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The role of the Mcm2 subunit in regulating the activities of the Mcm2-7 helicase

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Biochemistry

The role of the Mcm2 subunit in regulating the activities of the Mcm2-7 helicase

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ABSTRACT

The transmission of genetic information from parental to daughter cells requires the faithful duplication of an organism's genome. Uncontrolled DNA replication can result in proliferative diseases, such as cancer. DNA replication requires a single-stranded DNA template to be produced from duplex DNA. In eukaryotes, DNA unwinding for replication is performed by the heterohexameric replicative helicase complex comprised of the minichromosome maintenance proteins 2 through 7.

Each of the Mcm2-7 subunits likely has a unique role in DNA binding and unwinding by the Mcm2-7 complex. The present study examines the role of the *Saccharomyces cerevisiae* Mcm2 subunit in regulating the activities of Mcm complexes. Using *in vitro* assays for DNA unwinding and DNA binding with *E. coli*-purified Mcm subunits, this work demonstrates that Mcm2 requires nucleotide to actively regulate DNA binding and unwinding by Mcm complexes. These studies define Mcm2 as an active regulatory subunit within the Mcm2-7 complex.

Mcm2-7 is also targeted by various kinases that presumably modulate its activities. The Dbf4-dependent kinase (DDK) comprised of the Cdc7 catalytic subunit and Dbf4 regulatory subunit is one such kinase. Here, the residues of Mcm2 targeted for phosphorylation by DDK were mapped using recombinant proteins and verified in cells. The effects of phosphomimetic substitutions at these positions on the activities of the Mcm2-7 complex were examined. Interestingly, the ATPase activity of Mcm2 of the phosphomimetic Mcm2 is lower compared to wild type. A version of Mcm2-7 containing the phopshomimetic mutant of Mcm2 had a higher affinity for DNA, which in turn inhibited DNA unwinding by the complex. The biological function of phosphorylation of Mcm2 by DDK in budding yeast was also examined using cells containing a version of *mcm2* that cannot be phosphorylated by DDK. This mutation rendered the cells sensitive to agents that cause DNA base damage. Additionally, the mutant allele interacted with genes involved in the DNA damage checkpoint as determined by synthetic genetic array analysis. In sum, a model in which DDK-dependent phosphorylation of Mcm2 regulates its ATPase activity to slow replication forks in the cell's response to DNA damage is proposed.

Keywords: Mcm2, Mcm2-7, helicase, Cdc7, Dbf4, DDK, ATPase, DNA replication, kinase, phosphorylation, cell stress, DNA damage, DNA damage response, checkpoint, budding yeast.

CO-AUTHORSHIP STATEMENT

The following thesis contains material from a previously published manuscript. Megan Davey is a co-author on all materials presented here and was responsible for supervising Brent Stead during his thesis. For all chapters in this thesis, Brent Stead wrote the draft and Megan Davey edited the manuscripts. Christopher Brandl is also an author on the works presented in Chapters 2, 3 and 4.

Brent Stead carried out all the experiments described in this thesis, prepared all the figures and tables with the following exceptions. Xiaoli Ma purified many of the wild type proteins and complexes used in these studies. Catherine D. Sorbara cloned and purified the mutant versions of Mcm3 and Mcm5 used in Chapter 2. Christopher Brandl carried out all tetrad dissections to generate strains in Chapters 2 and 3 and in the verification of the SGA analysis in Chapter 4. Matthew Sandre contributed to analyzing the phenotypes of dissected tetrads in Chapter 4.

DEDICATION

This thesis is dedicated to my mother. Her love and support have made it possible to realize my dreams.

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This thesis would not have been possible with the supervision, guidance and support of my supervisor Megan Davey over the past five years. My sincerest thanks for all the thought-provoking discussions and assistance with all aspects of my project.

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LIST OF ABBREVIATIONS AND NOMECLATURE

µCi	Microcurie
µg, mg	Microgram, milligram
µl, ml	Microlitre, millilitre
µM, mM	Micromolar, millimolar
5-FOA	5-Fluorootic Acid
5-FU	5-fluorouracil
AAA+	ATPases associated with a variety of cellular activities
ADP	Adenosine diphosphate
ARS	Autonomously replicating sequence
ATM	Ataxia telangiectasia, mutated
ATP	Adenosine triphosphate
ATPγS	Adenosine 5′-[γ-thio]triphosphate
ATR	ATM and Rad3-related
BER	Base excision repair
bp	Base pair
BPV	Bovine papilloma virus
BSA	Bovine serum albumin
Cdc	Cell-division cycle
CDK	Cyclin-dependent kinase
Cdt	Cdc10-dependent transcript 1
CEN	Centromeric
Chk	Checkpoint kinase
CK2	Casein kinase 2
CMG	Cdc45-Mcm2-7-GINS
cpm	Counts per minute
Ctf	Chromosome transmission fidelity
Dbf	DumbBell Forming
Dbp	DNA Polymerase B
Ddc	DNA Damage Checkpoint
DDK	Dbf4-dependent kinase

DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotides
DSB	Double strand break
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
EXT	Exterior
G1	Gap 1
G2	Gap 2
Gal	Galactose
GINS	Go, Ichi, Nii, and San; five, one, two, and three in Japanese
Glu	Glutamic acid
GO	Gene Ontology
HeLa	Human cervical cancer cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HR	Homolgous recombination
HRP	Horseradish peroxidase
HU	Hydroxyurea
kDa	Kilodalton
Leu	Leucine
mA	Milliamps
MALDI	Matrix-assisted laser desorption/ionization
Mat	Mating type
Mcm	Minichromosome maintenance
Mec	Mitosis entry checkpoint
MMS	Methyl methanesulfonate
Mrc	Mediator of the Replication Checkpoint
mtMCM	Mcm complex from *Methanobacterium thermoautotrophicum*
NP-40	Nonyl phenoxypolyethoxylethanol
NT	N-terminal

ORI	Origin
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein Kinase A
P-loop	Phosphate binding loop
pmol	Picomole
Pre-IC	Pre-initiation complex
Pre-RC	Pre-replicative complex
PS1	Pre-sensor 1
Psf	Partner of Sld Five
Rad	Radiation sensitive
RNR	Ribonucleotide reductase
ROS	Reactive oxygen species
RPA	Replication protein A
S phase	Synthesis phase
SDS	Sodium dodecyl sulphate
Sld	Synthetic lethal with Dbp11
Spo	Sporulation
ssDNA	Single-stranded DNA
SSL	Synthetic sick or lethal
SsoMCM	Mcm complex from *Sulfolobus solfataricus*
SV40 LTAg	Simian virus 40 large T-antigen
Tel	Telomere maintenance
Tor	Target of rapamycin
Tris	tris(hydroxymethyl)aminomethane
Ura	Uracil
UTR	Untranslated region
V	Volts
YPD	Yeast Peptone Dextrose
YPGal	Yeast Peptone Galactose

CHAPTER 1 INTRODUCTION

1.1 DNA replication in eukaryotes

The cell cycle is comprised of a series of events that culminates in the division of a cell into two daughter cells. A cell's genome is duplicated prior to division such that the genetic information is passed from the parental to the daughter cells[1]. The cell cycle is subdivided into the interphase, during which the cell acquires nutrients and duplicates its genome, and the mitosis phase when cell division occurs (the yeast cell cycle is reviewed in ref. 2). The interphase is further subdivided into the Gap 1 (G1), Synthesis (S) and Gap 2 (G2) phases (Figure 1-1; ref. 2). It is during the synthesis phase that DNA replication occurs. Studies in budding yeast have provided invaluable information about the progression of the cell cycle. Indeed, the cell division cycle (Cdc) factors that control cell cycle progression were first characterized in budding yeast by Leland Hartwell[3]. He later shared a Nobel Prize in Medicine with Paul Nurse and Timothy Hunt for their work on the control of the cell cycle.

DNA replication is a tightly controlled process that faithfully duplicates an organism's genome (reviewed in refs. 4; 5; 6). Aberrations in DNA replication can alter cell proliferation and result in disease states such as cancer[7; 8; 9]. A complex series of protein-protein and protein-DNA interactions, as well as post-translational modifications of replication proteins is required for the accurate duplication of the DNA[4; 5; 6]. Checkpoints in the cell cycle also dictate the competency of the cell for DNA replication[10]. Additionally, throughout DNA replication, factors are constantly surveying for damage to the DNA which ultimately triggers elaborate mechanisms to halt DNA replication, repair the damage and resume DNA replication after repair[10]. All these levels of control are potential points to regulate the activities of the replication machinery.

During G1 phase of the cell cycle (Figure 1-1), replication factors assemble sequentially at DNA sequences termed origins of replication (origins; reviewed in refs. 11; 12; 13). Origins are said to be "licensed" for replication by the ordered assembly of the pre-replication complex (pre-RC; Figure 1-1). Licensing of origins requires the stable association of replication licensing factors, and cells will not initiate DNA replication at an origin if it is not licensed[14; 15]. First, the origin-recognition complex (ORC) comprised of the proteins Orc1 through 6 recognizes and binds to origins of replication[16]. The DNA sequences at origins in most eukaryotes lack any consensus, except for being A/T-rich[17; 18]. Origins in the budding yeast *Saccharomyces cerevisiae* however have well-defined autonomously-replicating sequences (ARS), so named for their ability to initiate DNA replication of exogenous DNA that otherwise lack replication origins[16; 19; 20; 21]. After ORC has bound to origins, Cdc6 is recruited. These factors assist in the final step of pre-RC assembly, which is the binding of the heterohexameric minichromosome maintenance (Mcm) 2-7 complex and the essential pre-RC component Cdt1[22]. After Mcm2-7 has bound, origins are licensed and ready for initiation[14; 15].

After assembly of the pre-RC, several factors are recruited to origins to form the pre-initiation complex (pre-IC; Figure 1-1). The essential serine/threonine kinase DDK (Dbf4-dependent kinase) comprised of the Cdc7 active subunit and Dbf4 regulatory subunit phosphorylates several subunits of the origin-bound Mcm2-7 complex[23; 24; 25; 26; 27; 28; 29; 30; 31]. This process is discussed in greater detail below. After modification of Mcm2-7 by DDK, the essential replication factor Cdc45 and a heterotetrameric complex comprised of Sld5p, Psf1p, Psf2p and Psf3p, termed the GINS complex (for the Japanese words for 5, 1, 2, 3; go ishi ni san) associate[32; 33; 34]. Together, the complex of Cdc45, the

Mcm2-7 complex and the GINS complex (also known as CMG) are believed to form the helicase holoenzyme and participate in DNA unwinding in eukaryotic cells[35]. Consistent with its role in unwinding DNA in cells, the budding yeast Mcm2-7 complex unwinds DNA *in vitro*[36]. Furthermore, association of Cdc45 and GINS activates the helicase activity of *Drosophila* Mcm2-7 *in vitro*[37]. Sld2 and Sld3 are also part of the pre-IC and are phosphorylated by the cyclin dependent kinase (CDK) which facilitates the recruitment of Dpb11[38; 39]. Dpb11 assists in recruiting CMG and polymerase ε[13; 38; 39; 40]. The recruitment of polymerase ε on the leading strand and polymerase δ on the lagging strand (among other factors) begins the S phase and polymerase δ and ε travel with advancing replication forks to synthesize the daughter strands of DNA (reviewed in ref. 41). DNA that is unwound during replication is stabilized by replication protein A (RPA), the eukaryotic equivalent of the bacterial single stranded binding protein[42].

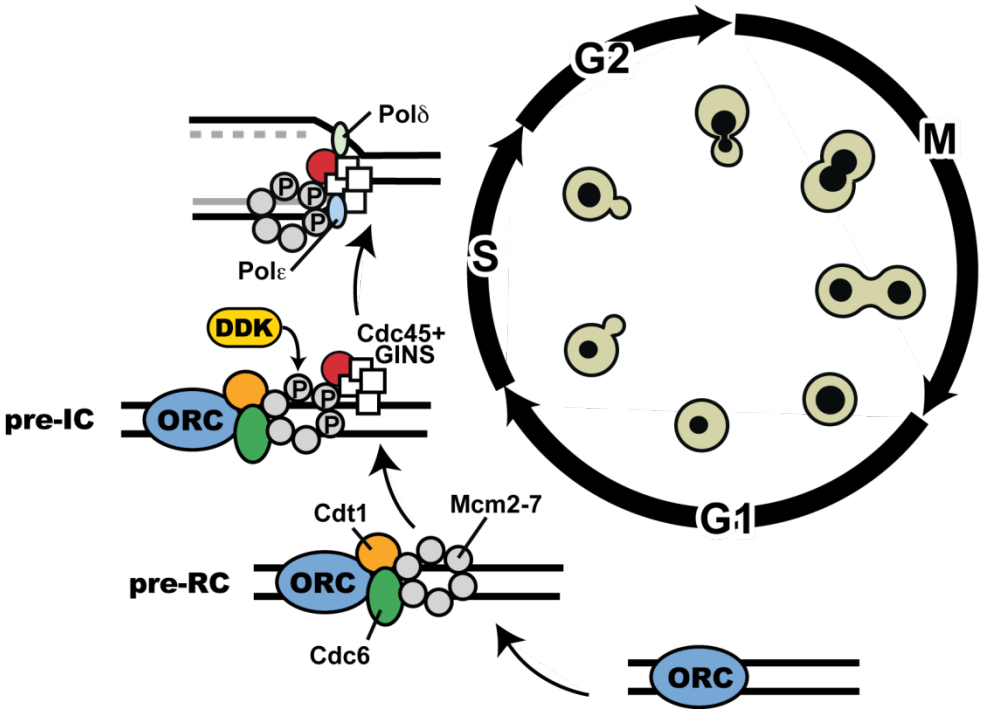


Figure 1-1 Assembly of the complexes necessary for DNA replication during the cell cycle of *Saccharomyces cerevisiae.* Depicted are the yeast cell cycle and the morphology of the budding yeast cell associated with each phase of the cycle. Details of the phases are provided in the main text. To the left are illustrations of the complexes involved in DNA replication associated with the stages of the cell cycle. Details of the constituents of each complex can be found in the main text. Some factors described in the main text are omitted for clarity of the figure.

1.2 Mechanisms of the minichromosome maintenance proteins 2-7

The minichromosome maintenance proteins 2 through 7 are members of the AAA+ (ATPases associated with a variety of cellular activities) superfamily of proteins. Members of this family convert the energy of ATP hydrolysis into mechanical force via conformational changes. Thus, as a helicase, Mcm2-7 couples the energy of ATP binding and hydrolysis to the mechanical force of unwinding duplex DNA during DNA replication. Consistent with the role of Mcm2-7 as the replicative helicase, each subunit of Mcm2 through 7 associates with origins of replication, is required for replication fork

progression and, additionally, complexes containing Mcms unwind DNA *in vitro*[36; 43; 44; 45; 46; 47; 48].

1.2.1 ATP site architecture of Mcms and related helicases

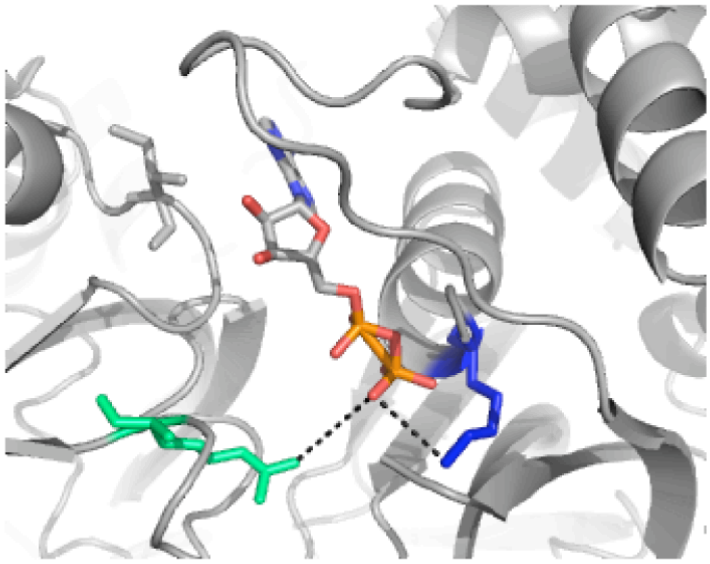
The ATP site of the Mcm proteins is found at the interface between subunits, a common feature of the AAA+ superfamily of proteins. One subunit binds nucleotide, including the phosphates by the phosphate binding loop (P-loop) and the other subunit provides residues important for the hydrolysis of the bound nucleotide[49; 50]. Figure 1-2A shows an example of the subunit interface between the Mcm2 and Mcm6 subunits. In this pair the Mcm2 subunit binds the nucleotide and the Mcm6 subunit contains the residues important for catalysis of the bound nucleotide, and these residues are also termed the "SRF motif" or arginine finger. In Archaea the replicative helicase is typically comprised of a complex of several copies of identical MCM subunits and has been well studied in *Methanobacterium thermoautotrophicum* (mtMCM) and *Sulfolobus solfataricus* (SsoMCM)[51; 52; 53; 54; 55; 56]. The structure of another AAA+ replicative helicase from the bovine papillomavirus (BPV), the E1 helicase, was solved as a hexamer in the presence of nucleotide and provides a structural view of a subunit interface[57]. Highlighted in Figure 1-2B is a conserved lysine of the P-loop (blue) and conserved arginine in the SRF motif (green) and ADP bound at the subunit interface. The amino acid sequences of the P-loop and SRF motif of AAA+ proteins are similar. Shown in Figure 1-2C is an alignment of the ATP sites of Mcm2 through 7 from *S. cerevisiae* and mtMCM and SsoMCM. Found within the P-loop of each Mcm subunit is an invariant lysine residue that is highlighted in the sequence alignment (Figure 1-2C). This lysine is analogous to the lysine highlighted in Figure 1-2B. The importance of this residue for Mcm function is evident in the fact that

substitution of this lysine with an alanine in any of the Mcm subunits is lethal in the organism *S. cerevisiae*[58]. Also highlighted in Figure 1-2C is the arginine of the SRF motif discussed above. A model of the subunit arrangement of Mcm2-7 is derived from the observed ATPase pairs, physical interaction of Mcm subunits and mutational studies (Figure 1-2D; refs. 36; 49; 50; 59).

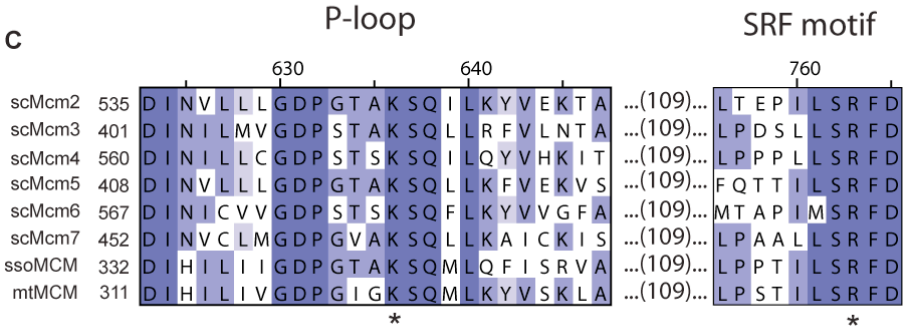
A


2
P-loop
SRF
6

B



C


P-loop
SRF motif
630
640
760
scMcm2 535 D I N V L L L G D P G T A K S Q I L K Y V E K T A ...(109)... L T E P I L S R F D
scMcm3 401 D I N I L M V G D P S T A K S Q L L R F V L N T A ...(109)... L P D S L L S R F D
scMcm4 560 D I N I L L C G D P S T S K S Q I L Q Y V H K I T ...(109)... L P P P L L S R F D
scMcm5 408 D I N V L L L G D P G T A K S Q L L K F V E K V S ...(109)... F Q T T I L S R F D
scMcm6 567 D I N I C V V G D P S T S K S Q F L K Y V V G F A ...(109)... M T A P I M S R F D
scMcm7 452 D I N V C L M G D P G V A K S Q L L K A I C K I S ...(109)... L P A A L L S R F D
ssoMCM 332 D I H I L I I G D P G T A K S Q M L Q F I S R V A ...(109)... L P P T I L S R F D
mtMCM 311 D I H I L I V G D P G I G K S Q M L K Y V S K L A ...(109)... L P S T I L S R F D
*
*

D


3
5
7
2
4
6

Figure 1-2 The ATP sites of replicative helicases and subunit arrangement of Mcm2-7. **A.** The ATP sites of Mcms are found at subunit interfaces. Depicted is one such interface formed by Mcm2 and Mcm6. One subunit (Mcm2) contains a phosphate binding loop (P-loop) and the other subunit (Mcm6) contains residues important for catalysis of the bound nucleotide (SRF motif). **B.** Crystal structure of the ATP site at a subunit interface in the BPV E1 hexamer (PDB ID=2GXA). Highlighted in green is a conserved lysine (K439) in the P-loop and in blue a conserved arginine (R538) in the SRF motif of a neighbouring subunit. **C.** A partial protein sequence alignment encompassing the P-loop and SRF motifs of budding yeast (sc) Mcm2 through 7 and the MCM subunits from the archaea *Methanobacterium thermoautotrophicum* (mtMCM) and *Sulfolobus solfataricus* (ssoMCM) is shown. Highlighted below the alignment (*) are two key residues in each motif, a conserved lysine in the P-loop and a conserved arginine, often termed the arginine finger, in the SRF motif. **D.** The subunit arrangement of the Mcm2-7 heterohexameric complex (C-terminal view) developed from mutational studies of the ATP sites of Mcms. Figure modified from ref. 49.

1.2.2 DNA binding and unwinding by helicases

The available x-ray crystallographic structures of the simian virus 40 large T-antigen (SV40 LTag), BPV E1 helicase, mtMCM and SsoMCM, coupled with biochemical analyses, have provided a great deal of insight into residues important for DNA binding and unwinding. In archaeal MCMs, residues in both the N- and C-termini make contacts with DNA. These residues form β-hairpin structural motifs (two anti-parallel β-strands connected by a linker of amino acids) and are highlighted in the crystal structure of near full-length mtMCM in Figure 1-3A[60]. In the archaeal MCMs, the β-hairpins make distinct contributions to DNA binding and unwinding. There are four β-hairpins in the SsoMCM crystal structure thought to be involved in DNA binding and unwinding: two N-terminal β-hairpins (Figure 1-3A; NT and H2I hairpins) and two C-terminal β-hairpins (Figure 1-3A; EXT and PS1 hairpins). Alignment of the protein sequences of the archaeal MCMs with those of the eukaryotic Mcm subunits reveals that the amino acid sequences of the H2I, EXT and PS1 hairpins share similarities, suggesting this mechanism of DNA binding is common among all Mcms (Figure 1-3B; ref. 61). The

NT hairpin sequence does not align well, but structural predictions suggest its existence in Mcms[62]. In the structures of the SV40 LTag helicase (also solved as a hexamer; Figure 1-3C) and BPV E1 helicase complex (Figure 1-3D), one can see the central channel of the hexamer into which the PS1 hairpins protrude (red). The structure of BPV E1 was solved in the presence of single stranded DNA (green; Figure 1-3D). The contributions of the PS1 hairpin to DNA unwinding are discussed below.

