample loading. (B) and (D), immunoprecipitation (IP) of Myc-tagged Dbf4 (B) or Cdc7 (D) was carried out in strains overexpressing HA-tagged Mcm2, Mcm3, Mcm4, Mcm5, Mcm6, or Mcm7. Shown are immunoblots of IP and supernatant (S) fractions detected with anti-HA or anti-Myc antibodies. 20 μg of input and one-fourth of the final bead suspension were loaded for the Dbf4 IP immunoblot, whereas 50 μg of input and one-fourth of the final bead suspension were loaded for the Cdc7 IP immunoblot. Ponceau S staining of the membrane was carried out to determine relative sample loading.



Appendix A Figure 2: An N-terminal Mcm2 region mediates interaction with Dbf4. (A) Mcm2WT and mutant cassettes used in this study. The location of the conserved MCM box, including Walker A, Walker B, and arginine finger motifs, is indicated. (B), (C), and (E), two-hybrid assays carried out using bait construct pEG-Dbf4. pJG-Mcm2 (WT), -Mcm2(1–504), -Mcm2(505–868), -Mcm2Δ63, and -Mcm2Δ2–4,10–63 were used as prey. The average of three replicates is shown ± S.D. (error bars). Immunoblot analyses to verify bait and prey expression were carried out as described for Fig. 3.1 (D) and (F), immunoprecipitation (IP) of Myc-tagged Dbf4. Shown are immunoblots of IP and supernatant (S) fractions detected with monoclonal anti-HA (Mcm2 detection) and anti-Myc antibodies (Dbf4 detection). 20 μg of input and one-fourth of the final bead suspension were loaded.



Appendix A Figure 3: Mcm2ΔDDD and Mcm4ΔDDD mutants have modest growth defects. CEN/ARS plasmid constructs YCplac111-Mcm2WT and -Mcm2ΔDDD were used to support growth in *mcm2::HIS3* background, whereas YCplac111-Mcm4WT and -Mcm4ΔDDD were used to support growth in a *mcm4::KanMX* background. (A) Cultures were grown in selective media and cell concentrations determined at the indicated time points. The average of three replicates is shown ± S.D. (*error bars*). The asterisk indicates a significant difference between Mcm4ΔDDD and Mcm4WT at the 8 h time point (paired Student's *t* test, $p < 0.05$). (B) Asynchronous (Async) cultures were arrested in α-factor (30 μg/ml) for 2.5 h followed by release into pheromone-free medium containing 50 μg/ml Pronase E (Sigma) with samples taken for FACS analysis. Areas representing S and G2/M phase cells for the released cells were determined using the WinMDI program and then converted to percentages of total cells in each sample as indicated.

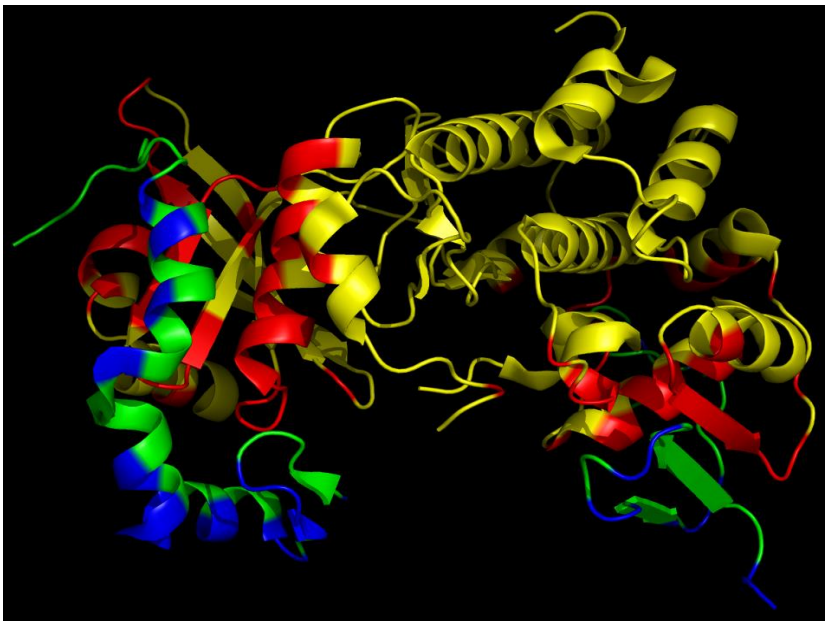


Appendix A Figure 4: Constitutive overexpression of Mcm2 or Mcm4 imparts sensitivity to genotoxic agents. Yeast strains were generated in which the endogenous promoter for genes encoding each of the Mcm2–7 subunits was individually replaced with the *GAL1* promoter. 10-fold serial dilutions of each *GAL1-MCM* overexpression strain were plated on YPG/R (2% galactose, 1% raffinose) containing the indicated concentrations of HU or MMS, at a starting concentration of $1x10^7$ cells/ml, and grown for 4 days at 30°C.

Appendix B: Chapter 4 Supplementary Material

Figure 1 contributed by Shaundrei Espiritu

A



B

Cdc7:

```
H.sapiens       89  LKHLIPTSHPIRIAAELQCLTVAGGQDNVMGVKYCFRKNDHVVIAMPYLEHESFLDILNS  148
                    LK  + TS P RI  EL  L +  G   V     + R  D V+  +PY  HE F
S.cerevisiae    75  LKKIYVTSSPQRIYNELNLLYIMTGSSRVAPLCDAKRVRDQVIAVLPYYPHEEFRTFYRD  134
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Dbf4:

```
H.sapiens       288  KEKKKKGYCECCLQKYEDLETHLLSEQHLSFAENDLNFEAIDSLIENLRF  336
                     +  K  GYCE C  KYE LE H++SE+H +FA+++  ++ +D ++  L F
S.cerevisiae    621  ETVKNSGYCENCRVKYESLEQHIVSEKHLSFAENDLNFEAIDSLIENLRF  680
```

Appendix B Figure 1: Bioinformatics analysis of Cdc7-Dbf4 Interface Residues. The Cdc7-Dbf4 interface residues were determined using a python script. (A) A human Cdc7-Dbf4 interface structure was generated using PyMol. Yellow/red represents Cdc7 amino acid region 89-148 and blue/green represents Dbf4 amino acids 288-336. Cdc7 sequences from human, chicken, fish, frog, yeast, and plant were chosen. The human Cdc7 and Dbf4 protein sequence was BLASTed to find homologs in each of these species. A multiple sequence alignment of Cdc7 and Dbf4 sequences was generated using MUSCLE (default parameters) as implemented in SeaView. Jalview was used to look at the MSA, reduce redundancy (if necessary) and check for conservation. The interface residues of Cdc7 and Dbf4, initially identified using the python script were checked for conservation among the species in the MSA. The Cdc7 residues were manually checked in the PyMol generated 3-D structure to see if they interact with residues of Dbf4, and vice versa. (B) Alignments of yeast and human Cdc7 and Dbf4 sequences revealed conserved interface residues (highlighted in red in both alignments). Residues of Cdc7 highlighted in a colour other than red are ones that show clear contacts with the same colour residue in the Dbf4 sequence (i.e. highlighted blue residue in Cdc7 shows contact with blue residue in Dbf4).