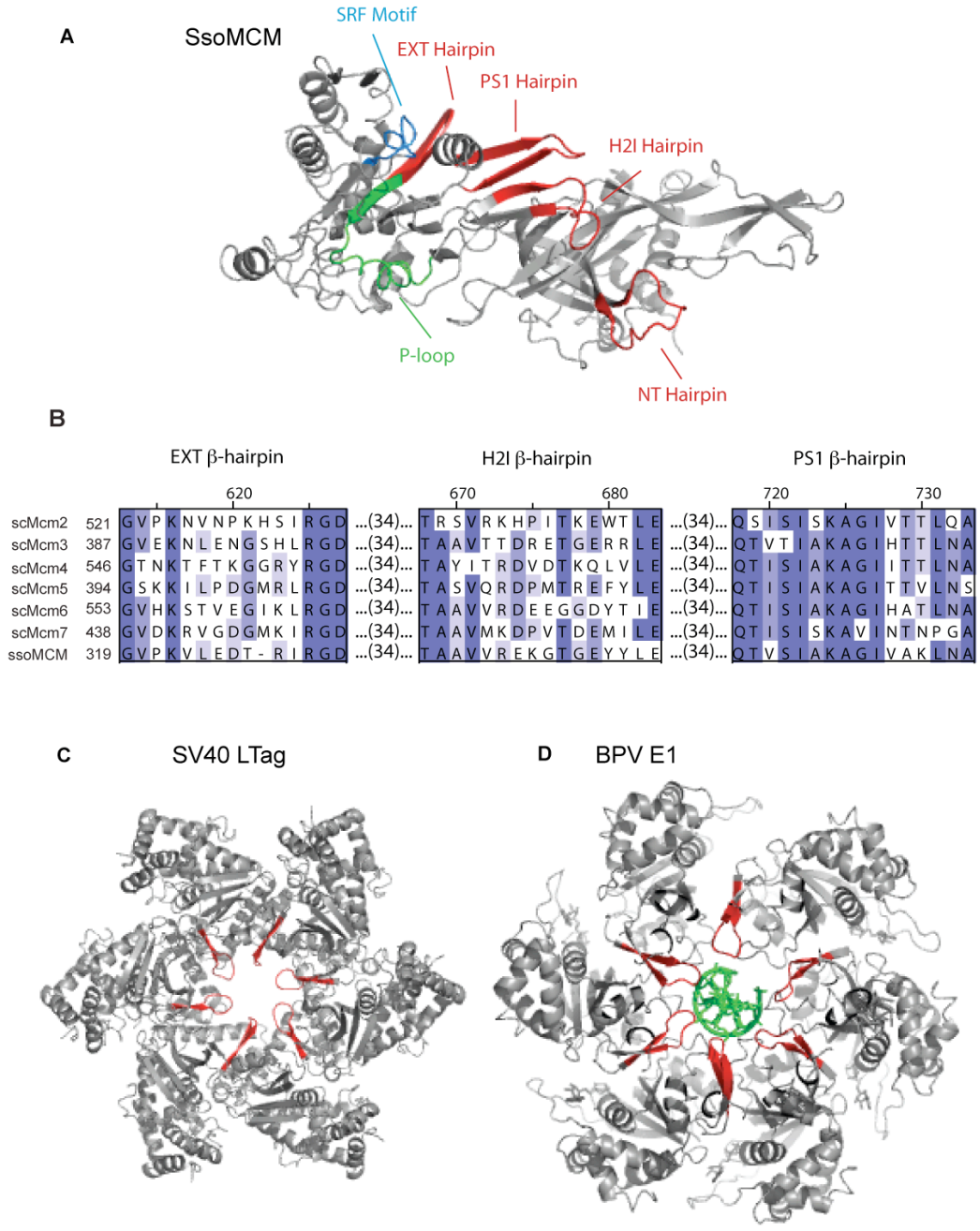


Figure 1-3 The β-hairpins and ATP site in the X-ray crystallographic structure of SsoMCM and hexamers of SV40 LTag and BPV E1. A. The structure of near full-length SsoMCM (PDB ID=3F9V). Highlighted are key structural features: the P-loop (green), the SRF motif (blue) and the various hairpin structures that contact DNA (red; EXT= external, PS=pre-sensor, NT=N-terminal, H2I=helix 2-insert). **B.** The structure of the helicase domain of the SV40 large T-antigen (PDB ID=1SVM). Highlighted in red are the PS1 β-hairpins. **C.** The structure of the E1 helicase was solved in the presence of ssDNA (PDB ID=2GXA). Highlighted in red are the PS1 β-hairpins located in the central channel of the helicase in close proximity to the bound DNA (green).

The additional β-hairpins observed in the crystal structures of SsoMCM also have roles in DNA binding and unwinding. The helix-2-insert hairpin (H2I) is predicted to protrude the furthest into the central channel in models of the hexameric structure of SsoMCM and is thus positioned close to DNA[60]. The position of the H2I hairpin is likely reflective of its critical role in DNA binding and DNA unwinding in the closely related archaeal mtMCM complex[63]. Curiously, mutations of the H2I β-hairpin increase the affinity of the mtMCM complex for ssDNA and dsDNA, but decrease DNA unwinding by the complex[63]. Recently, mutational studies of the EXT hairpin have also been shown to affect DNA binding and unwinding[64]. The NT hairpin also projects into the central channel of SsoMCM and mtMCM[54; 55]. Mutations of the NT-hairpin proportionally decrease DNA binding and unwinding by SsoMCM[54]. Structure-guided alignments predict that the NT hairpin exists in Mcm4 and Mcm5[62]. Mutational studies of the NT hairpins in Mcm4 and Mcm5 demonstrate the requirement for this motif in origin binding[62].

1.2.3 Translocation of helicases

Evidence of movement of the domain containing the PS1 β-hairpins with respect to DNA being coupled to the hydrolysis of ATP is found in studies of the structure of the homohexameric E1 helicase of the bovine papillomavirus[57; 65; 66]. The position of the PS1 β-hairpin with respect to DNA is dependent on the ATP-binding status of a subunit[57]. The domains containing the PS1 β-hairpins of the BPV E1 hexamer had different positions relative to the DNA sequentially around the ring as the subunits varied from the ATP, ADP and apo forms successively around the hexameric ring[57]. One can envision these conformations like a spiral staircase in the central channel of the hexamers. Likewise, the

structures of the SV40 LTag in the ATP, ADP and apo form also demonstrate the movement of the domain containing the PS1 β-hairpins when bound to nucleotide[67; 68]. A concerted movement of the PS1 β-hairpins in response to ATP hydrolysis in the ring has been proposed to "sweep" ssDNA through the central channel of this helicase.

1.2.4 Models of DNA binding by eukaryotic Mcms

Mcm complexes binding to DNA is a necessary prelude to DNA unwinding. Complexes of Mcms, like other ring-shaped helicases, are proposed to bind DNA by encircling it within a central channel. Mcm4/6/7 likely uses a "ring-maker" mechanism of DNA binding[69]. In a ring-maker mechanism, the hexameric helicase forms around the DNA from smaller oligomers[70]. Mcm4/6/7 is thought to be a trimer in the absence of ATP. In the presence of ATP, a shift to hexamer-sized Mcm4/6/7 promotes the binding to linear or circular single stranded DNA (Figure 1-4A; ref. 69). In contrast, incubation of Mcm4/6/7 with ATP to promote formation of hexamers before incubation with DNA inhibits the ability of the complex to bind to single stranded DNA[69]. A ring-breaker mechanism for DNA binding by Mcm2-7 has been proposed[71; 72] in which a transient gap forms in the helicase to allow passage of DNA into the central channel[70]. A putative 'gate' at the Mcm2/5 interface opens to allow the passage of single stranded DNA into the central channel of Mcm2-7 (Figure 1-4B). Mutational analysis at the Mcm2/5 interface demonstrates that mutation of the P-loop of Mcm5 opens the gate whereas a mutation of the Mcm2 SRF motif closes the gate[36]. The ATPase activity of neighbouring subunits is proposed to influence the opening and closing of the gate, including hydrolysis at the Mcm2/6 and Mcm3/5 interfaces[71].

The pre-RC components ORC, Cdc6 and Cdt1 influence the oligomeric structure and DNA binding properties of Mcm2-7[73; 74]. Mcm2-7 exists as a hexamer in solution, but incubation with the other pre-RC components and origin DNA results in two Mcm2-7 hexamers loaded head-to-head by their N-terminal faces[73; 74]. The double hexamer can slide along DNA, but does not do so directionally[73]. Since replication forks are bidirectional, it is speculated that once activation by DDK and association of the pre-IC components occurs, single hexamers may unwind DNA. Alternatively, DNA unwinding may be performed by double hexamers and this model is discussed in greater detail below.

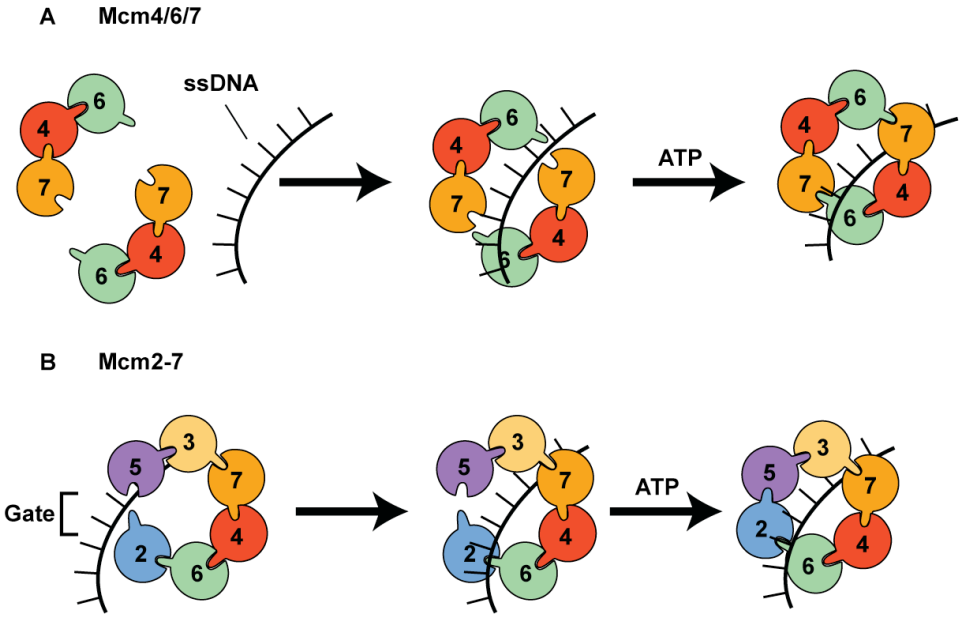


Figure 1-4 Models of DNA binding by eukaryotic Mcm complexes A. The proposed "ring-maker" mechanism used by Mcm4/6/7 to bind DNA. Shown is the putative the ATP-dependent transition of trimers to hexamers which likely promotes binding of DNA in the central channel of Mcm4/6/7. **B.** The proposed ring-breaker or 'gate' mechanism used by Mcm2-7 to bind DNA. A putative gate between the Mcm2/5 subunit interface allows the passage of DNA into the central channel of Mcm2-7. ATP hydrolysis by neighbouring subunits influences the activity of the gate[71].

1.2.5 Mechanisms of DNA unwinding by Mcm2-7 and other helicases

DNA unwinding by complexes of Mcm proteins was first noted with a heterohexameric sub-complex comprised of Mcm4, Mcm6 and Mcm7 originally isolated from HeLa cells[47]. The Mcm4/6/7 complex has proved a valuable tool in studying how complexes of Mcms unwind DNA[47; 69; 72; 75; 76; 77; 78; 79; 80; 81]. More recently, DNA unwinding by a variety of Mcm complexes has been observed including Mcm2-7[36; 37; 48; 82]. The mechanism by which Mcm2-7 unwinds DNA is presently unknown. Studies of other replicative helicases, including SV40 LTag, BPV E1 and the archaeal MCM complexes have generated multiple models of DNA unwinding by hexameric helicase complexes. First is the "ploughshare" model in which a ploughshare is dragged behind the hexamers to split the strands of DNA (Figure 1-5A; ref. 83). The lack of identifiable structures that could perform this function in the structure of SsoMCM and mtMCM make this an unlikely mechanism by which Mcm2-7 unwinds DNA[54; 60]. Second, a rotary pump model in which the hexamers are anchored and twist the DNA to introduce negative supercoiling, and thus unwind DNA has also been proposed (Figure 1-5B; ref. 84). This model was partially based on the observation that more Mcm subunits are bound to chromatin than are required for replication initiation (reviewed in ref. 46). In budding yeast, *Drosophila*, and *Xenopus* however, the concentrations of Mcms can be reduced without compromising DNA unwinding[85; 86; 87]. Additionally, it has now been shown that the excess Mcms are required to license dormant origins in the cell stress response weakening the argument for the rotary pump model[88; 89; 90]. These dormant origins are utilized during the recovery from the S phase checkpoint[88; 89; 90]. Furthermore, the ploughshare and rotary pump models were proposed at a time before DNA unwinding by the Mcm2-7 complex had been detected[36].

The structures of archaeal Mcms reveal two putative channels that can accommodate DNA (Figure 1-5C; ref. 60). A central channel can accommodate either dsDNA or ssDNA and two side channels can accommodate ssDNA. These channels are also seen in electron micrograph images of Mcm2-7 bound to origin DNA[73; 74]. As mentioned earlier, in the context of the pre-RC the Mcm2-7 complex encircles dsDNA as a dodecamer of two hexamers contacting each other via their N-terminal faces (Figure 1-5D; refs. 73; 74). This has implications for how Mcm complexes may unwind DNA and the mechanisms presented here take this into consideration (Figures 1-5E through J). In the "steric exclusion model", ssDNA passes through the central channel of the helicase and the other strand is excluded (Figure 1-5E). Translocation of the helicase in the 3' to 5' direction along the single-strand DNA unwinds duplex regions upstream. The Mcm4/6/7 helicase has been shown to unwind synthetic fork substrates 3' to 5' *in vitro* by this mechanism[78]. The crystal structure of BPV E1 bound to single-strand DNA also suggests a steric exclusion model is used by that helicase (Figure 1-5E; ref. 57). In the double hexamer steric exclusion model, the ssDNA that passes through the central channel exits via one of the side channels (Figure 1-5F). In the double side extrusion model, dsDNA enters the central channel, and one ssDNA passes through one side channel and the other ssDNA passes through the second side channel (Figure 1-5G & H). These models can be thought of as a modified version of the ploughshare model where the ploughshare is likely the H2I β-hairpin[91]. This mechanism has been proposed previously for the SV40 LTag[67; 68]. The single side-channel extrusion model predicts that dsDNA is accommodated in the first part of the central channel, after which one ssDNA then passes through one side channel and the other continues through the latter part of the central channel (Figure 1-5I). Lastly, the asymmetric looping model proposes that one

strand of ssDNA is extruded through one side channel and the second ssDNA is extruded through a putative C-terminal side channel (Figure 1-5J). Common to these mechanisms is the importance of coupling ATP binding and/or hydrolysis to changes in DNA binding and unwinding by the complex[92].

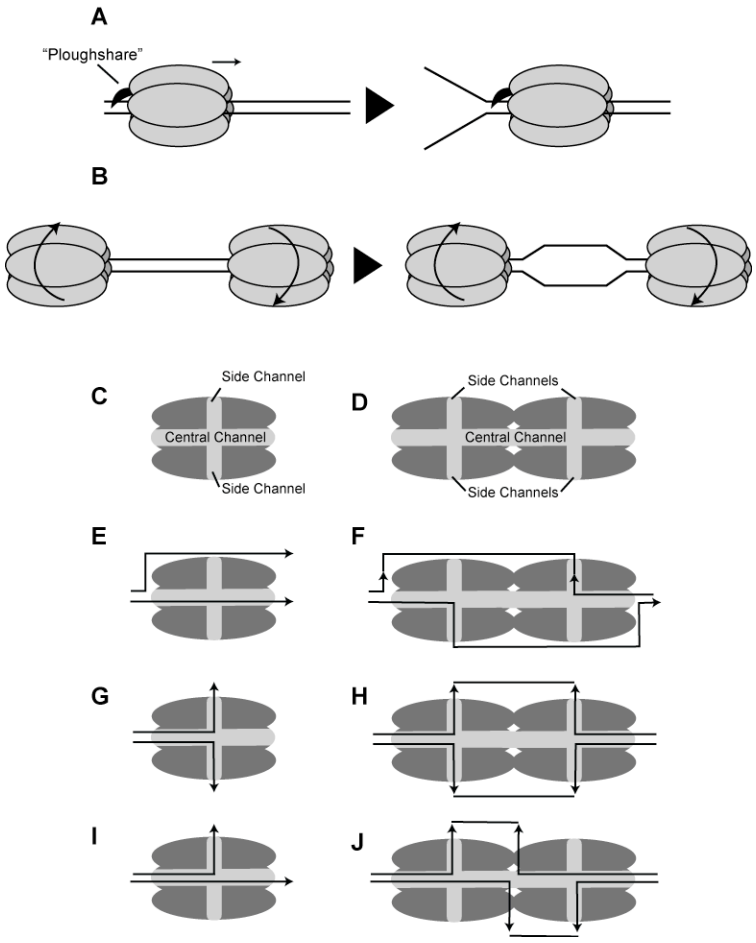


Figure 1-5 Potential mechanisms of DNA unwinding by Mcm helicases. Details of each model are given in the main text. **A.** The ploughshare model depicting a hexameric helicase with a ploughshare dragging behind it to separate dsDNA. **B.** The rotary pump model depicting single hexamers twisting dsDNA to unwind it. **C & D.** Schematic representations of a cross-section of a single hexameric Mcm complex (C) and a double hexameric Mcm complex (D). The central channel and two side channels are depicted in the lighter shade. **E-J.** Models of DNA unwinding by Mcm complexes. The steric exclusion model for a single hexamer (E) and double hexamers (F). The double side extrusion model of a single hexamer (G) and double hexamers (H). The single side extrusion model (I) and asymmetric looping model (J). (C-J) adapted from ref. 60.

1.2.6 Other functions of Mcms

In addition to their role as the replicative helicase, Mcm2 through 7 have potential functions in other cellular processes. The Mcm2 and Mcm5 subunits interact with and are required for transcriptional activation of RNA Pol II-mediated transcription[93; 94] and in human cells, Mcm5 is implicated in interferon-gamma-induced transcription[95]. Furthermore, subunits of Mcm2-7 interact with histones[96; 97; 98], chromatin remodelling factors[99] and checkpoint and recombination proteins. Recently, whole animal studies in mice show that decreasing the levels of Mcm subunits result in genomic instability and cell proliferation defects which are exacerbated by cell stress[100].

1.3 Regulation of Mcms

1.3.1 Role of the Mcm subunits in DNA unwinding

Each of the Mcm2 through 7 subunits is essential for DNA replication and replication fork progression (reviewed in ref. 46). Early biochemical studies with human, mouse and budding yeast Mcm4/6/7 demonstrated that Mcm2, Mcm3 and Mcm5 inhibit DNA unwinding by Mcm4/6/7[47; 96; 101; 102; 103]. One explanation for this observation is that the Mcm2, Mcm3 and Mcm5 subunits of Mcm2-7 regulate DNA unwinding as opposed to participating directly in the catalysis of DNA unwinding. Since the ATP sites of Mcms are essential, this model predicts that ATP hydrolysis by Mcm2 is required for its regulatory function. Chapter 2 of this thesis explores the active role of ATP hydrolysis by Mcm2 in regulating DNA binding by complexes of Mcms.

1.3.2 Cyclin-dependent kinases limit DNA replication to once per cell cycle

The subunits of Mcm2-7 are modified by multiple kinases (See Figure 1-6A for an overview). In the G1 phase of the cell cycle, Cyclin-dependent kinases (Cdk) 1 and 2 phosphorylate Mcms and contribute to limiting DNA replication to once per cell cycle[75; 76; 104; 105; 106; 107; 108; 109]. Modification of budding yeast Mcm subunits that are not origin-bound causes those subunits to be excluded from the nucleus[110]. Conversely, in human cells, phosphorylation of Mcm3 by Cdk1 promotes its incorporation into the Mcm2-7 complex and subsequent binding to chromatin[111]. The difference in these observations remains to be reconciled. However, these are not the essential functions of Cdk in DNA replication. Sld2 and Sld3 are essential replication proteins and phosphorylation of Sld2 and Sld3 by Cdk is required for the recruitment of Dpb11[39; 112; 113]. Dpb11, in turn, recruits polymerase ε to the origin[40; 114; 115].

1.3.3 Checkpoint kinases and casein kinase 2 phosphorylate Mcms

Mcms are also subject to modification by other kinases. Studies of human cell lines using siRNA depletion and phospho-specific antibodies show that ATM (ataxia telangiectasia mutated; Tel1 in yeast) phosphorylates the Mcm3 subunit and ATR (ataxia telangiectasia and rad3 related; Mec1 in yeast) phosphorylates the Mcm2 subunit in response to ionizing radiation and HU[116]. Similarly in *Xenopus*, Mcm2 is phosphorylated by ATR and ATM in the response to DSB[117]. Although not modified by ATM or ATR, a physical interaction between human Mcm7 and ATR has been reported and is important for the S phase checkpoint[116]. Additionally, in the presence of HU, Mcm4 in HeLa cells is phosphorylated consecutively by ATR and Cdk2. Phosphorylation by Cdk2 has been shown to inhibit DNA unwinding by the Mcm4/6/7 complex[118].

Using *in vitro* kinase assays and mass spectrometry, casein kinase 2 (CK2) phosphorylation sites in human Mcm2 were identified[29]. CK2 is involved in a wide variety of cellular processes including cell cycle progression, transcription, apoptosis and viral infections (reviewed in ref. 119). The functional relevance of this modification has not been explored. Taken together, these studies demonstrate that Mcm2-7 can serve as a substrate for multiple kinases. The effect that each of these phosphorylation events has on the activities of Mcm2-7 awaits further studies.

1.3.4 DDK modifies Mcm subunits

The Dbf4-dependent kinase, DDK comprised of the Cdc7 catalytic subunit and Dbf4 regulatory subunit is essential for replication initiation and is also required during the DNA damage checkpoint[30; 31; 120; 121; 122; 123; 124; 125]. DDK modifies at least the Mcm2, Mcm4 and Mcm6 subunits of Mcm2-7[23; 24; 25; 26; 27; 28; 29; 30; 31]. Modification of Mcm4 during initiation facilitates the recruitment of the essential replication factor Cdc45 in both humans and yeast (Figure 1-6B; refs. 30; 31; 120). In fact, in budding yeast, modification of Mcm4 is thought to be the essential function of DDK since phosphomimetic substitution of the DDK target sites of Mcm4 support growth in the absence of both the *cdc7* and *dbf4* genes[120].

DDK also phosphorylates Mcm2. In human cells several studies mapped the phosphorylation sites of Mcm2 by DDK, however there is disagreement as to the identity of the sites and mutations at these different positions have different effects on human cell lines[29; 126; 127]. Tsuji *et al.* demonstrated that alanine substitutions of three DDK target serine residues in the N-terminus of Mcm2 are unable to rescue a replication defect in HeLa cells[126]. Another report suggests that DDK modification of Mcm2 is responsible for

exit from cellular quiescence[127]. In budding yeast, Bruck and Kaplan (2009) demonstrated that the expression of a version of Mcm2 that cannot be phosphorylated by DDK is not viable[28]. In Chapter 3, I provide evidence that under control of the endogenous *MCM2* promoter or the *MCM6* promoter, this mutation supports viability. Growth defects of alanine substitutions of the Mcm2 DDK phosphosites are only observed when the cell is exposed to DNA damage. Chapter 4 of this thesis explores the role of DDK-dependent regulation of Mcm2 in response to DNA damage.

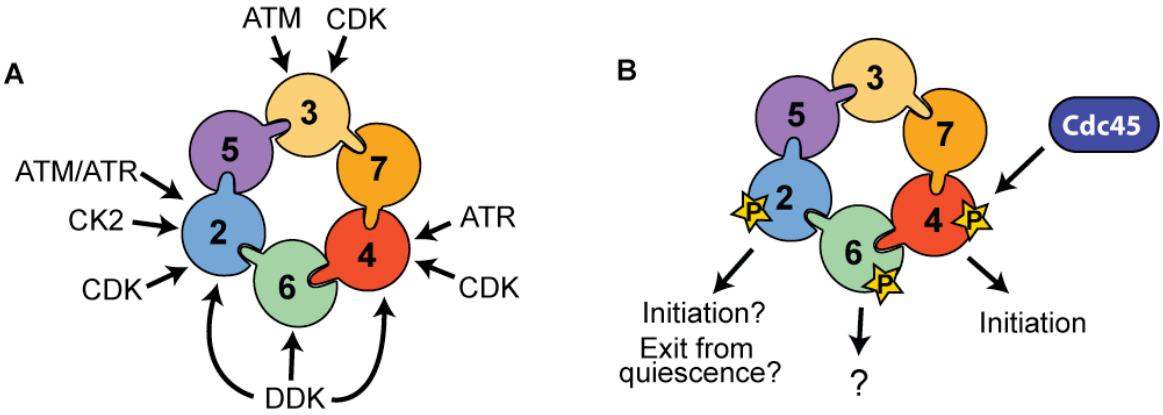


Figure 1-6 Overview of kinases known to target the Mcm2-7 complex and the potential cellular functions of DDK phosphorylation. A. Kinases that modify the subunits of Mcm2-7. CDK modifies Mcm2, Mcm3 and Mcm4 to inhibit re-replication. The Mcm3 subunit is modified by ATM (Tel1 in yeast) during the stress response in human cells. Casein kinase 2 (CK2) in human cells also modifies the Mcm2 subunit and the consequences of this event are currently unknown. The checkpoint kinases ATM (Tel1) and ATR (Mec1 in yeast) modify *Xenopus* Mcm2 in response to DNA damage. DDK modifies the Mcm2, Mcm4 and Mcm6 subunits. **B.** The consequences of the modification of Mcm2, Mcm4 and Mcm6 subunits by DDK. Phosphorylation of Mcm4 is required for the initiation of replication. The functional significance of the phosphorylation of the Mcm6 subunit is presently unknown. Studies from budding yeast and humans suggest that the modification of the Mcm2 subunit may be required for replication initiation[28; 126]. In human cells, phosphorylation of Mcm2 is also thought to be involved in the exit from cellular quiescence[127].

1.4 The cell's response to replicative stress and DNA damage

1.4.1 DNA damage and the S phase checkpoint

Cells are constantly inundated with DNA damage and events causing cellular stress. If left unrepaired, deleterious mutations of the genome can result in diseases and/or cell death. Eukaryotic cells have developed intricate mechanisms to remedy such errors and one such mechanism is the S phase checkpoint. DNA damage can occur from many environmental factors, for instance the exposure of cells to chemicals such as the DNA-alkylating agent methyl methanesulfonate (MMS), or exposure to ionizing radiation[128; 129]. When such errors are encountered, the cell pauses DNA replication in order to repair the damaged DNA, ensuring deleterious mutations are not passed on to subsequent generations of the cell.

The present discussion will focus on the response to DNA damage in budding yeast; however the proteins and processes involved are generally well conserved in eukaryotes[130]. It is also important to note that the S phase checkpoint consists of several stages: the establishment of the checkpoint, the maintenance of the response and finally recovery from the S phase checkpoint[131]. In budding yeast, damage is surveyed by a series of S phase checkpoint sensor proteins, including Ddc1/Rad17/Mec3 (homologs of the 9-1-1 complex in humans), Rad24 and Rad9 (See Figure 1-7 for an overview; refs. 132; 133; 134; 135). These proteins communicate with transducers of the S phase checkpoint, such as the Mec1/Tel1 complex and Rad9 in budding yeast, which elicit various responses through their respective kinase activities (note that Rad9 has been implicated as both a sensor and a transducer; refs. 133; 136; 137; 138). Central to this pathway is the activation of the effector kinase Rad53 by phosphorylation (Reviewed in

refs. 131; 139). Rad53, in turn, phosphorylates many targets to cause cell cycle arrest, repress transcription of cyclins and stabilize replication forks[131]. Other kinases, such as Chk1, can carry out these functions[131]. However, a recent report shows that the activation of the checkpoint can be Rad53-independent; Mec1/Tel1 can activate Chk1 independently of Rad53[140]. The different pathways are likely triggered by different damage signals and the consequences of their activation also differ.

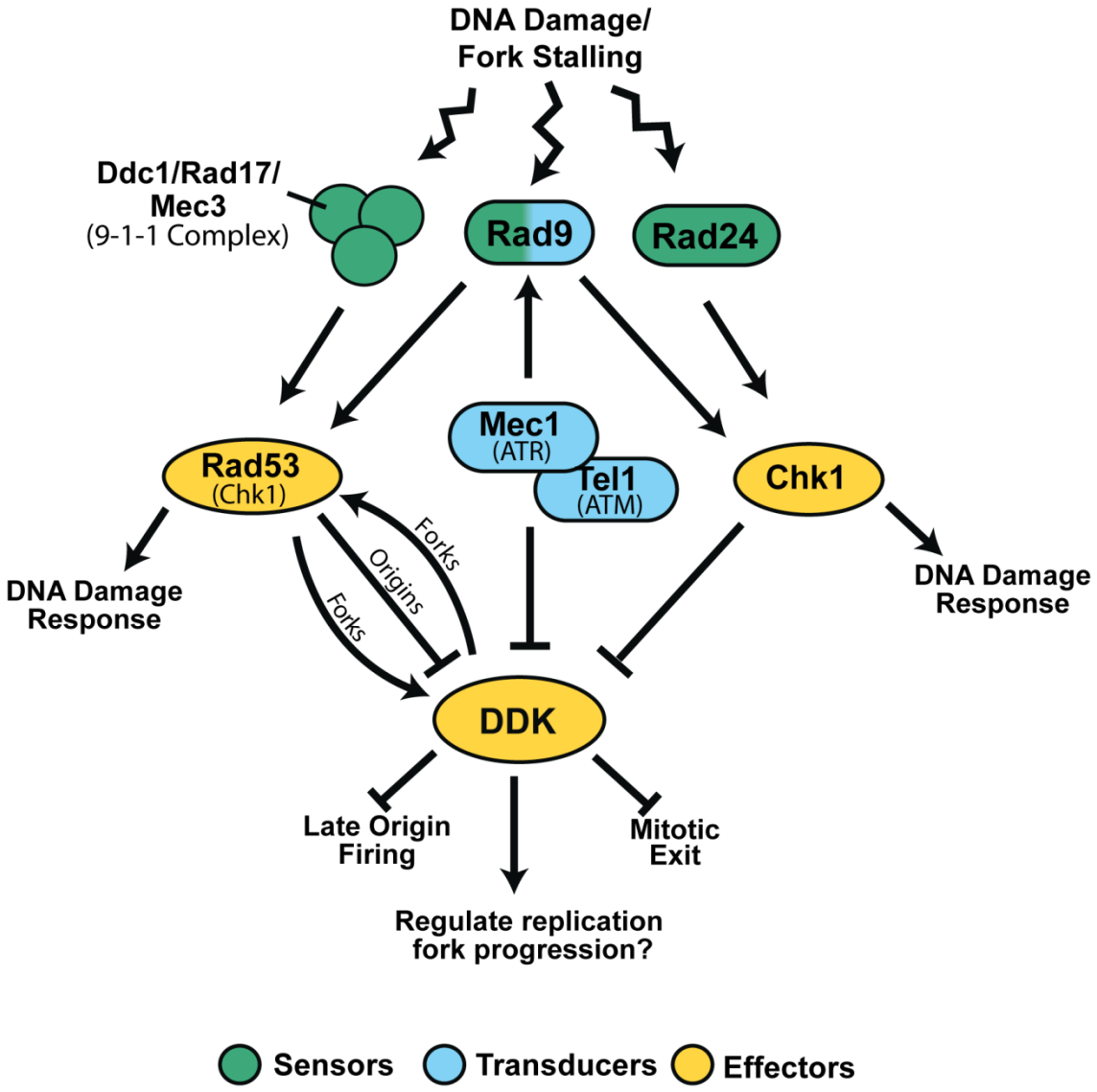


Figure 1-7 Overview of the yeast DNA damage checkpoint and the involvement of DDK. Sensors such as Rad9, Ddc1/Rad17/Mec3 and Rad24 detect DNA damage and replication fork blockage. These sensor proteins then transmit signals to transducers of the checkpoint including Rad53 and the Mec1/Tel1 complex. The transducers then activate the effector kinases (Chk1 and DDK, among many others) to elicit various responses such as replication fork pausing and invoking the DNA repair pathways. Shown in parentheses are the mammalian orthologs of the yeast proteins. Note that Chk1 is the functional homolog of Rad53 in mammalian cells and Chk2 is its sequence homolog.

Many cellular stresses induce the checkpoint and/or invoke DNA repair mechanisms. N-methylated DNA adducts, such as those caused by MMS, are repaired by base excision repair (BER; ref. 141). Defective BER can itself be cytotoxic and result in double strand breaks (DSBs) that are repaired by homologous recombination[142]. Oxidative DNA damage is also repaired via BER[143; 144]. Ionizing radiation causes DSBs that are repaired primarily by homologous recombination (HR) and also by non-homologous end joining, which fuses two ends of DNA together[145; 146; 147]. Moreover, treatment of cells with hydroxyurea (HU) inhibits ribonucleotide reductase resulting in the induction of the S phase checkpoint which then pauses replication fork progression[121; 148]. Treatment of cells with HU also strongly promotes homologous recombination, likely to bypass the increased number of collapsed replication forks in HU treated cells[149; 150].

1.4.2 Replication fork pausing and restart

Replication fork pausing requires the Mrc1-Tof1-Csm3 complex in budding yeast[151; 152; 153]. A direct interaction between Mcm6 and the Tof1 protein of the fork pausing complex has been detected implicating the Mcm2-7 complex in fork pausing and/or fork restart[154]. Many factors in addition to Mrc1 and Tof1 are important for the maintenance of stable replication forks. Replication factors, histones and chromatin remodelling factors, as well as post-translational modifications of these factors, all contribute to the establishment and maintenance of replication forks (Reviewed in ref. 155).

After repair of the DNA damage, the DNA damage checkpoint signal must be attenuated for cell growth to continue. The central checkpoint kinase Rad53 must be dephosphorylated and the protein phosphatases Ptc2 and Ptc3 perform this function in budding yeast[156; 157; 158]. The mechanism(s) by which DNA unwinding at a stalled replication fork is resumed is presently unknown.

1.4.3 DDK functions in the cell's response to DNA damage

DDK acts in most, if not all, stages of the S phase checkpoint (Figure 1-7). The Dbf4 subunit of DDK is modified by Rad53, an event that inhibits the firing of late origins[122; 123; 159; 160]. Rad53-dependent phosphorylation of Dbf4 decreases its association with origins. The activity of DDK is also required after replication initiation to maintain stable replication forks; mutations that destabilize replication forks have an increased sensitivity to genotoxic stress when the activity of the homolog of Cdc7 in *Schizosaccharomyces pombe*, Hsk1 is decreased by mutation[161]. Moreover, cells containing mutations in *cdc7* or *dbf4* are sensitive to HU treatment, suggesting that DDK has an active role in the S phase checkpoint[39; 112; 113; 120]. Lastly, DDK is also involved in translesion DNA synthesis[162; 163; 164]. In this capacity, Cdc7 functions in the RAD6 epistasis group in the response to UV- and MMS-induced DNA damage[163]. The function of DDK in translesion DNA synthesis remains to be determined.

1.4.4 Mcms in the S phase checkpoint

In addition to being targeted by the checkpoint kinases ATR and ATM in human cells as discussed above, Mcms have other roles in the S phase checkpoint. The Mcm subunits are present in copy numbers in the cell that exceed the number of replication

origins[165; 87; 166; 167; 168; 169]. In human cells and *Xenopus*, the excess Mcms are thought to license dormant origins in the response to cell stress[88; 89; 90].

1.5 Other cellular functions of DDK

A recent report demonstrates that the Cdc7 homolog in fission yeast phosphorylates Ams2, a regulator of histone expression, targeting it for degradation after the initiation of S phase[170]. Precedence for regulation of histones by DDK is also found in the studies of Baker *et al.* (2010) who demonstrated that Cdc7 directly phosphorylates histone H3 during DNA replication, an event thought to be important for genome integrity and for DNA repair[171]. Additionally, DDK binds to Cdc5, a polo-like kinase that regulates mitotic progression and cytokinesis[172]. Cells containing a mutant version of *dbf4* that abolishes a Dbf4-Cdc5 interaction aberrantly segregate chromosomes after nuclear misorientation[172].

The initiation of meiosis is also dependent on DDK[173; 174; 175; 176]. In this process, DDK phosphorylates Mer2, an accessory factor for Spo11[173; 174]. Spo11 is an endonuclease that generates DSBs necessary for meiotic recombination[177]. DDK also regulates the localization of the monopolin complex as well as the polo-like kinase cdc5 which are required for the proper orientation of chromosomes at the kinetochore[175; 178]. Collectively, it is evident that DDK has a wide array of cellular functions throughout the cell cycle to promote timely and accurate DNA replication as well as roles in meiosis and mitosis.

1.6 Yeast as a model organism

Fundamental processes, such as DNA replication, share a marked degree of similarity between distant species from budding yeast to humans. These similarities make studying DNA replication in yeast an attractive system to aid in understanding these processes compared to studies in more complex systems because of the relative simplicity of manipulating the yeast genome. When deprived of nutrients, a diploid *S. cerevisiae* cell can undergo sporulation to produce four haploids that can multiply and divide similarly to the parental cell. If a mutation is introduced into a gene of one chromosome of a diploid yeast cell and that cell is sporulated, two of the spores derived from that cell will contain a wild type copy of the gene and the other two spores will contain the mutant version of the gene[179; 180]. Thus, the ability of the yeast to live in the haploid state allows for easy analysis of mutations in genes including study of gene mutations in the absence of the wild type copy of the gene[181], generating haploid deletions of non-essential genes[182; 183] and facilitating synthetic lethal and suppression studies[184], all of which are employed in this thesis.

In addition, simple mating crosses of two haploid yeasts can generate diploid strains containing multiple genetic events, a process exploited in synthetic genetic array (SGA) analysis[184]. In such an analysis, each non-essential gene is replaced by a drug-selectable or nutrient-selectable marker. A query strain, such as one containing a genetic mutation of interest, can be systematically crossed with all non-essential genes to determine the combinatorial or "synthetic" effects of the query mutation coupled with a the gene deletion. Synthetic lethal interactions or suppression of phenotypes can yield valuable information into the cellular processes in which a gene may function. SGA

analysis is used in this thesis to explore the biological role of phosphorylation of Mcm2 by DDK. Additionally, genome-wide strain collections are commercially available and relatively easy to manipulate on a large scale. The Magic Marker yeast collection employed in this thesis allows for the generation of haploid yeast cells from diploid cells by nutrient marker and drug-resistance selection steps[185]. Specifically, the mating type **a** locus (*MFA1*) is linked to the *HIS3* gene allowing selection of *MAT* **a** cells after sporulation of diploid cells and selection on media lacking histidine.

1.7 Scope of Thesis

The Mcm2-7 complex is widely accepted as the eukaryotic replicative helicase, however detailed studies about the regulation of its activities and the contributions of each subunit to the catalytic activity of the complex have not been fully explored. Based on the evidence that Mcm2 inhibits DNA unwinding by Mcm4/6/7[47; 96; 101; 102; 103], I hypothesize that the Mcm2 subunit functions as an active regulator of the activities of Mcm2-7. Furthermore, DDK modifies Mcm2[23; 24; 27; 29; 126; 127] providing a potential mechanism to regulate the activity of Mcm2. Thus, I propose that the DDK-dependent phosphorylation of Mcm2 modulates its activities. Lastly, DDK's essential function in yeast cells is to phosphorylate Mcm4 during replication initiation[120]. DDK however, also functions in the cell's response to DNA damage. I theorize that phosphorylation of Mcm2 by DDK is required for the cell's response to DNA damage. Understanding how DNA replication is regulated is crucial in understanding how aberrations in the process can lead to disease states, such as cancer.

I present evidence in Chapter 2 that the Mcm2 subunit of the Mcm2-7 complex serves as a regulator of DNA binding by Mcm complexes rather than participating

directly in the catalysis of DNA unwinding. Using nucleotide analogues and an ATP site mutant of *mcm2* I demonstrate that Mcm2 requires nucleotide for its regulatory function. The regulatory function is dependent upon ATP hydrolysis by Mcm2. This regulatory activity is independent of Mcm3 and Mcm5, which are two other subunits thought to be important for regulation of Mcm2-7.

In Chapter 3, I examine the consequences of DDK-dependent phosphorylation of Mcm2 on its *in vitro* activities. Specifically, I demonstrate that serine to glutamic acid mutations in Mcm2 provide a phosphomimetic phenotype for DDK-dependent phosphorylation. I purify an *E. coli*-expressed version of the Mcm2 phosphomimetic mutant and conduct detailed biochemical analyses of its activities. A complex of Mcm2-7 containing the mutant version of Mcm2 unwinds DNA poorly but has a higher affinity for ssDNA, much like ATP site mutants of Mcm2. I show through *in vitro* and *in vivo* methods that phosphorylation of Mcm2 regulates its ATPase activity.

The work presented in Chapter 4 explores how phosphorylation of Mcm2 by DDK is required for the response to DNA base damage. Through the use of a genome-wide screen for synthetic lethal and suppressor interactions with a phosphosite mutant of Mcm2, genes are identified that are involved in the cell's response to DNA damage. Cells containing a version of Mcm2 that cannot be phosphorylated by DDK are sensitive to agents that damage DNA bases. Collectively, these data suggest a role for Mcm2 in the cell's response to DNA damage.

Cdc7 has quickly become a heavily studied target of anti-cancer therapeutics largely due to its dual roles in both replication initiation and genome maintenance[186; 187;

[188]. Potential molecules that are typically ATP analogues inhibiting the active site of Cdc7 are currently under pharmacological investigation and have been found to be effective in inhibiting tumour growth in animal models[189; 190; 191]. Chapter 5 proposes a comprehensive model of the regulation of DNA unwinding during the S phase through the DDK-dependent phosphorylation of Mcm2 in response to DNA damage summarizing the works in this thesis. These studies have broad implications on the understanding of the regulation of DNA replication and must be considered when exploring DDK as a potential anti-cancer target.

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CHAPTER 2 ATP HYDROLYSIS BY MCM2 IS REQUIRED TO REGULATE DNA BINDING BY MCM COMPLEXES[a]

2.1 Introduction

The ability of Mcm2 to inhibit Mcm4/6/7 is the starting point for the studies presented in this chapter[1; 2; 3]. Current models suggest that Mcm2 modulates the helicase activity of Mcm4/6/7 by disrupting the oligomeric structure of the complex. The functional form of Mcm4/6/7 is likely a hexameric ring comprised of two Mcm4/6/7 trimers that binds to DNA by encircling it within a central channel[1; 2; 4; 5; 6; 7; 8]. This mode of DNA interaction is likely important for DNA unwinding[6; 9; 10; 11]. Based on the observation that Mcm2 interacts with Mcm4/6/7 in the absence of ATP and disrupts the hexameric structure to form tetramers containing Mcm2, 4, 6 and 7[1; 2; 3], current models propose that modulation of Mcm4/6/7 activity by Mcm2 occurs by a "passive" process and is simply a consequence of the interaction between Mcm2 and Mcm4/6/7.

Here, I examine the mechanism of Mcm2 modulation of Mcm4/6/7 activities and relate these findings to the Mcm2-7 complex from *S. cerevisiae*. Specifically, I tested the hypothesis that Mcm2 inhibits Mcm4/6/7 activity via a passive mechanism using a Mcm2 mutant ($Mcm2_{K549A}$) that was severely compromised in its ability to hydrolyze ATP with the expectation that the mutant would inhibit Mcm4/6/7 activity. Surprisingly, I found that $Mcm2_{K549A}$ was unable to inhibit DNA unwinding by Mcm4/6/7. This same mutant interacted with Mcm4/6/7, suggesting that association of Mcm2 with Mcm4/6/7 is not sufficient for Mcm2 to exert its effects. Rather, based on observations with ATP analogues and $Mcm2_{K549A}$, I suggest that the ADP-bound state of Mcm2 is required for

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inhibition of DNA binding and thus DNA unwinding by Mcm4/6/7. The relevance of this result to the intact complex was apparent from the finding that the Mcm2-7 complex containing $Mcm2_{K549A}$ in place of wild type Mcm2 possesses a higher affinity for single stranded DNA than wild type Mcm2-7. I suggest that ATP hydrolysis at the Mcm2 site modulates Mcm2-7 DNA binding by a mechanism in which the ATP binding status of Mcm2 is communicated to other Mcm subunits.

2.2 Materials and Methods

2.2.1 Materials

Sources of reagents were Sigma Aldrich for ATP, ADP and ATPγS ($\geq$ 99 % purity for each); New England BioLabs for molecular biology enzymes; and Integrated DNA Technologies for helicase substrates, unless otherwise indicated. M13mp18 single stranded DNA was phenol extracted from PEG-precipitated phage[12] and is greater than 95 % circular single stranded DNA. Purification of individual Mcm subunits was carried out as described previously[13], with the exceptions described below. For Mcm6, the EAH Sepharose step was omitted. Mcm4/6/7 was reconstituted from individual subunits as described in Kaplan et al[6]. Mcm2-7 was reconstituted from individual subunits as described[13] except that a Mono S column was used as an extra purification step (Buffer H, 20 ml 0-500 mM NaCl gradient) before the MonoQ column and the gel filtration column was omitted. For ATPases, Mcm6 was purified by gel filtration as described in Ref. 13. Molar concentrations of proteins are expressed as monomer for Mcm2 and Mcm6, dimer for Mcm3/5 and hexamer for Mcm4/6/7 and Mcm2-7.

2.2.2 Plasmids

mcm2$_{K549R}$ was generated by amplifying a portion of the *MCM2* coding region from pET11a-*MCM2*[13] using the mutagenic primer, 5'-d(CCAGGTACCGCCAGATCTCA)-3'and a second primer, 5'-d(TCGGGCTTTGTTAG)-3' with Elongase polymerase (Invitrogen). A second round of amplification used the megaprimer product[14] generated in the first reaction and 5'-d(AATTGTGAGCGGATAACAATTCCCCTC)-3' to amplify the *MCM2* coding region containing the K549R mutation as well as NdeI and BamHI sites at the 5' and 3' ends. *mcm2*$_{K549A}$ was generated similarly, except that the mutagenic primer was 5'-d(GATCCAGGTACCGCCGCATC)-3'. The mutant PCR products were digested with BamHI and NdeI and ligated into the same sites of pET24a (Novagen) to yield pET24a-*mcm2*$_{K549R}$ and pET24a-*mcm2*$_{K549A}$. Mutations were confirmed by sequencing the *MCM2* coding region (Robarts Research Institute DNA Sequencing Facility).

pMD279, for disruption of *MCM2,* is YIplac211[15] with the 349 bp intergenic region upstream of *MCM2* as well as the *MCM2* coding sequence in which nucleotides 876-2211 were replaced with a 1.3 kb BsaI fragment encoding *HIS3*. pMD240 allows expression of genes from the *MCM6* promoter in yeast. Nucleotides 120907 to 122297 of *S. cerevisiae* chromosome VII were amplified using the primers 5'-d(TATATCTGCAGCAACAAGCCTAATTC)-3' and 5'-d(ATAATCTAGACATATGAAAAAAACCAGTTTTAACCT). The resulting 1.4 kbp PCR fragment was digested with PstI and XbaI and inserted into the same sites of YCplac111. The 2.8 kbp NdeI-BamHI fragment from each of pET11a-*MCM2*, pET24a-

mcm2$_{K549R}$ and pET24a-*mcm2*$_{K549A}$ was inserted into the same sites of pMD240 to yield pMD269, pMD270 and pMD271, respectively.

mcm5$_{K422A}$ was generated from pET16b-*MCM5*[13] using the mutagenic primer 5'd(GATCCAGGTACCGCCGCTAGCCAACTATTGAAATTTGTG)-3' and a second primer 5'd(GCATATAAGGCCTTATCCAGTG)-3'. The resulting 1 kb fragment was digested with KpnI and StuI and then inserted into the same sites of pET16b-*MCM5* to yield pET16b-*mcm5*$_{K422A}$. The mutation was confirmed by sequencing the *MCM5* ORF across the amplified region.

2.2.3 Yeast strains

MCM2 was disrupted in yeast using two-step gene replacement[16]. pMD279 was linearized by digestion with EcoNI and transformed into BY4743 (*MAT*a/α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0*; ref. 17). Ura+ colonies were grown on YPD and then on synthetic minimal media containing 5-FOA to select cells that had lost *URA3*. Colonies that grew on 5-FOA were plated on media lacking histidine, resulting in YMD33. Disruption in one of the *MCM2* loci was confirmed by PCR. After transforming YMD33 with pMD269, the strain was sporulated, tetrads dissected and Leu+, His+ spores selected, yielding YMD46. To carry out plasmid shuffling[18], the *LEU2* marker on pMD269 in YMD46 was switched to *URA3* by transforming YMD46 with the XhoI-HpaI fragment of pLU12 [19] generating YMD54.

2.2.4 Mcm2 purification

Mcm2 proteins were purified as described[13] with an appended step to remove a nuclease contaminant. Mcm2-containing fractions from the MonoQ step were pooled (approximately 3 ml, 2.7 mg/ml) and dialyzed overnight against Buffer H (20 mM

HEPES-NaOH pH 7.5, 0.1 mM EDTA, 2 mM DTT, and 10 % (v/v) glycerol). The dialysate was applied to a 10 ml ssDNA-Sepharose column[20], washed with 100 ml of Buffer H and eluted with a 100 ml, 0-500 mM NaCl gradient.

2.2.5 Mcm3^{PK} and Mcm4^{PK}

A Protein Kinase A recognition motif was fused to Mcm4 by inserting the 3.1 kb NdeI-ClaI fragment from pET11a-*MCM4*[13] into the same sites of pHKEp[21]. Similarly, the 2.9 kb NdeI-BlpI fragment from pET21b-MCM3[13] was inserted into the same sites of pHKEP[21]. Mcm4^{PK} purified similarly to untagged Mcm4[13], however a ssDNA-Sepharose chromatography step was inserted between the Heparin agarose and Mono S steps. Pooled fractions from the Heparin agarose column (100 ml, 0.2 mg/ml) were applied to a 20 ml ssDNA Sepharose column equilibrated in Buffer H. The column was washed with 200 ml of Buffer H and eluted with a 200 ml, 0-500 mM NaCl gradient. Mcm4^{PK}-containing fractions were dialyzed against Buffer H and applied to a Mono S 5/50 GL column (GE Healthcare) as described previously[13].

Mcm3^{PK} was purified similarly to tagged Mcm6[13], with the following modifications. The Mcm3^{PK}-containing fractions from the Ni-affinity chromatography column were dialyzed against Buffer H and applied to a 15 ml SP Sepharose column (GE Healthcare) equilibrated in Buffer H and eluted with a 0-500 mM NaCl gradient in Buffer H. Mcm3^{PK}-containing fractions were pooled and dialyzed against Buffer A then applied to a MonoQ 10/100 GL column (GE Healthcare) and eluted with an 80 ml, 0-500 mM NaCl gradient. Both Mcm3^{PK} and Mcm4^{PK} reconstituted similarly to untagged Mcm3 and Mcm4 into Mcm4/6/7 and Mcm2-7, respectively. The activity of Mcm4^{PK}/6/7 was

comparable to Mcm4/6/7 in DNA unwinding assays and the activity of Mcm2-7^{3PK} was similar to Mcm2-7 in ATPase assays.

2.2.6 ATP hydrolysis

ATP hydrolysis by Mcm2 (mutant and wild type) in the presence and absence of Mcm6 as well as Mcm4/6/7 with and without Mcm2 was assayed using thin layer chromatography. Each 15 μl reaction contained 1 mM [γ^{32}P]-ATP (20 Ci/mmol; Perkin Elmer Life Sciences), 20 mM Tris-HCl pH 7.5, 10 mM magnesium acetate and 2 mM DTT as well as 1 μM each of Mcm2 (wild type or mutant) and/or Mcm6 for Mcm2/6 analysis or 200 nM Mcm4/6/7 and/or 400 nM Mcm2 for Mcm4/6/7 analysis. At the indicated times, 2 μl of each reaction in the Mcm2/6 analysis was removed and quenched with 2 μl of 50 mM EDTA, pH 8. Mcm4/6/7 analyses were performed over 90 minutes, with 30-minute intervals. One μl was spotted onto polyethyleneimine cellulose thin layer chromatography sheets (EM Science), which were developed in 0.6 M potassium phosphate pH 3.4 for 10 min. The dried TLC sheets were exposed to a PhosphorStorage screen before scanning with a Storm 860 scanner (GE Healthcare). The "volume" of the spots corresponding to P_i and ATP were used to determine the extent of hydrolysis. A background volume was subtracted and the molar concentration of P_i produced was determined by: 10^3 x (volume P_i)/(volume P_i + volume ATP). To calculate ATP hydrolysis rates of the Mcm2/6 complexes, the background levels of hydrolysis by the individual subunits were subtracted. For the Mcm4/6/7 + Mcm2 reactions, the background levels of hydrolysis by Mcm2 were subtracted. The rate of ATP hydrolysis was determined by linear regression of plots of time x pmol P_i/pmol protein using Prism 5 software (GraphPad Software). The rates are the mean of three replicate experiments.

2.2.7 DNA unwinding

DNA unwinding measurements were performed with a DNA substrate containing 30 nucleotides of duplex with 60 nucleotides of single stranded DNA on one strand and a 5' biotin tag on the other as described in ref. 6. Primer 2T 5'-d(ATGTCCTAGCAAGCCAGAATTCGGCAGCGTC-$(T)_{60}$)-3' was 5'-end labeled with ^{32}P and annealed to 1B 5'-biotin-d(GACGCTGCCGAATTCTGGCTTGCTAGGACAT)-3' by boiling 2 min in 10 mM Tris-HCl pH 8.5, 30 mM sodium citrate and 300 mM NaCl and then slow cooling to room temperature. Each reaction (6 μl) contained 20 mM Tris-HCl pH 7.5, 10 mM magnesium acetate, 100 μM EDTA, 5 mM DTT, 5 mM ATP, 67 nM streptavidin, 1 nM substrate with 100 or 200 nM Mcm4/6/7 (as indicated) and the indicated concentrations of Mcm2 (mutant or wild type) and Mcm3/5 (mutant or wild type). The mixtures were incubated at 37ºC for 10 min and then unwinding was stopped by the addition of 1 μl Proteinase K (10 mg/ml) for 2 min at 37°C followed by the addition of 1.5 μl of 20 mM EDTA pH 8.0, 1 % SDS, 0.02 % bromophenol blue, 5 % (v/v) glycerol and 0.02 % xylene cyanol FF. The samples were analyzed by native (TBE) PAGE (8 %) and dried gels were exposed to PhosphorStorage screens before scanning with a Storm 860 scanner (GE Healthcare). The volume of the bands corresponding to ssDNA product and dsDNA substrate as well as a background volume were used to calculate the extent of DNA unwinding after subtracting the background volume: % DNA unwound = volume ssDNA/(volume ssDNA + volume dsDNA) x 100.

2.2.8 Affinity Chromatography

Mcm4/6/7 (600 nM) was incubated on ice for 15 min with or without equimolar Mcm2 or $Mcm2_{K549A}$ in 100 µl of 5 mM imidazole, 20 mM Tris-HCl pH 7.9, 5 mM ATP and 100 mM NaCl. Forty µl of HIS-Select Nickel Affinity resin (50 % slurry in the same buffer; Sigma Aldrich) was added and the mixtures were incubated for a further 10 min. Resin was pelleted by brief centrifugation at 1000 rpm in a table top centrifuge. Approximately 40 % of the input protein bound to the resin. Supernatant was removed and the resin washed 5 times with 100 µl of 5 mM imidazole, 20 mM Tris-HCl pH 7.9, 5 mM ATP and 350 mM NaCl and eluted with 20 µl of SDS loading dye. Ten percent of the load, 8 % of the wash and 40 % of the eluate were analyzed by SDS-PAGE (6 %) stained with Coomassie Brilliant Blue R-250. In addition, the eluate was probed with anti-Mcm2 antibody (Santa Cruz Biotechnology, sc6680) using rabbit anti-goat IgG conjugated to horse radish peroxidase (Sigma Aldrich) and chemiluminescence (Pierce) to detect the signal.

2.2.9 Labeling of $Mcm4^{PK}$/6/7 and Mcm2-7^{3PK}

$Mcm4^{PK}$ /6/7 (100 pmol) or Mcm2-7^{3PK} (50 pmol) was radiolabeled by incubation with 50 µCi [γ^{32}P]-ATP (3000 Ci/mmol; Perkin Elmer Life Sciences), 12.5-25 units of Protein Kinase A catalytic subunit (from bovine heart; Sigma Aldrich), 20 mM Tris-HCl pH 8.0 and 20 mM magnesium acetate for one hour at 21ºC. Labeled protein was separated from [γ^{32}P]-ATP using a 5 ml Sephadex G-25 column (GE Healthcare) equilibrated in Buffer A containing 100 mM NaCl and 50 µg/ml BSA. The labeled complexes typically had specific activities of 1-1.5 µCi/pmol complex. Greater than 99 % of unincorporated ^{32}P-ATP is removed by gel filtration.

2.2.10 DNA Binding

DNA binding assays were performed in 100 μl buffer containing 10 mM Tris-HCl pH 7.5, 10 mM magnesium acetate, 100 μM EDTA and 2 mM DTT as well as 5 mM ATP, ADP or ATPγS, as indicated. When added, each assay contained 10 pmol of Mcm2 or $Mcm2_{K549A,}$ 1 pmol of $Mcm4^{PK}/6/7$, 100 fmol of Mcm2-7^{3PK} and/or $Mcm2_{K549A}$-7^{3PK}. Proteins, buffer and nucleotide were incubated on ice for 10 min before addition of M13mp18 ssDNA (2 pmol (as M13 DNA molecule) in the Mcm4/6/7 assays and the indicated amounts for Mcm2-7 assays in 1.5 μl) and the mixtures were incubated for 10 min at 37 ºC. Two μl of the reaction were removed for liquid scintillation counting (load) and the remainder applied to a 5-ml 4 % plain agarose bead column (Agarose Bead Technologies) equilibrated in Buffer A containing 100 mM NaCl, 10 mM magnesium acetate, and 40 μg/ml BSA. The column was eluted with the same buffer and 8 drop fractions (~200 μl) collected. Twenty μl of the indicated fractions was analyzed by liquid scintillation counting. The amount (in fmol) of labeled $Mcm4^{PK}/6/7$ or Mcm2-7^{3PK} in each fraction was determined by: [cpm fraction * (fraction volume/fraction volume counted)]/[(cpm load/fmol in load) * (load volume/load volume counted)]. A representative graph from at least three replicates of each experiment is shown.

2.3 Results

2.3.1 Purification and reconstitution of Mcm subunits and complexes

The Mcm2-7 complex contains six ATP sites encoded by each of the subunits that are important for the function of this complex[22]. To determine the effects of ATP hydrolysis by Mcm2 on its potential function as a regulator, I mutated the invariant lysine residue (K549) of the Mcm2 P-loop, a motif found in many nucleotide binding proteins[23;

[24], to alanine and aginine. Previous studies have indicated that the *mcm2*$_{K549A}$ allele does not support viability[25; 26]. Viability of the *mcm2*$_{K549R}$ mutant has not yet been tested and is discussed further below. Mcm2, Mcm2$_{K549A}$, Mcm2$_{K549R}$, Mcm6 and Mcm6$_{R708A}$ were purified as described in Material and Methods and an aliquot of the final purification step of each protein was examined on Coomassie Blue R250-stained SDS gels (Figure 2-1A). The purified proteins did not contain contaminating ATPase, nuclease or helicase activities that would interfere with my assays (Figure 2-2B, Figure 2-5A and data not shown). Mcm complexes were assembled from individual subunits as described previously[6; 13]. Peak fractions from the final column step of each reconstitution are shown in Figure 2-1B. The components of co-migrating or closely migrating bands within the Mcm complexes were confirmed by Western blotting and/or MALDI mass spectrometry (data not shown). For the wild type Mcm4/6/7 and Mcm2-7 complexes, ATPase and DNA unwinding assays were performed on peak fractions to confirm that the complexes had formed (data not shown). Note that the addition of 100 mM sodium glutamate to the assays containing Mcm2-7 was required to detect DNA unwinding, consistent with a previous study[27].

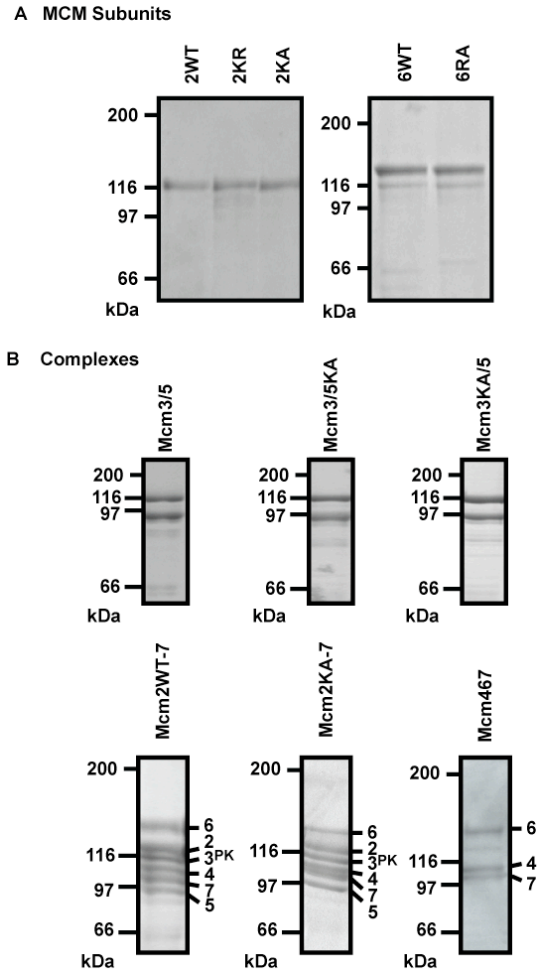


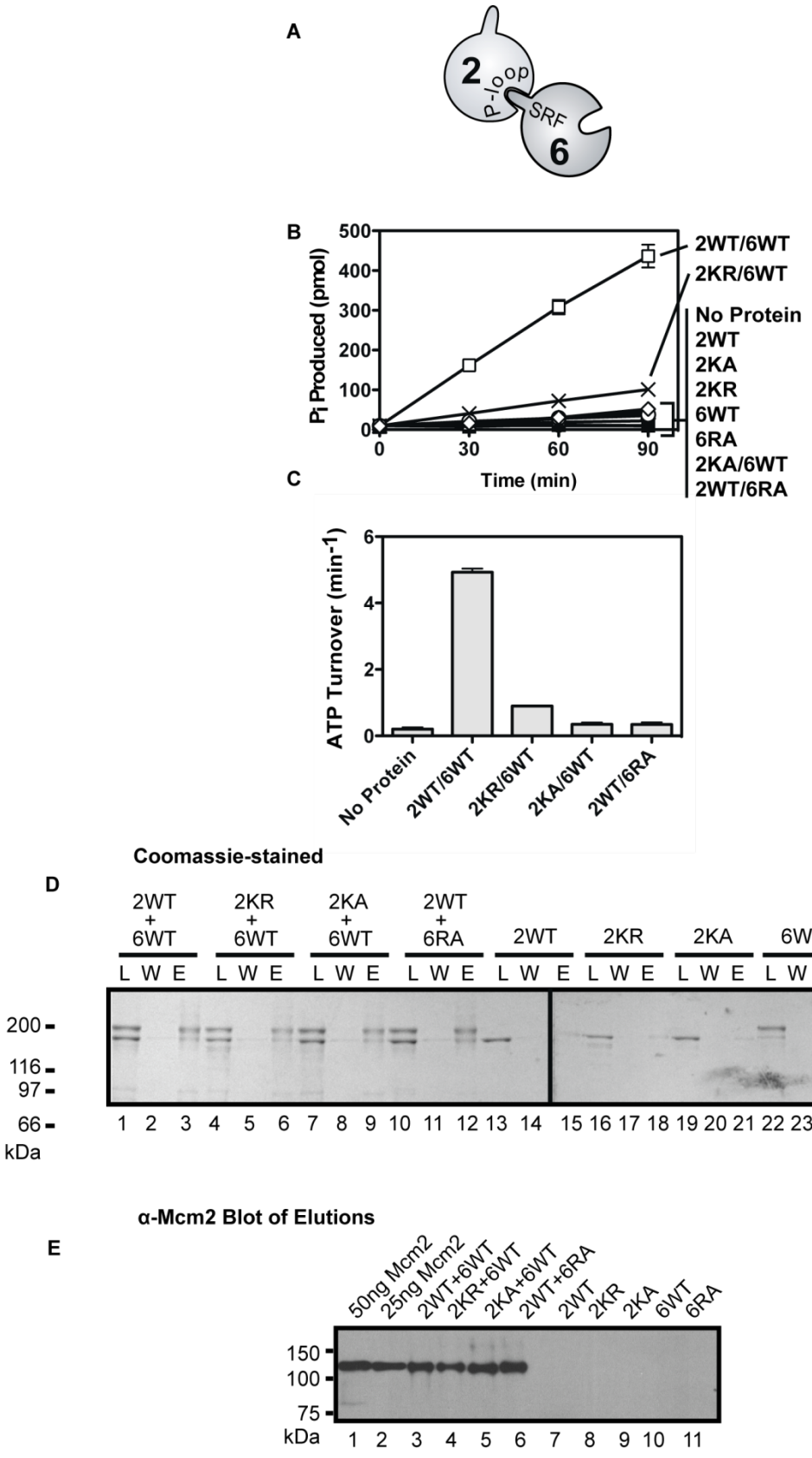
Figure 2-1 Coomassie Blue R250 stained SDS polyacrylamide (6%) gels of the Mcm mutant proteins and complexes. The migration of size standards and individual Mcm proteins are on the left and right of each panel, respectively. Shown in **(A)** are Mcm2, $Mcm2_{K549A}$, $Mcm2_{K549R}$, Mcm6 and $Mcm6_{R708A}$. **B.** Mcm complexes used in this study are shown.

2.3.2 Mutation of the Mcm2/6 interface disrupts ATP hydrolysis

ATP hydrolysis by Mcm2 is dependent on Mcm6[13; 25]. The ATP site of Mcm2/6 is at the interface of the two proteins, with a conserved arginine residue from Mcm6 contributing to hydrolysis of ATP bound to Mcm2 (Figure 2-2A; ref. 13). $Mcm2_{K549A,}$ $Mcm2_{K549R}$ and wild type Mcm2 were purified identically from *Escherichia coli* expression strains and ATP hydrolysis assayed. Neither Mcm2 nor Mcm6 alone

hydrolyzed ATP at rates that were significantly above background levels seen in the absence of protein ($\leq 0.5 \pm 0.1$ min^{-1}; Figure 2-2B). However, when Mcm2 and Mcm6 were combined, ATP hydrolysis roughly 10-fold above levels with individual proteins was detected (4.9 ± 0.2 min^{-1}; Figure 2-2B and C). These results confirmed that ATPase activity requires both Mcm2 and Mcm6 and verified there were no contaminating ATPases in the individual preparations. Substitution of $Mcm2_{K549A}$ for wild type Mcm2 had a severe effect on ATP hydrolysis, with a rate near background levels (0.3 ± 0.1 min^{-1}). Also consistent with the proposed arrangement in Figure 2-2A, mutation of the conserved catalytic arginine in Mcm6 to an alanine ($Mcm6_{R708A}$) had the same effect on ATP hydrolysis as the $Mcm2_{K549A}$ mutant. $Mcm6_{R708A}$ in combination with Mcm2 hydrolyzed ATP with a rate near background levels (0.3 ± 0.1 min^{-1}; Figure 2-2B and C). A more conservative substitution of lysine 549 of Mcm2 to an arginine ($Mcm2_{K549R}$) had a less drastic effect on ATP hydrolysis, reducing the rate to ~18 % of the activity with wild type Mcm2 (0.9 ± 0.0 min^{-1}; Figure 2-2B and C). My Mcm2/6 ATPase analyses indicate that both the conserved lysine 549 of Mcm2 and arginine 708 of Mcm6 are required for ATP hydrolysis by Mcm2/6. The reduced hydrolysis observed with each of the mutants may be due to a direct effect on the hydrolysis of the ATP and/or due to reduced ATP binding. I detected interactions between Mcm2 and Mcm6 with each of the mutant proteins, indicating that the lack of ATPase activity is not due to lack of protein-protein interaction. Mcm2-Mcm6 interaction was examined to determine whether the deficiencies in ATPase activity observed with the Mcm2 and Mcm6 mutants could be explained by a failure of the mutant proteins to interact. I took advantage of a 10 X histidine tag on the N-terminus of Mcm6 that allows Mcm6 to bind to nickel (Ni) affinity

resin. In contrast, Mcm2 does not bind to resin (Figure 2-2D). However, when Mcm6 is present, Mcm2 is retained on the resin (Figure 2-2D and E). Consistent with a previous study[28], a substoichiometric amount of Mcm2 co-purifies with Mcm6 via affinity chromatography. When $Mcm2_{K549A}$, $Mcm2_{K549R}$ or $Mcm6_{R708A}$ were substituted in place of their wild type counterparts, interaction between Mcm2 and Mcm6 was still observed (Figure 2-2D and E). This experiment indicates that interaction between Mcm2 and Mcm6 is not disrupted by the mutations and that the change in activity observed with these mutations is not due to disruption of protein-protein interactions.


A
2
P-loop
SRF
6
B
Pi Produced (pmol)
500
400
300
200
100
0
0
30
60
90
Time (min)
2WT/6WT
2KR/6WT
No Protein
2WT
2KA
2KR
6WT
6RA
2KA/6WT
2WT/6RA
C
ATP Turnover (min-1)
6
4
2
0
No Protein
2WT/6WT
2KR/6WT
2KA/6WT
2WT/6RA
Coomassie-stained
D
2WT + 6WT
2KR + 6WT
2KA + 6WT
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200
116
97
66
kDa
6
2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
α-Mcm2 Blot of Elutions
E
50ng Mcm2
25ng Mcm2
2WT+6WT
2KR+6WT
2KA+6WT
2WT+6RA
2WT
2KR
2KA
6WT
6RA
150
100
75
kDa
1 2 3 4 5 6 7 8 9 10 11

Figure 2-2 Mutations at the Mcm2/6 interface reduce ATP hydrolysis by Mcm2/6. A. Schematic of the Mcm2/6 ATPase site, located at the subunit interface, is shown. The P-loop of Mcm2, which includes the invariant lysine residue (549), is indicated. "SRF" represents conserved residues in Mcm6 likely required for ATP hydrolysis by Mcm2[13]. **B.** ATP hydrolysis by Mcm2 ("2WT" filled square), $Mcm2_{K549A}$ ("2KA" filled triangle), $Mcm2_{K549R}$ ("2KR" filled circle), Mcm6 ("6WT" filled diamond), $Mcm6_{R708A}$ ("6RA" empty triangle), Mcm2 + Mcm6 ("2WT/6WT" empty square), $Mcm2_{K549A}$ + Mcm6 ("2KA/6WT" empty circle) Mcm2K549R + Mcm6 ("2KR/6WT" X) and Mcm2 + $Mcm6_{R708A}$ ("2WT/6RA" empty diamond) was measured. Reactions were quenched at 30, 60 and 90 min and μM P_i produced at each time point was determined. The reactions were performed in triplicate and the mean and standard deviation of each time point is plotted. The standard deviation bars are often eclipsed by the symbols. Addition of single stranded DNA did not affect ATP hydrolysis (data not shown) as previously observed[13; 25]. **C.** The rates less the background hydrolysis of the individual proteins from part (B) are shown with the standard deviation. **D-E.** Mcm2 and Mcm6 interaction was assayed using Ni affinity chromatography and the His-tag on Mcm6. **D.** Coomassie-stained gels of the load ("L") final wash ("W") and elution ("E") from each experiment. Lanes 1-3 contain wild type Mcm2 and Mcm6; lanes 4-6, $Mcm2_{K549R}$ (2KR) with wild type Mcm6; lanes 7-9, $Mcm2_{K549A}$ with wild type Mcm6; lanes 10-12 wild type Mcm2 with $Mcm6_{R708A}$; lanes 13-15 Mcm2 only; lanes 16-18, $Mcm2_{K549R}$ only; lanes 19-21, $Mcm2_{K549A}$ only; lanes 22-24, Mcm6 only; and lanes 25-27, $Mcm6_{R708A}$ only. The migration of molecular size markers through the gel is on the left and the migration of Mcm2 and Mcm6 on the right. **E.** Western blot of the elutions from the experiment in (A) using anti-Mcm2 antibody.

2.2.3 $Mcm2_{K549A}$ interacts with Mcm4/6/7

Previous studies in *S. pombe* and mammalian systems have demonstrated that Mcm2 forms a complex with Mcm4/6/7 in the absence of ATP[1; 2; 3]. To determine whether $Mcm2_{K549A}$ is altered in its interaction with Mcm4/6/7, I examined Mcm2's interaction with Mcm4/6/7 by taking advantage of a histidine-tag on the N-terminus of Mcm6[13] that allows Mcm4/6/7 to bind nickel (Ni) affinity resin (Figure 2-3A, lane 3). Mcm2 only binds Ni resin if it is bound to Mcm4/6/7 (Figure 2-3A, lanes 6 and 9, Figure 2-3B, lanes 3 and 5). Equimolar ratios of Mcm2 (monomer) and Mcm4/6/7 (hexamer) with 5 mM ATP were used in these assays. When the binding experiment was repeated with $Mcm2_{K549A}$, a comparable level of interaction between $Mcm2_{K549A}$ and Mcm4/6/7 was detected (Figure 2-3A, lane 12 and Figure 2-3B, lane 6). The ability of $Mcm2_{K549A}$ to

interact with Mcm4/6/7 was confirmed by co-elution of $Mcm2_{K549A}$ with Mcm4/6/7 through gel filtration columns in the presence and absence of ATP (Figure 2-3C through G). If Mcm2 interacts with Mcm4/6/7 then one would expect Mcm2 to elute from the gel filtration column earlier in the presence of Mcm4/6/7 than in its absence. In the absence of Mcm4/6/7, Mcm2 eluted in fractions 20 to 42 (Figure 2-3C). The Mcm2 peak was broad, diluting the protein below levels easily detectable by Coomassie stain and therefore Western blotting was used to detect Mcm2 in experiments with Mcm2 alone. A control experiment with $Mcm2_{K549A}$ alone indicated that it eluted similarly to wild type Mcm2, except that there was a slight tendency to aggregate, as indicated by the small proportion of $Mcm2_{K549A}$ in the void and very early fractions (Figure 2-3D). There was no discernible change to the elution profile of Mcm4/6/7 when Mcm2 was added (compare Figure 2-3E and F). However, the elution profile of Mcm2 was altered dramatically. The Mcm2 peak was more defined and it eluted earlier from the gel filtration column (fraction 14), co-eluting with Mcm4/6/7 as detected on Coomassie-stained gels (Figure 2-3F) and by Western blotting with anti-Mcm2 antibody (data not shown). The elution of $Mcm2_{K549A}$ was also shifted in the presence of Mcm4/6/7 (Figure 2-3G), similar to wild type Mcm2 with Mcm4/6/7. Interaction of Mcm2 with Mcm4/6/7 was also detected in the absence of nucleotide (data not shown). Densitometric analysis of the gel filtration fractions indicates that the ratio of Mcm2 to Mcm4, 6, or 7 is 1:1, even when excess Mcm2 was used (data not shown). Furthermore, the ratio of Mcm2 to Mcm4/6/7 was identical with $Mcm2_{K549A}$. These observations confirmed that $Mcm2_{K549A}$ interacts with Mcm4/6/7 and does so under helicase assay conditions. The resolution of the gel filtration column in the size range of Mcm4/6/7 hexamers and Mcm2/4/6/7 tetramers (414-630 kDa) is limited and the elution profile of Mcm4/6/7 with or without Mcm2 is relatively

broad, thus I made no conclusions about the size of the Mcm complexes from these experiments. More importantly, I was able to conclude that there was little or no difference in the elution profile of Mcm4/6/7 with wild type or mutant Mcm2 over a range of protein concentrations (for example, up to 8-fold excess Mcm2; data not shown).

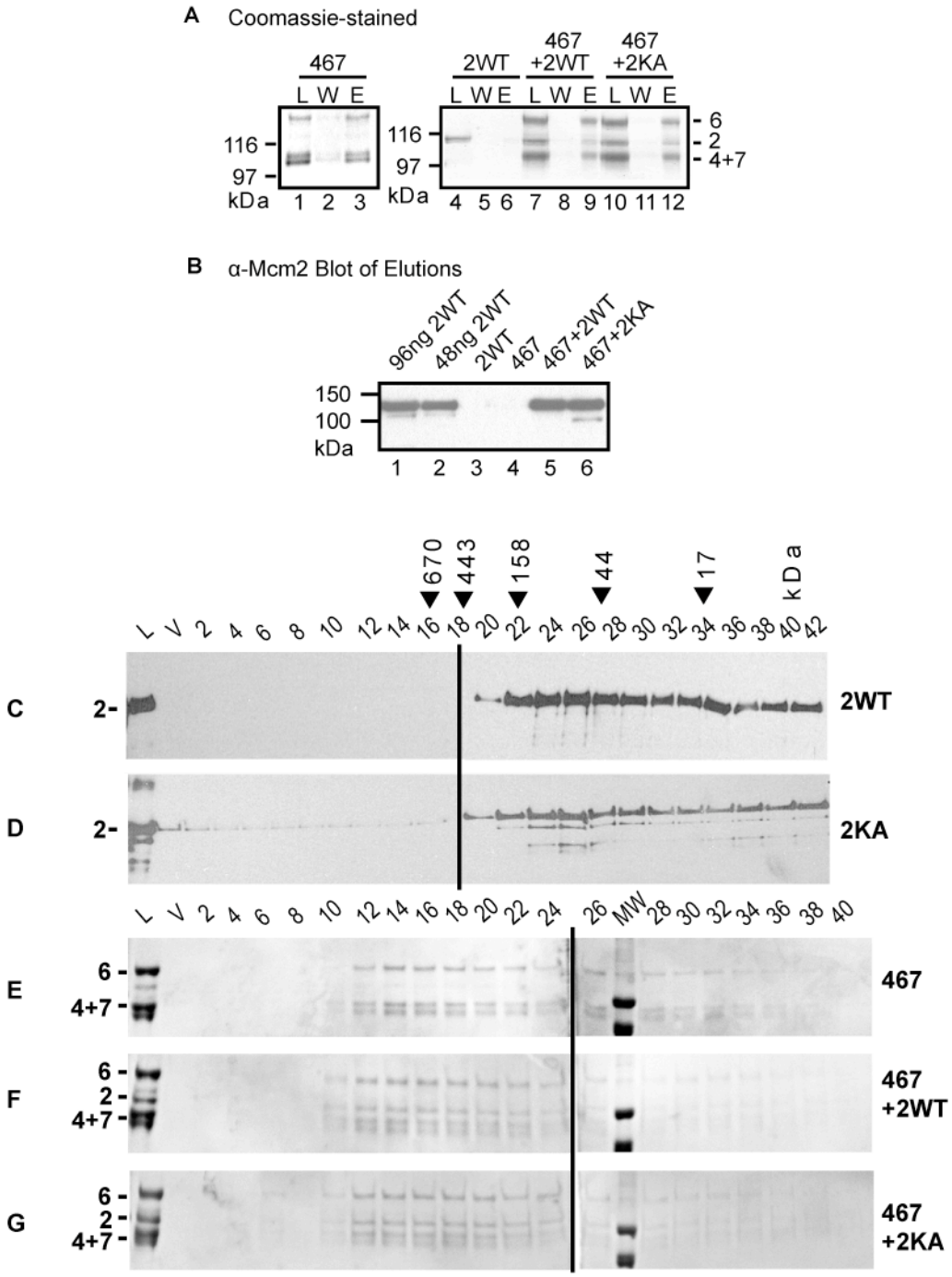

Figure 2-3 Interaction of Mcm2 with Mcm4/6/7. A. Affinity chromatography-based assay for Mcm4/6/7 interaction with Mcm2. Mcm4/6/7 and/or Mcm2 (mutant or wild type) were mixed and then bound to nickel resin. The resin was washed and then eluted with SDS loading dye. Ten percent of the load ("L"), 8 % of the wash ("W") and 40 % of the elution ("E") for each experiment are shown. Mcm2 flows through the column when analyzed alone (not shown). The Mcm4/6/7 alone experiment (lanes 1-3) was analyzed on a separate gel. **B.** Western blot analysis of the elutions from (A). Anti-Mcm2 antibody was used to confirm the presence or absence of Mcm2 (wild type or mutant) in the elutions of the experiments in (A). Lanes 1 and 2 are controls with known amounts of Mcm2, lanes 3 through 6 are the elutions of the affinity resin experiments. The input protein(s) are shown above the lane. The migrations of size markers through the gel are shown on the left. **C-G.** Gel filtration analysis of Mcm2 interaction with Mcm4/6/7 was performed at 4ºC in Buffer A containing 100 mM NaCl, 10 mM magnesium acetate and 5 mM ATP using a 2.4 ml Superose 6 PC 3.2/30 column (GE Healthcare). Fifty µl of 1.2 µM Mcm4/6/7 and/or 1.2 µM Mcm2 (mutant or wild type) were incubated on ice for 15 min in gel filtration buffer before centrifugation at 4ºC at 13,000 rpm for 10 min. The supernatant was applied to the gel filtration column and a void volume (0.8 ml) followed by 50 µl fractions were collected. Proteins were detected either by Western blotting using anti-Mcm2 antibody (C and D) or by Coomassie-staining of SDS polyacrylamide gels (E, F and G). A minor contaminant migrates between Mcm6 and Mcm2 through the gel in the load, but is not detected in the purified fractions. The fraction numbers are indicated above the gels as well as the load (L) and the void (V). The elution positions of size standards (thyroglobulin, 670 kDa; apoferritin, 443 kDa; bovine gamma globulin, 158 kDa; ovalbumin, 44 kDa; and myoglobin, 17 kDa) are indicated above the fractions.

2.2.4 $Mcm2_{K549A}$ reduces ATP hydrolysis by Mcm4/6/7

A study with *S. pombe* proteins has shown that Mcm2 almost completely inhibits ATP hydrolysis by Mcm4/6/7[1]. When I tested the effects of Mcm2 on ATP hydrolysis by *S. cerevisiae* Mcm4/6/7, addition of Mcm2 decreased ATP hydrolysis to 68% of the activity of Mcm4/6/7 in the absence of Mcm2 (40.6 ± 6.0 min^{-1} without Mcm2 and 27.8 ± 5.5 min^{-1} with Mcm2; Figure 2-4). Increasing the molar ratio of Mcm2 to Mcm4/6/7 did not increase the inhibition (data not shown). To examine whether ATP hydrolysis contributed by Mcm2 masks any reduction of Mcm4/6/7 activity, I used the mutant version of Mcm2. In the presence of $Mcm2_{K549A}$, ATP hydrolysis by Mcm4/6/7 was reduced to 11.5 ± 5.1 min^{-1} (Figure 2-4). These results suggest that Mcm2 reduces ATP

hydrolysis by Mcm4/6/7, even in the absence of ATP hydrolysis by Mcm2. Furthermore, the inhibition of Mcm4/6/7 by $Mcm2_{K549A}$ confirms the ability of the mutant to interact with Mcm4/6/7.

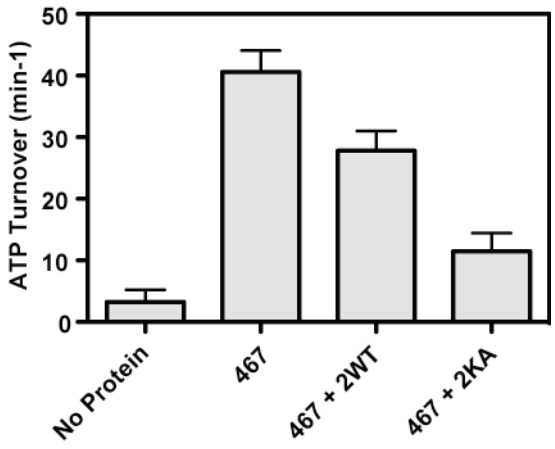

Figure 2-4 Effect of Mcm2 on ATP hydrolysis by Mcm4/6/7. ATP hydrolysis was measured with Mcm4/6/7 in the presence and absence of Mcm2 or $Mcm2_{K549A}$ as described in "Materials and Methods". Three separate experiments were performed and the rates of hydrolysis were determined, less background hydrolysis by Mcm2 or $Mcm2_{K549A}$ and plotted with the standard error of the mean. Addition of ssDNA did not affect activity (data not shown).

2.2.5 Inhibition of DNA unwinding by Mcm4/6/7 requires ATP binding and/or hydrolysis by Mcm2

Mcm2 inhibits DNA unwinding by purified Mcm4/6/7 complexes from a variety of eukaryotic systems[1; 2; 6; 29; 30]. Current models suggest a passive mechanism of inhibition, simply requiring interaction of Mcm2 and Mcm4/6/7[1; 2]. In contrast, active regulation of Mcm4/6/7 DNA unwinding would require the essential ATP site of Mcm2. I examined regulation of DNA unwinding by Mcm4/6/7 using oligonucleotide substrates established previously[6]. DNA unwinding is detected as the appearance of single stranded DNA (ssDNA) product accompanied by the loss of double stranded DNA (dsDNA)

substrate on native polyacrylamide gels (Figure 2-5A). To examine whether mutation of the essential Mcm2 ATP site affected its ability to modulate Mcm4/6/7 helicase activity, wild type Mcm2 (lanes 4-6) or $Mcm2_{K549A}$ (lanes 7-9) was titrated into a constant concentration of Mcm4/6/7. The percent DNA unwound was calculated as the proportion of total DNA that migrates as ssDNA on the gel. DNA unwinding was inhibited at all concentrations of Mcm2 assayed, reaching a maximum at equimolar ratios of Mcm2 monomer to Mcm4/6/7 hexamer (Figure 2-5B; circles). In contrast, $Mcm2_{K549A}$ was unable to inhibit DNA unwinding by Mcm4/6/7 (Figure 2-5B; squares). Neither wild type Mcm2 nor $Mcm2_{K549A}$ contained contaminating helicase or nuclease activities that would interfere with these results (Figure 2-5A; lanes 10 and 11). I concluded that ATP binding or hydrolysis by Mcm2 is required for regulation of DNA unwinding by Mcm4/6/7. Furthermore, these results indicate that $Mcm2_{K549A}$ is a valuable tool to evaluate models of the mechanism by which Mcm2 inhibits helicase activity.

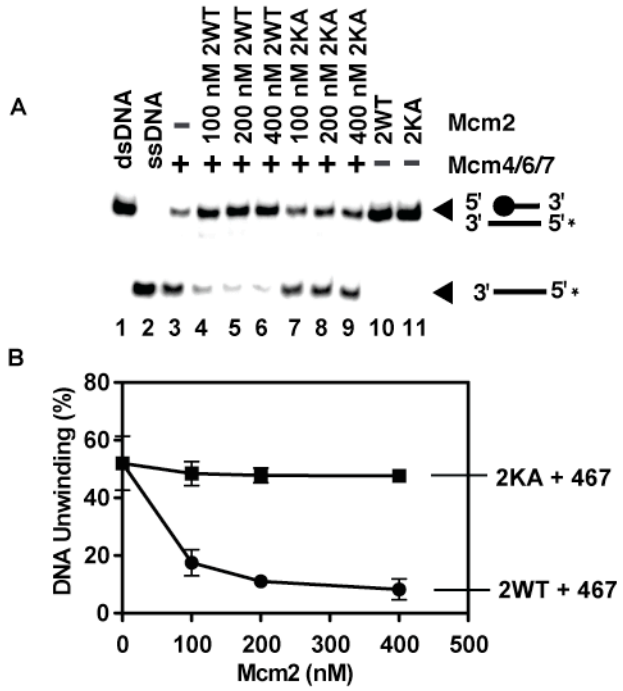

Figure 2-5 Mcm4/6/7 DNA unwinding inhibition by wild type and mutant Mcm2. A. DNA unwinding in the presence of mutant and wild type Mcm2 was examined. The indicated concentrations of wild type Mcm2 ("2WT") or $Mcm2_{K549A}$ ("2KA") were mixed with 200 nM Mcm4/6/7 and assayed for DNA unwinding. **B.** The percentage of DNA unwinding was calculated from four replicates and the amount of ssDNA produced from total DNA.

2.2.6 DNA binding by Mcm4/6/7

Mcm2 may inhibit the helicase activity of Mcm4/6/7 by interfering with DNA binding by Mcm4/6/7[7; 29; 31], a necessary prelude to DNA unwinding. Before examining whether $Mcm2_{K549A}$ inhibits single stranded DNA (ssDNA) binding by Mcm4/6/7, I first established a ssDNA-binding assay. I used a gel filtration based approach to examine binding of Mcm4/6/7 to circular DNA similar to assays previously used to examine DNA binding by the *E. coli* replicative helicase, DnaB[32]. One advantage of this assay is that it utilizes circular DNA and thus helicases cannot translocate off the ends, as may occur on linear DNA. I used a gel filtration column in which DNA and any bound protein elute at a

relatively small volume, while free protein elutes later. I radiolabeled Mcm4/6/7 by engineering a Protein Kinase A (PKA) target site on the N-terminus of Mcm4. The protein, $Mcm4^{PK}$ was purified, reconstituted into a complex with Mcm6 and Mcm7 then radiolabeled to high specific activity using [γ ^{32}P]-ATP and PKA. When $Mcm4^{PK}$/6/7 was applied to the gel filtration column in the absence of ssDNA, the majority of the protein eluted in fractions 19-29 (Figure 2-6A; triangles). Mixing $Mcm4^{PK}$/6/7 and M13mp18 ssDNA followed by gel filtration resulted in most $Mcm4^{PK}$/6/7 co-eluting with DNA in earlier fractions (10-15), indicating that DNA binding by $Mcm4^{PK}$/6/7 had occurred (Figure 2-6A; squares). The elution profile of ssDNA through the gel filtration column was confirmed by agarose gel electrophoresis in a separate experiment and the presence of the Mcm6 and Mcm7 subunits in the ssDNA-containing fractions was verified by Western blotting of sample experiments (data not shown). I also examined whether $Mcm4^{PK}$/6/7 binds to circular dsDNA, using a 7.3 kb double stranded plasmid in place of M13mp18 and observed that $Mcm4^{PK}$/6/7 does not bind dsDNA to the same extent as ssDNA (Figure 2-6A; circles), consistent with a previous study[5].

The experiments described above were performed in the presence of 5 mM ATP. When ATP was omitted from the DNA binding assays, very little binding of $Mcm4^{PK}$/6/7 to ssDNA was detected (Figure 2-6A; circles). I also examined the effects of ADP and the slowly hydrolyzed analogue, ATPγS on DNA binding by $Mcm4^{PK}$/6/7. When ADP was provided to $Mcm4^{PK}$/6/7 in place of ATP, binding was observed, but was slightly reduced to 80 ± 10 % of $Mcm4^{PK}$/6/7 bound in the presence of ATP (Figure 2-6C; circles). In contrast, there was no decrease in ssDNA binding by Mcm4/6/7 when ATPγS was provided in place of ATP (Figure 2-6D; circles). Very little $Mcm4^{PK}$/6/7 was observed in

the early fractions in the absence of ssDNA with any of the nucleotides (Figure 2-6A and data not shown). In summary, I have shown that Mcm4/6/7 binds to ssDNA circles in a nucleotide-dependent manner and that DNA binding with ATP and ATPγS is slightly greater than with ADP.

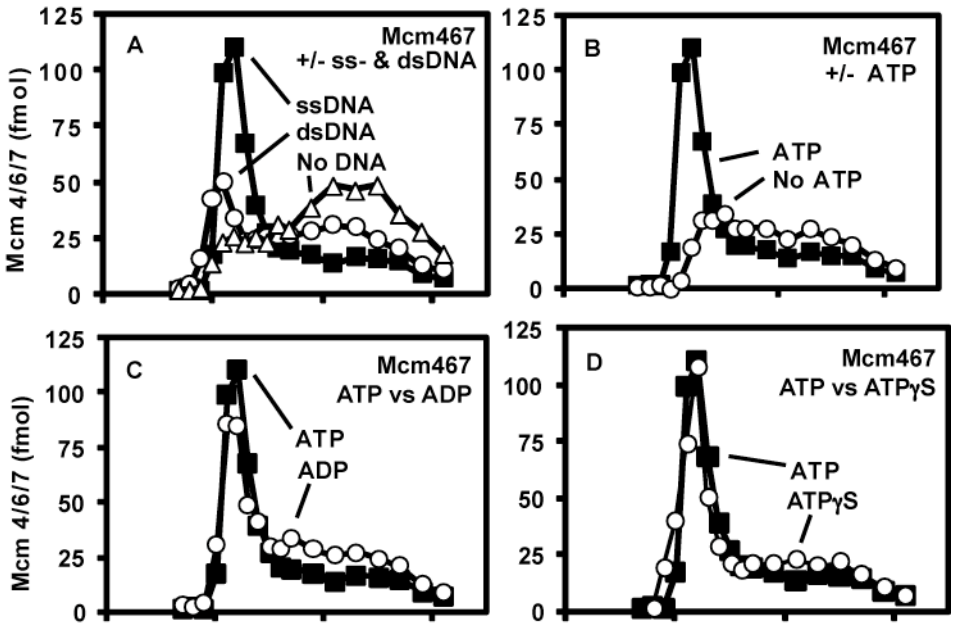

Figure 2-6 Binding of Mcm4/6/7 to single stranded DNA. DNA binding was measured in a gel filtration based assay as described in "Materials and Methods". Plots indicate the amount of $Mcm4^{PK}/6/7$ in each fraction as determined by scintillation counting of ^{32}P-labeled $Mcm4^{PK}$. **A.** Binding of $Mcm4^{PK}/6/7$ to ssDNA (square) and dsDNA (circle) in the presence of ATP is shown. $Mcm4^{PK}/6/7$ in the presence of ATP but in the absence of DNA is also indicated (triangle). **B.** $Mcm4^{PK}/6/7$ binding to ssDNA in the presence (square) and absence (circle) of 5 mM ATP. **C.** Comparison of $Mcm4^{PK}/6/7$ binding to ssDNA with ATP (square) or ADP (circle), and (**D)** with ATP (square) or ATPγS (circle). Note that Mcm4/6/7 binding to ssDNA was examined using a range of protein and DNA concentrations and representative assays are shown with the purpose of demonstrating the effect of Mcm2 on Mcm4/6/7 interaction with ssDNA (as described below). Relative binding of Mcm4/6/7 to ssDNA with ATP, ADP and ATPγS was similar under all conditions tested.

2.2.7 Mcm2 requires ATP hydrolysis to inhibit ssDNA binding by Mcm4/6/7

In the presence of ATP, Mcm2 reduced ssDNA binding of $Mcm4^{PK}$/6/7 to 65 ± 8 % the level seen when Mcm2 is absent (Figure 2-7A; circles). I also examined the effects of ADP and ATPγS on the ability of Mcm2 to inhibit ssDNA binding by Mcm4/6/7. In the presence of the poorly hydrolyzed ATP analogue, ATPγS Mcm2 did not inhibit DNA binding of $Mcm4^{PK}$/6/7 (Figure 2-7B; circles). This suggests that ATP hydrolysis by Mcm2 is required for inhibition of Mcm4/6/7. ATPγS is likely bound by Mcm2 since it inhibits ATP hydrolysis by Mcm2/6 (data not shown). In contrast, ADP supported Mcm2-mediated inhibition to the same extent as was observed with ATP (reduction to 65 ± 9 % of the amount of $Mcm4^{PK}$/6/7 bound to ssDNA when Mcm2 is absent; Figure 2-7C, circles). Thus, it appears that the ADP-bound state of Mcm2 is required to inhibit Mcm4/6/7 binding to ssDNA. When ATPγS is provided, inhibition may not occur since it cannot be hydrolyzed to ADP. An alternative explanation is that the different nucleotides exert their effects on Mcm4/6/7 by changing its susceptibility to Mcm2. To differentiate between these scenarios, I returned to the Mcm2 mutant and examined its ability to regulate ssDNA binding by Mcm4/6/7. $Mcm2_{K549A}$ did not inhibit DNA binding of $Mcm4^{PK}$/6/7 in the presence of ATP (Figure 2-7A; triangles). This observation suggests that ATP hydrolysis by Mcm2 is required for inhibition. However, the same result would occur if $Mcm2_{K549A}$ were defective in nucleotide binding. To test this possibility I examined the ability of $Mcm2_{K549A}$ to inhibit DNA binding by $Mcm4^{PK}$/6/7 in the presence of ADP. Recall that ADP supports inhibition by wild type Mcm2. $Mcm2_{K549A}$ inhibited $Mcm4^{PK}$/6/7 binding to ssDNA to 69 ± 6 % the level seen in the absence of Mcm2 (Figure 2-7C; triangles), which is approximately the same extent as seen with wild

type Mcm2 (compare circles to triangles in Figure 2-7C), therefore $Mcm2_{K549A}$ is not defective in nucleotide binding. Thus, I conclude that ATP hydrolysis by Mcm2 is required for it to inhibit DNA binding and unwinding by Mcm4/6/7.

I tested whether Mcm2 associates with DNA-bound Mcm4/6/7 by performing quantitative Western blot analysis on the fractions from DNA binding experiments with Mcm4/6/7 and mutant or wild type Mcm2. Mcm2 co-eluted with M13 and $Mcm4^{PK}$/6/7 in amounts proportional to the amount of $Mcm4^{PK}$/6/7 (Figure 2-7D). These results emphasize that interaction of Mcm2 with Mcm4/6/7 is not sufficient for inhibition of DNA binding by Mcm4/6/7. Mcm2 may affect Mcm4/6/7 binding to ssDNA by removing bound Mcm4/6/7 from ssDNA. To test this idea, I bound $Mcm4^{PK}$/6/7 to ssDNA and isolated the complex from free protein using gel filtration. Equal portions of the peak fraction were incubated either with Mcm2 or with buffer and the mixtures were then subjected to a second gel filtration column. Regardless of whether Mcm2 was present, $Mcm4^{PK}$/6/7 was retained on the DNA after the second gel filtration column, suggesting that Mcm2 cannot remove $Mcm4^{PK}$/6/7 from DNA once it is bound (Figure 2-7E). Since the amount of protein and DNA that is applied to the second gel filtration column differs from our "standard" conditions, I confirmed that Mcm2 is able to inhibit DNA binding by $Mcm4^{PK}$/6/7 using the same amounts of protein and DNA as were applied to the second gel filtration column (Figure 2-7F). Thus, I concluded that Mcm2 is unable to remove $Mcm4^{PK}$/6/7 already bound to ssDNA. This finding suggests that Mcm2 inhibition of Mcm4/6/7 ssDNA binding occurs before or during formation of a Mcm4/6/7-ssDNA complex.

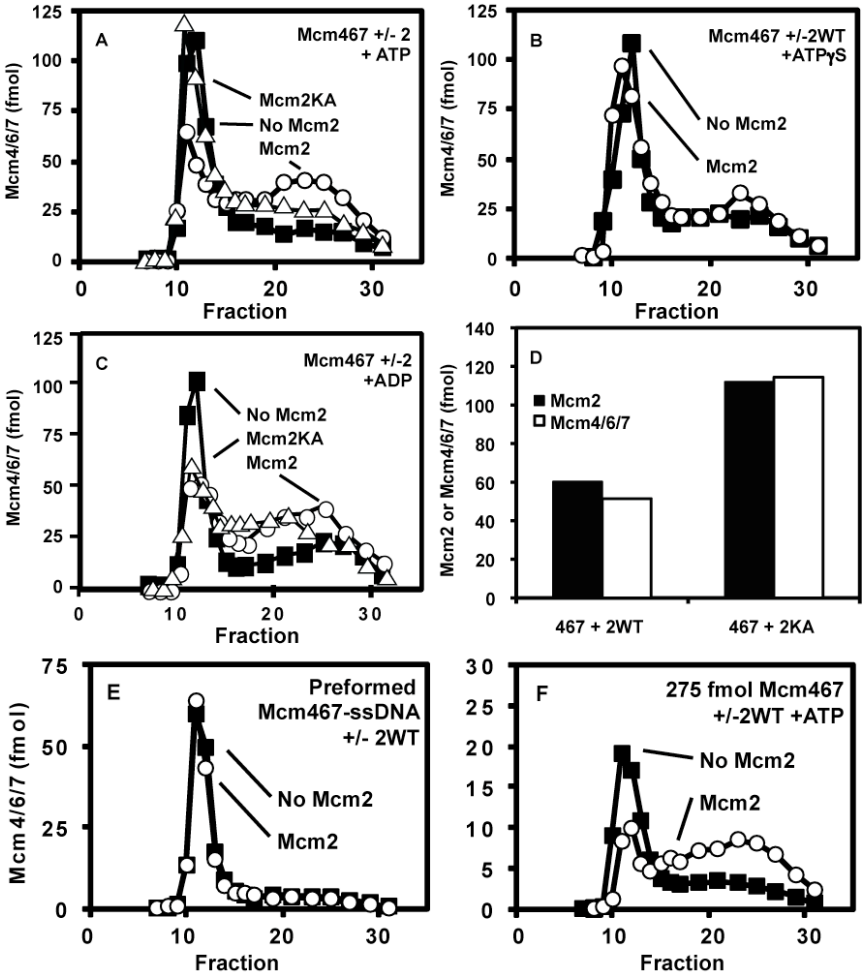

Figure 2-7 Mcm2 inhibits single stranded DNA binding by Mcm4/6/7. DNA binding was measured in a gel filtration based assay. Plots indicate the amount of $Mcm4^{PK}$/6/7 in each fraction. **A.** $Mcm4^{PK}$/6/7 binding to ssDNA absence (square) of and in the presence of wild type (circle) or mutant (triangle) Mcm2 with 5 mM ATP. **B & C.** $Mcm4^{PK}$/6/7 binding to ssDNA in the presence (circle) and absence (square) of 10 pmol Mcm2 with (B) 5 mM ATPγS and (C) $Mcm4^{PK}$/6/7 binding to ssDNA absence (square) of and in the presence of wild type (circle) or mutant (triangle) Mcm2 with 5 mM ADP. **D.** Quantitative Western blot analysis of Mcm2 (mutant or wild type) in DNA binding experiments with 5 mM ATP. The amount of Mcm2 determined from the blots as well as the amount of $Mcm4^{PK}$/6/7 determined by scintillation counting in the peak fraction (fr 11) from the experiments in d and e are shown. The amount of Mcm2 that binds to M13 in the absence of Mcm4/6/7 was subtracted. DNA binding was measured in a gel filtration based assay. Plots indicate the amount of $Mcm4^{PK}$/6/7 in each fraction. **E.** $Mcm4^{PK}$/6/7 (4 pmol) was incubated with ssDNA (8 pmol) and applied to a gel filtration column. The peak fraction was determined and reactions containing $Mcm4^{PK}$/6/7-DNA complex were split in equal parts. A ~30-fold molar excess of Mcm2 (circle) was added to one half and an equal volume of buffer to the other (square) and binding to ssDNA was redetermined by gel filtration. **F.** As a control, the same amount of $Mcm4^{PK}$/6/7 and ssDNA (275 fmol of each) used in (E) was incubated in the presence (circle) or absence (square) of a ~30-fold molar excess of Mcm2.

2.2.8 $Mcm2_{K549A}$ alters DNA binding of the Mcm2-7 complex

DNA unwinding *in vivo* requires all six Mcm2-7 subunits, therefore I tested whether Mcm2 ADP-mediated regulation of DNA binding applied to this complex. I reconstituted both a wild type Mcm2-7 complex and a complex containing the $Mcm2_{K549A}$ mutant ($Mcm2_{K549A}$-7) and compared their activities. Given that Mcm2 inhibits Mcm4/6/7 DNA binding, but Mcm2 $_{K549A}$ does not (which can also be stated as $Mcm2_{K549A}$/4/6/7 has a higher affinity for DNA than Mcm2/4/6/7), I anticipated that $Mcm2_{K549A}$-7 would have enhanced DNA binding compared to wild type Mcm2-7. The Mcm2-7 complexes were formed with a PKA target sequence on the Mcm3 subunit. The PKA tag did not affect activity of the complex and allowed labeling to high specific activity with ^{32}P (data not shown). I assayed for DNA binding by Mcm2-7^{3PK} and $Mcm2_{K549A}$-7^{3PK} using gel filtration. Both Mcm2-7^{3PK} and $Mcm2_{K549A}$-7^{3PK} require nucleotide to bind ssDNA and do not bind dsDNA (Figure 2-8A through E; data not shown). I next tested the relative affinities of Mcm2-7^{3PK} and $Mcm2_{K549A}$-7^{3PK} for ssDNA binding in the presence of ATP by decreasing the amount of DNA in the assay with a constant concentration of Mcm2-7. Decreasing the ssDNA concentration from 100 fmol to 50 fmol resulted in an 8-fold reduction in DNA binding by Mcm2-7^{3PK} (Figure 2-8C through F). In contrast, $Mcm2_{K549A}$-7^{3PK} bound DNA robustly at all concentrations of DNA tested, including at a level of DNA that Mcm2-7^{3PK} bound very poorly (50 fmol; Figure 2-8E and F). Consistent with my DNA binding studies of Mcm4/6/7, mutation of the ATP site in Mcm2 enhances DNA binding of Mcm complexes.

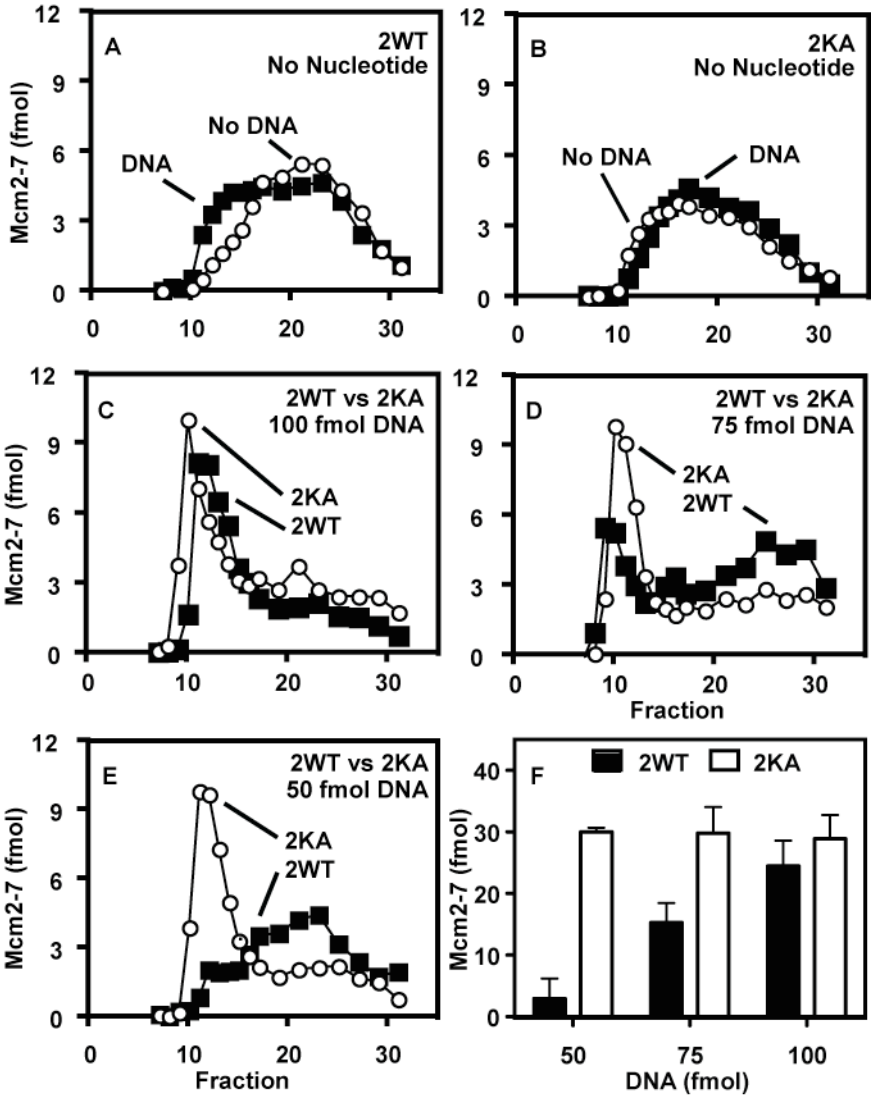

Figure 2-8 DNA binding by $Mcm2_{K549A}$-7 is enhanced compared to Mcm2-7. Mcm2-7^{3PK} and $Mcm2_{K549A}$-7^{3PK} were reconstituted from pure subunits. **A** & **B.** DNA binding of Mcm2-7 complexes was measured using gel filtration as described in "Materials and Methods." Binding of Mcm2-7^{3PK} (A**)** and $Mcm2_{K549A}$-7^{3PK} (B) in the absence of nucleotide was measured in the presence (square) and absence (circle) of ssDNA. **C-E.** DNA binding by Mcm2-7^{3PK} (square) or $Mcm2_{K549A}$-7 (circle) was measured with either 100 fmol (part C), 75 fmol (part D) or 50 fmol (part E) of ssDNA in the presence of ATP. **F.** The amount of DNA binding by Mcm2-7^{3PK} or $Mcm2_{K549A}$-7^{3PK} was quantified by summing the amount of Mcm2-7^{3PK} found in the peak fractions (fractions 10 through 15). The amount of Mcm2-7^{3PK} in the peak fractions for three replicates each at DNA concentrations of 50 fmol, 75 fmol and 100 fmol, less the amount of protein found in the early fractions in the absence of ssDNA, were quantified and plotted.

2.2.9 An Mcm2 mutant with minimal ATP hydrolysis regulates Mcm4/6/7 and supports viability

The observation that ATP hydrolysis by Mcm2 regulates DNA binding by Mcm2-7 raises the possibility that ATP hydrolysis by Mcm2 has a regulatory role and not a direct role in catalysis of DNA unwinding by Mcm2-7. Support for this idea comes from the observation that $Mcm2_{K549A}$ does not hydrolyze ATP yet is able to support DNA unwinding by Mcm2-7, albeit at a lower level than wild type Mcm2-7[27]. If ATP hydrolysis by Mcm2 is regulatory, then one might expect that an intermediate level of hydrolysis would be sufficient to support its activity. To explore this idea, I used the $Mcm2_{K549R}$ mutant which had a less drastic effect on ATP hydrolysis than $Mcm2_{K549A}$ when mixed with Mcm6 (Figure 2-2B & C). I examined whether the mutations in Mcm2 affect viability of cells on rich media. I compared the growth of *S. cerevisiae* when plasmids bearing *MCM2*, $mcm2_{K549R}$ or $mcm2_{K549A}$ were present as the sole copy of *MCM2* after shuffling out a wild type copy of *MCM2* present on a *URA3* plasmid. Strains containing wild type *MCM2* or $mcm2_{K549R}$ were able to grow on plates containing 5-FOA, which selects for loss of the *URA3*-containing plasmid bearing *MCM2* (Figure 2-9A). As observed previously[25; 26], $mcm2_{K549A}$ was unable to support growth. The lower level of ATP hydrolysis observed with $Mcm2_{K549R}$ was sufficient for viability at all temperatures tested (Figure 2-9A) and is consistent with the ability of this mutant to modulate Mcm4/6/7 DNA unwinding to the same extent as wild type Mcm2 (Figure 2-9B). These data suggest that ATP hydrolysis by Mcm2 is essential for its regulatory function, but only a minimal amount of ATP hydrolysis by Mcm2 is required for this role.

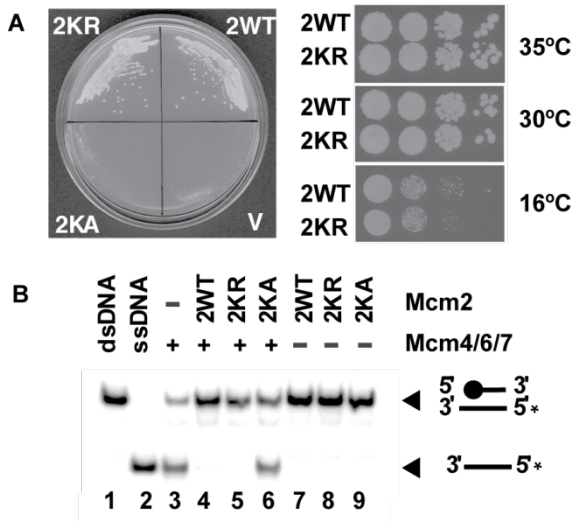

Figure 2-9 Minimal ATP hydrolysis by Mcm2 supports yeast cell viability and Mcm2. A. Viability of wild type *MCM2* (2WT), $mcm2_{K549R}$ (2KR) and $mcm2_{K549A}$ (2KA) were determined by plasmid shuffling on media containing 5-FOA. "V" indicates a vector control with no *MCM2* insert. Growth of shuffled strains containing *MCM2* (WT) or $mcm2_{K549R}$ (KR) at 16ºC, 30ºC and 35ºC is shown on the right. **B.** DNA unwinding in the presence of mutant and wild type Mcm2 was examined. Wild type Mcm2, $Mcm2_{K549R}$ or $Mcm2_{K549A}$ were mixed with equimolar (200 nM) Mcm4/6/7 and assayed for DNA unwinding. Shown is a representative PhosphorImager scan. Lanes 1 and 2 are dsDNA substrate and ssDNA product, respectively and their migrations through the gel are shown on the right. The substrate includes a 5' biotin on the top strand, as indicated by the black sphere. The proteins added to each assay are indicated at the top of the gel and the lane numbers are shown underneath.

2.2.10 Mcm3/5 does not restore the activity of $Mcm2_{K549A}$

DNA unwinding by Mcm4/6/7 is also inhibited by Mcm3/5 in *S. pombe*[1] and humans[7]. In these cases, Mcm2 and Mcm3/5 appear to function independently[1]. To address this question with the *S. cerevisiae* proteins, I examined the ability of Mcm3/5 to inhibit DNA unwinding by Mcm4/6/7 in the presence and absence of wild type and mutant Mcm2. I found that Mcm3/5 inhibits DNA unwinding by Mcm4/6/7, but not to the same extent as the equivalent molar concentration of Mcm2 (assuming a Mcm3/5 dimer and a Mcm2 monomer; Figure 2-10A, compare lanes 4 and 6). When both Mcm2 and Mcm3/5 were added to Mcm4/6/7, DNA unwinding was inhibited further (Figure 2-10A, lane 7). However, when $Mcm2_{K549A}$ and Mcm3/5 were added to Mcm4/6/7, the extent of Mcm4/6/7 inhibition was the same as observed for Mcm3/5 on its own (Figure 2-10A, lane 8). These results indicate that Mcm3/5 is unable to compensate for the defect in $Mcm2_{K549A}$ and that Mcm3/5 functions independently of Mcm2 to modulate Mcm4/6/7 activity.

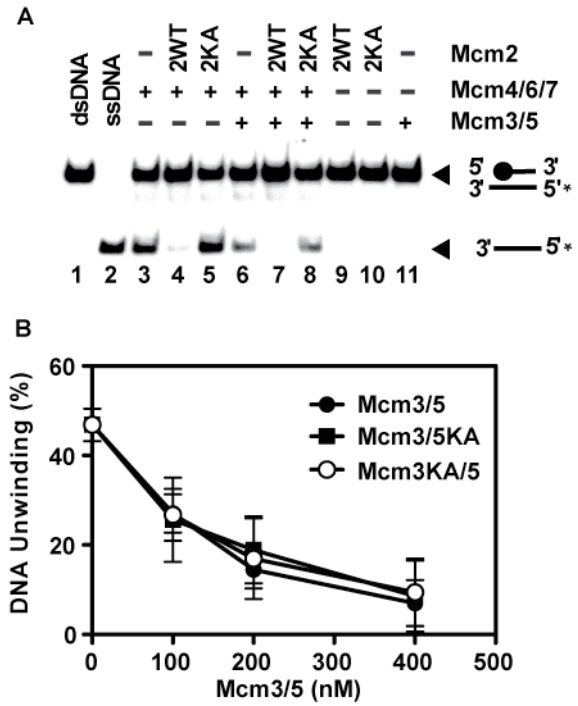

Figure 2-10 Inhibition of Mcm4/6/7 by Mcm3/5. A. Mcm2 and/or Mcm3/5 inhibition of DNA unwinding by Mcm4/6/7 was analyzed. Mcm2, Mcm3/5 and $Mcm2_{K549A}$ (100 nM) were mixed as indicated with 100 nM Mcm4/6/7 and DNA unwinding was assayed. The migrations of dsDNA substrate and ssDNA product are indicated to the right of the gel. **B.** DNA unwinding by Mcm4/6/7 was examined in the presence or absence of increasing amounts of Mcm3/5 (filled circle), $Mcm3_{K415A}/5$ (empty circle) or $Mcm3/5_{K422A}$ (square). The means of three experiments are plotted.

2.2.11 P-loop mutations do not affect inhibition by Mcm3/5

I utilized P-loop mutations in Mcm3 and Mcm5 to determine whether the ATP sites of these proteins are important for their ability to inhibit Mcm4/6/7. $Mcm3_{K415A}/5$ and $Mcm3/5_{K422A}$ were titrated into DNA unwinding assays containing Mcm4/6/7 (Figure 2-10B). Both $Mcm3/5_{K422A}$ and $Mcm3_{K415A}/5$ inhibit DNA unwinding by Mcm4/6/7 to the same extent as wild type Mcm3/5. This result suggests that ATP binding or hydrolysis is not important for the regulation of Mcm4/6/7 by Mcm3/5. It should be noted that the lysine to alanine mutations in Mcm3 and Mcm5 used here are inviable in yeast[25]. These

results also suggest that the ADP-mediated inhibition of Mcm4/6/7 is unique to the Mcm2 subunit.

2.3 Discussions

By examining the effects of Mcm2 on the activities of Mcm4/6/7, I have shown that Mcm2 has an essential role in regulating DNA binding by Mcm complexes and that a minimal level of ATP hydrolysis by Mcm2 is required for this activity. Specifically, Mcm2 inhibition of ssDNA binding by Mcm4/6/7 occurs in the presence of ATP and ADP but not with ATPγS. Inhibition with a Mcm2 mutant unable to hydrolyze ATP occurs in the presence of ADP and not ATP. Since the mutant ($Mcm2_{K549A}$) interacts with Mcm4/6/7 but does not inhibit Mcm4/6/7, interaction alone is not sufficient for Mcm2 to exert its effects. Rather, my results imply that Mcm2 communicates its nucleotide binding status to the other subunits. The altered properties of $Mcm2_{K549A}$ were also observed in Mcm2-7, indicating that my findings are relevant to the Mcm complex *in vivo*. Furthermore, the requirement for only a minimal level of ATP hydrolysis by Mcm2 suggests that Mcm2 plays a regulatory rather than direct catalytic role in DNA unwinding by Mcm2-7.

2.3.1 ATP sites in Mcm proteins

The ATP sites of Mcm2-7 are located at the interface between subunits, with one subunit binding nucleotide and the other providing a *trans*-acting catalytic residue, the arginine finger[13; 28]. A study on *S. sulfolobus* MCM has indicated that additional *trans*-acting residues, conserved in eukaryotic Mcm proteins, are important for the function of the helicase[33]. Furthermore, structural studies on the viral SF3 helicases, E1 from papillomavirus and large T antigen (Tag) from simian virus 40 (also AAA+ proteins),

illustrate that these *trans*-acting residues undergo conformational changes dependent on the nucleotide bound to the neighboring subunit[34; 35]. Interestingly, it has been noted that some *trans*-acting residues that contact the phosphates of ATP bound to the neighboring subunit are adjacent to β hairpin structures that interact with ssDNA in the E1 structure and undergo positional changes in response to ATP binding and hydrolysis[33; 35]. Therefore, binding of nucleotide to one Mcm subunit has the potential to affect the conformation and activity of the adjacent subunit through *trans* interactions of specific residues with the phosphate moieties of the nucleotide. It is therefore reasonable to envision possible mechanisms by which Mcm2 could communicate its nucleotide bound state to Mcm4/6/7 and other subunits of Mcm2-7 to affect ssDNA binding by these complexes.

2.3.2 DNA binding by Mcm4/6/7

In contrast to my findings, Mcm4/6/7 did not bind circular ssDNA in a previous study[5]. This may be due to differences in the point of addition of nucleotide since I have found that incubation of Mcm4/6/7 with nucleotide before DNA interferes with DNA binding[4]. I also note that there are differences in the ability of ADP to support DNA binding by Mcm4/6/7 in the two studies. These are not easily explained, but I note that the type of assay utilized and the preparation of proteins were not identical in the two studies.

2.3.3 Mechanism of Mcm2 inhibition of Mcm4/6/7

Mcm2 was previously thought to inhibit Mcm4/6/7 by disrupting the oligomeric structure of Mcm4/6/7[1; 2]. Here, I have shown that interaction with Mcm4/6/7 is not sufficient for Mcm2 to exert its effects and thus the model that Mcm2 acts solely by disrupting a Mcm4/6/7 hexamer is not upheld. Indeed, $Mcm2_{K549A}$ interacts with Mcm4/6/7 to the same extent as wild type Mcm2 suggesting that similar-sized complexes are formed with mutant and wild type Mcm2. I have considered the different mechanisms that may account for the ability of Mcm2 to disrupt ssDNA binding by Mcm4/6/7. The simplest explanation is that Mcm2 could compete with Mcm4/6/7 for binding to DNA. However, I detected only very weak ssDNA binding by Mcm2 (consistent with a previous study[36]) such that only 1/20 ssDNA molecules were bound by Mcm2 under the conditions used in my assays (data not shown). I have also shown that Mcm2 is unable to remove Mcm4/6/7 from DNA once it is bound. Therefore, Mcm2 appears to act before or when Mcm4/6/7-ssDNA complexes are formed whereby interaction of the ADP-bound form of Mcm2 with Mcm4/6/7 results in a conformation of Mcm4/6/7 incapable of binding ssDNA.

The inability of $Mcm2_{K549A}$ to inhibit ssDNA binding and helicase activities of Mcm4/6/7 are likely closely related. However, it is interesting to note that Mcm2 inhibition of DNA binding was not as robust as its inhibition of DNA unwinding. This difference may reflect a disparity in the dynamic ranges of the assays and/or the dilution of protein complexes that occurs in the gel filtration assays. Alternatively, there may be additional mechanisms by which Mcm2 regulates Mcm4/6/7.

The effects of ATP hydrolysis by Mcm2 on DNA binding by Mcm4/6/7 are presumably communicated allosterically. I argue that the effects of ATP hydrolysis by Mcm2 on DNA binding are not communicated through changes in ATP binding and hydrolysis by Mcm4/6/7 since $Mcm2_{K549A}$ inhibits Mcm4/6/7 ATPase activity but not DNA binding. In addition, Mcm4/6/7 ssDNA binding is only slightly affected by ATP hydrolysis. Rather, ATP hydrolysis by Mcm2 may affect the conformation of one or more subunits in Mcm4/6/7 thereby reducing DNA binding.

2.3.4 Mcm4/6/7 inhibition by Mcm3/5

DNA unwinding by Mcm4/6/7 is also inhibited by Mcm3/5 (this study and refs. 1; 7). However in contrast to Mcm2, mutation of the Mcm5 and Mcm3 P-loops did not interfere with their abilities to regulate Mcm4/6/7. Consistent with previous studies, I have shown that Mcm3/5 and Mcm2 act independently. Interestingly, the Schwacha group has shown that the Mcm2 and Mcm3 P-loops are not essential for DNA unwinding by Mcm2-7[27] even though mutation of the conserved P-loop lysine to alanine in Mcm2 or Mcm3 results in inviability (as does mutation of Mcm5; ref. 25). My observation that of these three Mcms only the Mcm2 P-loop regulates DNA binding is consistent with other observations that have led to the suggestion that each ATP site within Mcm2-7 contributes differently to the activity of the complex[25; 27; 28; 37].

2.3.5 Mcm2 function within Mcm2-7

The ability of Mcm2 to regulate Mcm4/6/7 is coincident with its ability to support yeast growth, thus the inhibitory role of Mcm2 likely has a role *in vivo*. It is important to note that each of Mcm2-7 are required for DNA unwinding during DNA replication and Mcm4/6/7 is not sufficient to support DNA replication[38; 39; 40], thus a biological role for

the effects of Mcm2 on Mcm4/6/7 is found within the context of Mcm2-7. I observed that a functional Mcm2 ATP site is required to modulate ssDNA binding by the Mcm2-7 complex. As with the inhibition of Mcm4/6/7 DNA binding, the ATP binding status of Mcm2 is likely relayed to other Mcm subunits within Mcm2-7 to alter DNA binding of the complex. I can envision Mcm2 affecting DNA binding by Mcm2-7 *in vivo* in two ways. One is during DNA unwinding and the other is within the activation process. In general, helicases are thought to function via repeated cycles of DNA binding, translocation and DNA release[11]. The translocation steps are coupled to ATP binding and hydrolysis, much like the effects of Mcm2 on DNA binding by Mcm4/6/7. Thus, during translocation, Mcm2 inhibition of DNA binding may be important for DNA release. It would therefore follow that mutations that severely impair ATP hydrolysis by Mcm2 would cripple DNA unwinding by Mcm2-7. Interestingly, $Mcm2_{KA}$-7 retains DNA unwinding activity, albeit at a reduced level compared to wild type Mcm2-7, unlike similar mutations in all other Mcm subunits except Mcm3[27]. Furthermore, $Mcm2_{K549R}$ with very low ATPase activity compared to wild type Mcm2 supports viability. The low catalytic rate of this mutant might suggest that the Mcm2 ATP site is not necessarily required for catalysis of helicase activity. Interestingly, Mcm2 is targeted by the Dbf4-dependent kinase (DDK) comprised of the Cdc7 catalytic subunit and Dbf4 regulatory subunit[41; 42; 43; 44]. Modification of Mcm2 by DDK may be important in modulating its regulatory activity.

2.5 References

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CHAPTER 3 REGULATION OF MCM2-7 ACTIVITY BY DDK PHOSPHORYLATION OF MCM2 OCCURS THROUGH MODULATION OF MCM2 ATPASE ACTIVITY

3.1 INTRODUCTION

The observation in Chapter 2 that ATP hydrolysis by Mcm2 regulates DNA binding and unwinding by Mcm complexes is the starting point of the studies in this chapter. I surmised that since Mcm2 functions as an active regulator of DNA binding and unwinding, its regulatory activity is likely controlled. Phosphorylation of a protein is one way in which the activity of a protein may be regulated.

Unlike other replicative helicases that contain six copies of identical subunits, minichromosome maintenance proteins 2 through 7 (Mcm2-7) is comprised of six distinct, but related subunits[1]. The requirement for six different subunits in eukaryotic cellular replication is thought to reflect individual roles for each subunit. For example, a subcomplex comprised of Mcm4, 6 and 7 is sufficient for DNA unwinding *in vitro*[2; 3; 4]. Mcm2 and Mcm3/5 inhibit DNA unwinding by Mcm4/6/7[2; 4], leading to the suggestion that Mcm4/6/7 comprises the catalytic core of the replicative helicase with Mcm2, 3 and 5 playing essential regulatory roles. This idea is consistent with the finding that, in contrast to wild type Mcm2, mutations that severely inhibit ATP hydrolysis by Mcm2 do not strongly affect DNA unwinding by Mcm2-7 in *Saccharomyces cerevisiae*[5; 6]. I have proposed that Mcm2 regulates DNA binding by Mcm2-7 during DNA unwinding since mutations that decrease ATP hydrolysis by Mcm2 increase binding to single stranded DNA[7].

Mcm2-7 is the essential target of the S-phase kinase, DDK[8; 9; 10; 11; 12; 13]. DDK activates DNA unwinding by Mcm2-7 *in vivo* thereby triggering origin firing throughout

S phase[9; 14; 15; 16; 17; 18; 19; 20]. Mcms were first thought to be targets for DDK based on the observation that a mutation in *mcm5* (*bob1*) allows growth with an otherwise lethal deletion of *cdc7*, the gene for the catalytic subunit of DDK[21]. Furthermore, several of the Mcm subunits are phosphorylated by DDK[8; 22; 23; 24; 25; 26; 27; 28; 29; 30]. It should be noted that Mcm5 is not a substrate for DDK and the suppressor activity of the *mcm5-bob1* mutation is mediated allosterically. Phosphorylation of Mcm4 is likely the essential event for the initiation of DNA replication; mutations that delete a portion of the N-terminus and/or replace phosphorylation sites with phosphomimetic aspartic acid residues in the N-terminus of Mcm4 bypass the requirement for *CDC7*[9]. Phosphorylation of human and budding yeast Mcm4 is implicated in recruiting the cell division cycle 45 protein (Cdc45) to complexes at replication origins, a necessary prelude to origin firing[8; 29; 31; 32].

Mcm2 is also phosphorylated by DDK[23; 27; 28; 30; 33; 34]. Multiple roles for the phosphorylation of Mcm2 by DDK have been postulated. One study using human cells suggested that phosphorylation of Mcm2 by DDK is required for initiation since serine to alanine substitutions of the DDK target sites of human Mcm2 results in a replication defect[33]. Another study suggested that DDK phosphorylation of Mcm2 is required for re-entry of quiescent cells into the cell cycle through control of Mcm2 association with chromatin[35]. Interpretation of these results is complicated by the fact that there is not agreement on the identity of DDK sites in human Mcm2[28; 30; 33]. In budding yeast, overexpression of Mcm2 in which a DDK phosphorylation site (S170) was replaced with alanine interfered with growth[27]. This observation prompted the authors to suggest that DDK phosphorylation of Mcm2 may be essential for replication initiation. However the result may be due to overexpression of the altered allele since strains containing deletions of the Mcm2 N-terminus that includes the S170 DDK site are viable[9].

Here, I show that DDK phosphorylation of Mcm2 at S164 and S170 is not essential for normal cell growth, but is required in response to DNA damage. Cells containing Mcm2 with alanines substitutions at the DDK sites at S164 and S170 are sensitive to caffeine and MMS. A version of Mcm2 in which S164 and S170 are replaced with glutamic acids confers a phosphomimetic phenotype; cells bearing $mcm2_{EE}$ are not sensitive to caffeine or MMS. Furthermore, the $mcm2_{EE}$ allele suppresses the HU and caffeine sensitivity of a strain deleted at *cdc7* (but rendered viable by *mcm5-bob1*). Binding to single stranded DNA is increased with Mcm2-7 containing phosphomimetic Mcm2 ($Mcm2_{EE}$-7), as observed with mutations that cause a reduction in ATP hydrolysis by Mcm2. Increased affinity of $Mcm2_{EE}$-7 for DNA results in decreased DNA unwinding. These effects are similar to Mcm2-7 containing $Mcm2_{K549R}$, a mutant severely affected for ATP hydrolysis. Indeed, $Mcm2_{EE}$ has reduced ATPase activity. Interestingly, similar to $mcm2_{AA}$ strains, $mcm2_{K549R}$ strains are sensitive to caffeine and MMS. Collectively, these results lead to a model in which DDK-dependent phosphorylation of Mcm2 controls Mcm2-7 activity via modulation of ATP hydrolysis by Mcm2 in the cell's response to DNA damage.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Caffeine, hydroxyurea, methyl methanesulfonate and ATP were purchased from Sigma Aldrich (≥ 99 % purity) and molecular biology enzymes from New England BioLabs, unless otherwise stated. Primers were purchased from Integrated DNA

Technologies. YPD is 1 % (w/v) Yeast Extract (Difco), 2 % (w/v) Bacto Peptone (Difco) and 2 % (w/v) Dextrose (Difco).

3.2.2 Plasmids

All DNA was amplified with Elongase polymerase (Invitrogen) and the primers in Table 3-1. Plasmids and their relevant characteristics are listed in Table 3-2. The full-length coding sequence of the *mcm2* mutants was amplified using a ~300 bp mutagenic PCR fragment as a megaprimer[36]. The resultant products were digested with NdeI and BamHI and ligated into the same sites of pET16b. The $mcm2_{AA}$ and $mcm2_{EE}$ mutations were generated similarly except that the mutagenic megaprimer was amplified from pET16b-$mcm2_{S164A}$ and pET16b-$mcm2_{S164E}$, respectively. pET24a-$mcm2_{EE}$ contained the NdeI/BamHI $mcm2_{EE}$ fragment from pMD337 inserted into the same sites of pET24a. The mutant *mcm2* genes were transferred from the pET16b vectors into the NdeI-BamHI sites of pMD240 for expression under control of the *MCM6* promoter in the *LEU2* centromeric plasmid, YCplac111[37]. Mutant versions of *mcm2* plasmid pMD240 containing S164E, S170E and K549R ($mcm2_{EE,KR}$; pMD423) or S164A, S170A and K549R ($mcm2_{AA,KR}$; pMD422) were generated by inserting the BsrGI-BamHI fragment of pET24a-$mcm2_{K549R}$ into the same sites of pMD388 and pMD328, respectively. Mutations were verified by DNA sequencing (London Regional Genomics Center). The plasmids, pMD367 and pMD371 contain $mcm2_{AA}$ and $mcm2_{EE}$, respectively, and were generated by transferring the SphI-BamHI fragments of pET16b-$mcm2_{AA}$ and pET16b-$mcm2_{EE}$ into the same sites of sites of YCplac211[37]. pMD408 is the XbaI-BamHI fragment of pET16b-*MCM2* in the same sites of YCplac211. pMD397 is the BglII-StuI fragment of pET16b-*bob1* in the same sites of YCplac211. *CDC7* was amplified with the indicated

oligonucleotides and the PCR fragment was digested with NdeI/BglII and inserted into the NdeI/BamHI sites of pCDF-Duet (Novagen). Similarly, a *DBF4* fragment generated by PCR was digested with NdeI/BamHI and then inserted into the same sites of pET16b (Novagen). Clones encoding N-terminal deletions of Mcm2 were generated by amplifying the corresponding portion of the *MCM2* coding region from pET11a-*MCM2* using the indicated primers (Table 3-1).

pMD407 is YCplac111 containing the *GAL10* promoter. Nucleotides 275864 to 276250 of *S. cerevisiae* chromosome II which contain the 5'-untranslated region of *GAL10* were amplified with the primers MD463 and MD464 using Elongase polymerase (Invitogen). The resultant PCR fragment was digested with BamHI and PstI and ligated into the same sites of YCplac111 generating pMD407. pMD444, pMD445 and pMD446 are the NdeI-SphI fragments of YCplac111-*MCM2*, -$mcm2_{S164}$ and -$mcm2_{S170A}$ in the same sites of pMD407, respectively.

Table 3-1. Oligonucleotides used in these studies.

Purpose	Name	Sequence
Low T_m primer for megaprimer mutagensis	MD329	CGGATAACAATTCCCC
High T_m primer for megaprimer mutagensis	MD330	CTCAGCTTCCTTTCGGGCTTTGTTAGC
mcm2$_{S164A}$ mutagenesis	MD343	GTCATCAGCATTCTC
mcm2$_{S170A}$ mutagenesis	MD344	CATGTCAGCCAATAG
mcm2$_{S164E}$ mutagenesis	MD361	CGTCATCTTCATTCTC
mcm2$_{S170E}$ mutagenesis	MD362	CCATGTCTTCCAATAG
Verification of *kanMX* insertion in *cdc7*Δ *bob1* strain	MD405	GGAATTCCCCGGGGACGGAGTTTTTTAGTCAGTTC
	MD230	CTGCAGCGAGGAGCCGTAAT
mcm2$_{\Delta 2\text{-}15}$ mutagenic	MD297	CGATTACATATGGAAAATGAGCTACCGCCATCC
mcm2$_{\Delta 2\text{-}25}$ deletion	MD298	CGATTACATATGCAGCAACATTTTAGAGGGGG
mcm2$_{\Delta 2\text{-}107}$ deletion	MD301	CGATTACATATGGAACGTCGCCGCATTGATGC
mcm2$_{\Delta 2\text{-}170}$ deletion	MD302	CGATTACATATGGACATGGACATTGACCCATTAAG
CDC7 cloning for expression in *E. coli*	MD218	GCAGCCAGATCTCAGACTAG
	MD219	GATATACATATGACAAGCAAAACG
DBF4 cloning for expression in *E. coli*	MD220	GGAGATATCATATGGTTTCTCCAACG
	MD221	GGATACTTTCCTAGGTGCACTTTACGTCG
GAL10 promoter cloning	MD463	GATCCATATGTATTGAATTTTCAAAAATTCTTACTTTTTTTTT
	MD464	TTTGTGGGGCCAGGTTAC

Table 3-2. Plasmids used in these studies.

Plasmid	Characteristic	Reference
pMD248	*MCM2* in pET16b	7
pMD311	$mcm2_{S164A}$ in pET16b	This study
pMD394	$mcm2_{S170A}$ in pET16b	This study
pMD318	$mcm2_{AA}$ in pET16b	This study
pMD334	$mcm2_{S164E}$ in pET16b	This study
pMD393	$mcm2_{S170E}$ in pET16b	This study
pMD337	$mcm2_{EE}$ in pET16b	This study
pMD353	$mcm2_{EE}$ in pET24a	This study
YCplac111	Leu-selectable CEN/ARS yeast expression vector	37
pMD240	YCplac111 containing the *MCM6* 5'UTR	7
pMD321	$mcm2_{S164A}$ in pMD240	This study
pMD395	$mcm2_{S170A}$ in pMD240	This study
pMD328	$mcm2_{AA}$ in pMD240	This study
pMD387	$mcm2_{S164E}$ in pMD240	This study
pMD396	$mcm2_{S170E}$ in pMD240	This study
pMD388	$mcm2_{EE}$ in pMD240	This study
YCplac211	Ura-selectable integration vector	37
pMD371	$mcm2_{EE}$ full-length coding region in YCplac211	This study
pMD367	$mcm2_{AA}$ full-length coding region in YCplac211	This study
pMD200	*bob1* in pET16b	M. O'Donnell
pMD397	*bob1* in YCplac211	This study
pMD303	*mcm2*Δ*2-15* in pET16b	This study
pMD304	*mcm2*Δ*2-25* in pET16b	This study
pMD305	*mcm2*Δ*2-107* in pET16b	This study
pMD306	*mcm2*Δ*2-170* in pET16b	This study
pMD309	$mcm2_{S105A}$ in pET16b	This study
pMD310	$mcm2_{S107A}$ in pET16b	This study
pMD281	*DBF4* in pET16b	This study
pMD295	*CDC7* in pCDFduet	This study
pMD407	YCplac111 containing the *GAL10* 5'UTR	This study
pMD444	*MCM2* in pMD407	This study
pMD445	$mcm2_{S164A}$ in pMD407	This study
pMD446	$mcm2_{S170A}$ in pMD407	This study

3.2.3 Yeast strains and manipulations

Two-step gene replacement[38] was employed to replace the *MCM2* allele in the parental BY4743 strain (*MATa/α his3Δ1 leu2Δ0 ura3Δ MET15/met15Δ0 LYS2/lys2Δ0*) with the mutant *mcm2* alleles. Briefly, the plasmids pMD367 ($mcm2_{AA}$) and pMD371 ($mcm2_{EE}$) were digested with BsrGI and PstI, respectively and transformed into BY4743 and plated on media lacking uracil. Ura+ transformants were streaked on media lacking uracil, then on YPD media and finally on media containing 5-FOA. Diploid colonies that survived were screened for the mutations by DNA sequencing, generating MDY104 ($MCM2/mcm2_{AA}$) and MDY106 ($MCM2/mcm2_{EE}$). Next, MDY104 and MDY106 were sporulated and tetrads dissected. MDY139 and MDY159 are spore colonies containing $mcm2_{AA}$ or $mcm2_{EE}$ as the sole copy of the *MCM2* gene, respectively, as verified by DNA sequencing.

To facilitate selection of cells containing the mutant versions of *mcm2*, the *URA3* gene was integrated downstream of the *mcm2* coding region. To do so, a YCplac211 vector containing *MCM2* (nucleotides 2023 to 2607; pMD408) was digested with MscI and transformed into MDY139 and MDY159, respectively, generating MDY169 and MDY191. Insertion of the *URA3* gene downstream of the *mcm2* coding region was verified by PCR. A strain wild type for *MCM2* (BY4741) was similarly tagged generating MDY167.

Two-step gene replacement was used to replace *MCM5* with *bob1*[21] in a *cdc7Δ* Magic Marker strain (BY23713; *MATa/α ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0/LYS2 met15Δ0/MET15 can1Δ::LEU2+-MFA1pr-HIS3/CAN1 CDC7/cdc7::kanMX*). BY23713 cells were transformed with YCplac211-*bob1* linearized with MscI. Haploids were then

generated through the use of "Magic Marker" technology[39]. MDY210 is one such *MAT***a** haploid that survived on media containing G418. The deletion of *cdc7* was verified by PCR using primers MD230 and MD405. The presence of the *bob1* allele was determined by DNA sequencing.

3.2.4 Mcm2 purifications

Plasmids encoding Mcm2 N-terminal deletions were transformed in to *E. coli* and the resulting strains were grown, harvested and cell lysates were prepared as described previously[7; 40] with one exception. The cells were resuspended in Buffer C (20 mM Tris-HCl pH7.9, 500 mM NaCl, 10 % glycerol (vol/vol)) with 5 mM imidazole. The resultant cell lysate was mixed in batch with 4 ml of a 50 % slurry of Ni-charged chelating Sepharose (Ni-Sepharose; GE Healthcare) equilibrated in Buffer C with 5 mM imidazole, washed with 10 ml of the same buffer followed by 20 ml of Buffer C with 60 mM imidazole. Bound proteins were eluted in Buffer C with 1 M imidazole and then dialyzed against Buffer A lacking DTT with 500 mM NaCl, followed by dialysis against Buffer A lacking DDT with 100 mM NaCl. After dialysis, DTT was added to a final concentration of 2 mM.

The Mcm2 point mutants were further purified by anion exchange chromatography. After elution from the Ni^{2+} resin, the protein was dialyzed against Buffer A and then applied to a 3 ml Fast-Flow Q column equilibrated in Buffer A with 50 mM NaCl. The column was washed with 30 ml of the same buffer then eluted with a 30 ml, 50-500 mM NaCl gradient in Buffer A. Protein-containing fractions, determined from Coomassie stained gels, were dialysed against Buffer A with 100 mM NaCl and stored at -80 ºC.

3.2.5 DDK Purification

Lysates of cells co-expressing Cdc7 and His-Dbf4 were prepared as for Mcm2 above. Lysates were then applied to a 20 ml Ni-Sepharose column equilibrated in Buffer C with 5 mM imidazole and washed with 400 ml of the same buffer. After washing the column with 200 ml of Buffer C with 30 mM imidazole, bound proteins were eluted with a 200 ml 30 mM-1M imidazole gradient in Buffer C. Protein-containing fractions were dialyzed against Buffer A lacking DTT with 50 mM NaCl. After dialysis, DTT was added to final concentration of 2 mM and the dialysate was then applied to a 1 ml Mono Q column equilibrated in Buffer A containing 50 mM NaCl. After washing the column with 5 ml of the same buffer, the retained proteins were eluted using a 50-500 mM NaCl gradient in Buffer A. The presence of Dbf4 and Cdc7 in the peak fractions was verified by Western blotting with anti-Dbf4 and anti-Cdc7 antibodies (Santa Cruz) and MALDI mass spectrometry (London Regional Functional Proteomics Facility). Peak fractions containing Cdc7 and Dbf4 were frozen at -80ºC for subsequent assays. Yields were approximately 100 µg DDK per L of cells.

3.2.6 Kinase Assays

Reactions (25 µl) contained the indicated amounts of DDK (as complex) and 500 ng of Mcm2 (wild type or mutant) in kinase buffer (40 mM HEPES-NaOH (pH 7.5), 0.5 mM EDTA, 2 mM DTT, 10 mM magnesium acetate, 80 µg/ml BSA) with 100 µM [γ^{32}P]-ATP (20 Ci/mmol). The mixtures were then incubated for 30 minutes at 30ºC then stopped by addition of sample loading buffer (50 mM Tris-HCl pH 6.8, 2 % (w/v) SDS, 0.1 % bromophenol blue, 10 % (v/v) glycerol, 100 mM DTT) and analyzed by SDS-PAGE (8 %) stained with GelCode Blue (ThermoFisher Scientific). The gel was imaged,

dried and exposed to a PhosphorStorage Screen before scanning using a Storm 860 scanner (GE Healthcare). The amount of Mcm2 in each lane of the GelCode Blue-stained gel was quantified using ImageQuant 5.2 software and normalized to wild type Mcm2 (GE Healthcare). The amount of ATP incorporated (pmol) was quantified using known amounts of [γ^{32}P]-ATP scanned in the same image.

3.2.7 Viability Assays

Viability assays in liquid media were performed as described previously[41]. Briefly, YMD167 and YMD169 were diluted to 1 X 10^6 cells/ml. Ten millilitres of culture was treated with MMS or hydroxyurea (amounts indicated) for 4 hr at 30°C with shaking. An untreated control for each strain was incubated similarly. After incubation, 50 µl of culture was removed, diluted 1000-fold and 50 µl plated on YPD media in triplicate. These plates were incubated for 2 days at 30°C and colonies counted using the Colony Counter tool in ImageQuant TL. Percent survival was calculated as the number of cells counted on the YPD plated from the treated culture as a percent of cells in the untreated control. The assay was performed in triplicate and the mean percent survival is reported with the standard deviation of the mean.

3.2.8 Proteins

Mcm2-7 was reconstituted from constituent subunits that were purified as previously described and contained a PKA-tag on Mcm3 for radiolabeling[7; 40]. In the final step Buffer A was used for the gel filtration (20 mM HEPES pH 7.5, 0.1 mM EDTA, 2 mM DTT, 100 mM NaCl and 10 % (v/v) glycerol). For ATPases with Mcm6 and Mcm2, an additional gel filtration step was required to remove a contaminating ATPase from the protein preparations as described[40].

3.2.9 ATP hydrolysis

ATPase assays were performed as described[7] with the following modifications. For ATPase rates, each 15-µl reaction contained 1 mM [γ^{32}P]ATP (20 Ci/mmol; Perkin Elmer Life Sciences), 20 mM Tris–HCl (pH 7.5), 10 mM $Mg(CH_3COO)_2$, and 2 mM DTT, as well as 1 µM Mcm2 and/or Mcm6. The reactions were quenched with an equal volume of 40 mM EDTA (pH 8) and 0.1 % SDS and the amount of Pi produced measured by thin layer chromatography as previously described[7]. The rates are the means of three replicates.

3.2.10 DNA unwinding

DNA unwinding was assayed using an oligonucleotide substrate (1 nM) containing a 5'-biotin and 3'-d(T_{60}) tail adjacent to a duplex region was incubated with Mcm2-7 at the concentrations indicated in 6 µl of reaction buffer (10 mM HEPES-NaOH, 10 mM $Mg(CH_3COO)_2$, 0.1 mM EDTA, 5 mM DTT, 40 mM NaCl and 5 % PEG 3350)[7]. For reactions containing DDK, the indicated amounts were incubated with 200 nM Mcm2-7 and ATP for 30 minutes at 30°C in the presence of an ATP-regenerating system (50 mM creatine phosphate and 2 µg/ml creatine phosphokinase) and reaction buffer. The oligonucleotide substrate (1 nM) was then added to commence the DNA unwinding reactions. The reactions were analyzed by native (Tris-borate-EDTA) PAGE (8 %) and the resultant gels were dried and exposed to a PhosphorStorage screen that was scanned using a Storm 860 (GE Healthcare). Percent DNA unwound = volume of ssDNA/(volume of ssDNA+volume of dsDNA) X 100. The percent DNA unwound (± standard error of the mean) was plotted as a function of the molar amount of Mcm2-7 in three separate replicates.

3.2.11 DNA binding by gel filtration

DNA binding by wild type and mutant Mcm2-7 complexes was measured in a gel filtration-based assay described previously[7]. The elution of ssDNA was determined by agarose gel electrophoresis on select experiments (data not shown). Co-elution of Mcm2, Mcm4, Mcm5, Mcm6 and Mcm7 with the radiolabeled Mcm3 was detected by Western blotting of representative experiments (data not shown).

3.2.12 DNA binding by electrophoretic mobility shift assays

For DNA binding by electrophoretic mobility shift assays (EMSAs), 1 nM of 5'-end ^{32}P-labeled poly-d(T_{60}) was incubated with the indicated amounts of wild type or mutant Mcm2-7 complexes for 10 minutes at 37°C in 6 µl of the reaction buffer used in the DNA unwinding reactions. Glycerol was added to a final concentration of 5 % (v/v) and the reaction was then applied to a 5 % native (Tris-borate-EDTA) polyacrylamide gel (19:1 acrylamide:Bis-acrylamide; BioShop Canada) containing 5 % glycerol, 0.1 % NP-40 and 10 mM $Mg(CH_3COO)_2$. The gel was resolved at 30 mA for 3 hours, dried and exposed to a PhosphorStorage screen and imaged on a Storm 860 scanner (GE Healthcare). The presence of Mcm2-7 in the protein-containing band was verified by Western Blotting for Mcm2, Mcm3 and Mcm7 (data not shown) and by colloidal Coomassie Blue staining of the gel.

3.3 RESULTS

3.3.1 DDK modifies Mcm subunits in vitro

In order to examine the phosphorylation of Mcm subunits by DDK *in vitro*, I purified the kinase from an *E. coli* strain co-expressing *S. cerevisiae* Cdc7 and His-tagged Dbf4. The resulting complex contained equal portions of Cdc7 and Dbf4 as determined

from Coomassie Blue R250 stained gels (Figure 3-1A). I used this preparation to examine the phosphorylation of Mcm subunits individually *in vitro* (Figure 3-1B). The Mcm proteins were purified from *E. coli* expression strains, so they are not phosphorylated. Incubation of each of the Mcm subunits with DDK and [γ-^{32}P]-ATP resulted in phosphorylated Mcm2, consistent with previous studies (Figure 3-1B, lane 1; refs. 22; 23; 28; 33). Other Mcms were phosphorylated to a lesser degree, with modification of Mcm4 being the next greatest (Figure 3-1B, lane 5). A longer incubation of Mcm2 with DDK resulted in the incorporation of approximately two phosphates per Mcm2 molecule (Figure 3-1C). Even within the Mcm2-7 complex, Mcm2 was the most highly modified subunit (Figure 3-1D). It should be noted that here, Mcm2-7 is not origin bound, which has been shown to affect target selection by DDK[26].

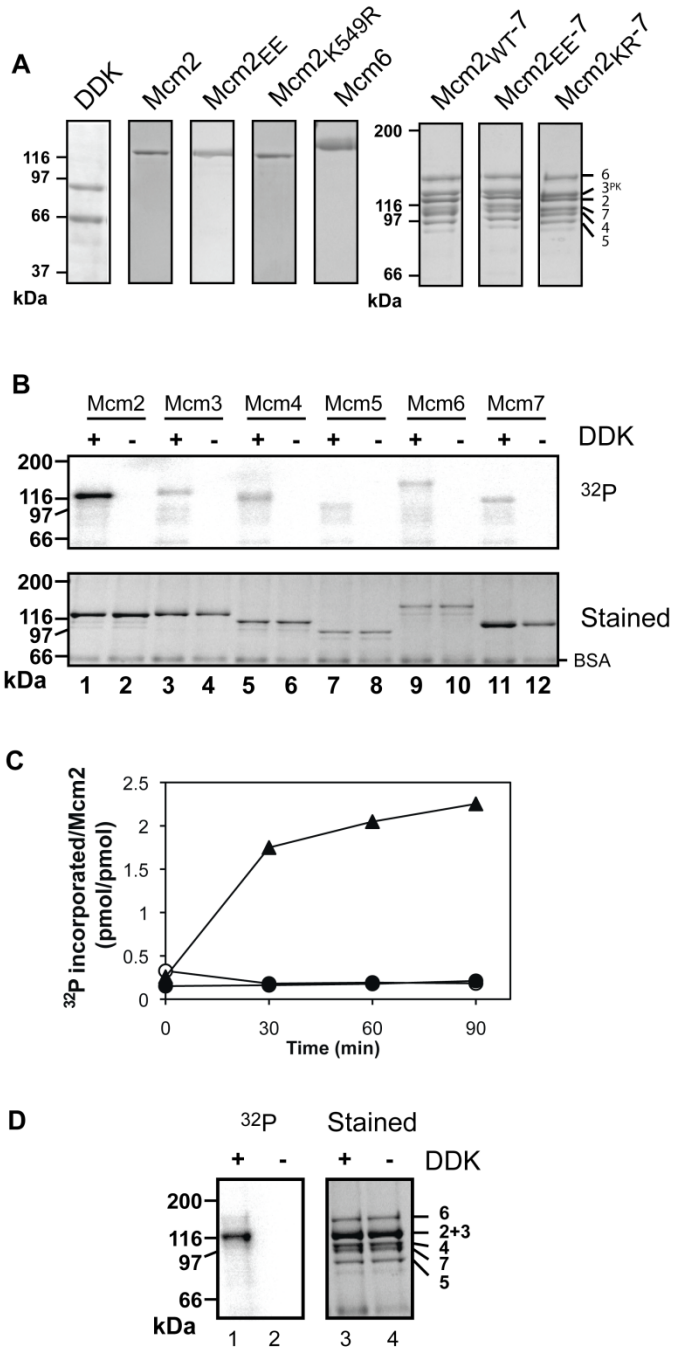


Figure 3-1 Proteins used in these studies and phosphorylation of Mcms by DDK. A. Coomassie-Blue stained gels of proteins used in these studies. Migration of size standards is indicated to the left. Mcm2-7 complexes were resolved on 6 % gels and the other proteins on 10 % gels. **B.** The ability of DDK to phosphorylate each of the Mcm subunits was assayed and the samples were analysed by SDS-PAGE. Top panel is a PhosphorImager scan of the gel. The bottom panel is a colloidal Coomassie-Blue stained image of the same gel. The migrations of size standards are indicated on the left of each

panel. **C.** A time course of Mcm2 phosphorylation. Mcm2 with DDK (triangles), DDK alone (filled circles) or Mcm2 alone (empty circles) is shown. **D.** DDK phosphorylation of Mcm2-7 complex. The left panel is a PhosphorImager scan and the right panel is a Coomassie Blue stained gel. The migration of size standards is indicated on the left and of each of the subunits (represented by number only) is indicated on the right.

3.3.2 DDK phosphorylates Mcm2 at serines 164 and 170 in vitro

Studies on human Mcm2 indicate that DDK phosphorylation sites are found in the N-terminus of Mcm2[28; 33]. Although the exact position of the sites is not conserved from yeast to humans, I thought it likely that DDK phosphorylation would also be confined to the N-terminus of Mcm2. Indeed, elimination of amino acids 2 through 170 of Mcm2 decreased phosphorylation to background levels (Figure 3-2B, lane 6). I further narrowed the location of the phosphorylation sites to a region between residues 108 and 170 by the observation that deletions of residues 2-107 did not greatly affect the extent of phosphorylation (Figure 3-2B; lanes 3-5).

Two serines are found between amino acids 108 and 170 of Mcm2: one at residue 164 and one at residue 170 (Figure 3-2C). I predicted that at least two residues would be phosphorylated by DDK since I observe a 2:1 ratio of ^{32}P incorporated per Mcm2 molecule (Figure 3-2D). I mutated each of these residues to alanines individually ($Mcm2_{S164A}$ and $Mcm2_{S170A}$) and in combination ($Mcm2_{AA}$). Mutation of either S164 or S170 to alanine was not sufficient for loss of DDK phosphorylation *in vitro* (Figure 3-2D; lanes 3 and 4). When both positions were changed to alanine, DDK phosphorylation of Mcm2 was lost (Figure 3-2D; lane 5). I concluded that S164 and S170 are sites for DDK phosphorylation *in vitro.* S164 and S170 were also identified as DDK sites by another group while my study was ongoing[27].

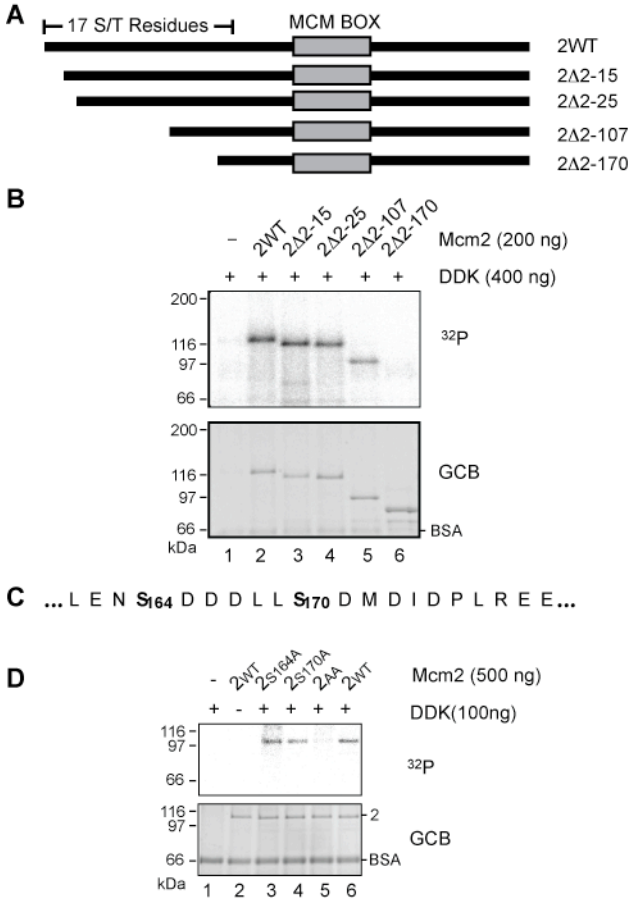


Figure 3-2 Mapping of Mcm2 phosphorylation sites. A. Schematics of full-length Mcm2 ("2WT") as well as the different deletions are shown. The position of the conserved MCM box is indicated. **B.** Phosphorylation assays of purified full-length Mcm2 (lane 2), and the Mcm2 deletion mutations (lanes 2-6) are shown. The stained gel and PhosphorImage are shown. **C.** The amino acid sequence of the region of Mcm2 comprising amino acids 161 to 180. **D.** A kinase assay was performed with 500 ng of purified Mcm2, $Mcm2_{S164A}$, $Mcm2_{S170A}$ or $Mcm2_{AA}$ in combination with 100 ng of DDK (lanes 3-6). Phosphorylated products were not detected with DDK alone (lane 1) or with Mcm2 alone (lane 2). "GCB" indicates the gel was stained with GelCode Blue.

3.3.3 Serines 164 and 170 of Mcm2 are not essential for normal S. cerevisiae growth

I tested whether loss of the DDK phosphorylation sites in Mcm2 affects growth using plasmid shuffling in a haploid strain deleted at *mcm2* but bearing a *URA3* plasmid

with wild type *MCM2*. The strain (MDY054) was transformed with *LEU2* plasmids encoding $mcm2_{S164A}$, $mcm2_{S170A}$, $mcm2_{S164A,S170A}$ ($mcm2_{AA}$) or wild type *MCM2* under control of the *MCM6* promoter. The resulting strains were then tested for growth on 5-FOA, which is metabolized to a toxic product by Ura3 thus testing whether the *mcm2* encoded on the *LEU2*-containing plasmid will support viability. All of the alleles including $mcm2_{AA}$ supported viability (Figure 3-3A). DNA sequencing analysis of the shuffled plasmids confirmed that the mutations were present (data not shown). The growth of strains bearing the mutant alleles was comparable to that of cells with wild type *MCM2* at 30ºC and 37ºC (Figure 3-3A). A slight reduction in growth was observed with $mcm2_{AA}$ at 16ºC whereas strains with the single mutations grew comparably to strains with wild type *MCM2* (Figure 3-3A). Similar results were observed in cells in which $mcm2_{AA}$ was integrated at the *MCM2* locus (data not shown). I concluded that S164 and S170 are not essential for yeast viability when the "normal" promoter is used.

In addition to its essential role in the initiation of DNA replication, DDK is also thought to participate in the S-phase checkpoint response[9; 41; 42; 43; 44; 45; 46; 47]. Notably, mutations in *S. cerevisiae* and *S. pombe* Dbf4/Dfp1 result in sensitivity to drugs that induce the S-phase checkpoint, such as hydroxyurea (HU) and methyl methanesulfonate (MMS; refs. 41; 47; 48). To determine whether phosphorylation of Mcm2 by DDK plays a role in checkpoint response, I subjected cells deleted at *mcm2*, but supported for growth by a plasmid-encoded copy of the *mcm2* alleles to HU or MMS. Strains containing $mcm2_{AA}$ are more sensitive than wild type *MCM2* strains to MMS but not HU (Figure 3-3B). When the strain containing $mcm2_{AA}$ was grown on YPD containing caffeine, its growth was also reduced (Figure 3-3C). Caffeine is a purine analog that inhibits phosphatidyl inositol 3 kinase (PI3K)-like kinases, including those involved in the S-

phase checkpoint (Tel1 and Mec1) and cell growth (TOR; refs. 49; 50; 51; 52). The inhibition of the checkpoint kinases likely causes DNA damage when caffeine is provided in the doses used here[49]. The sensitivity to caffeine was only observed in cells bearing an alanine mutation at both S164 and S170 (*mcm2*$_{AA}$); the single mutations at either of these positions did not affect sensitivity to caffeine (Figure 3-3C), suggesting that, at least with respect to caffeine sensitivity, these residues are functionally redundant. In the studies of Bruck and Kaplan, *mcm2*$_{S170A}$ was lethal when expressed from the *GAL10* promoter. I also observed lethality with *mcm2*$_{S170A}$ when expressed from the *GAL10* promoter, suggesting that the differing observations were not due to strain backgrounds but rather to overexpression of *mcm2*$_{S170A}$ (Figure 3-3D).

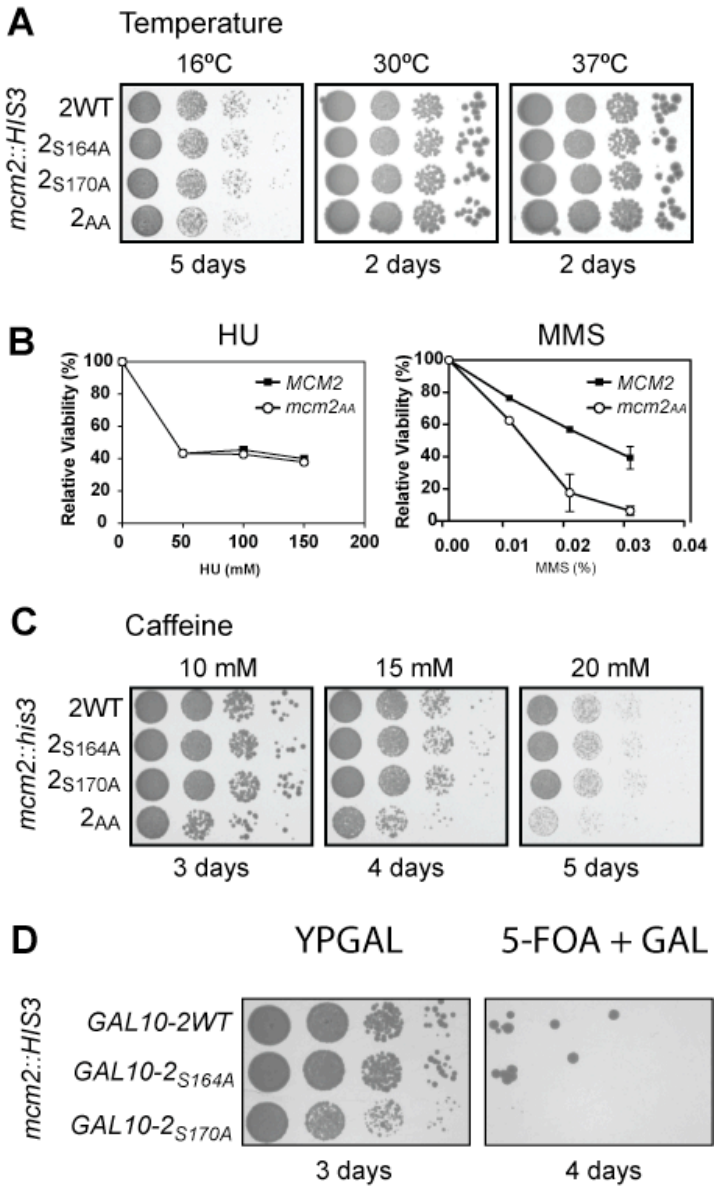


Figure 3-3 Strains containing Mcm2 phosphorylation sites mutants are viable. A. The growth of haploid *mcm2*Δ strains bearing *CEN/ARS* plasmids encoding *MCM2*, $mcm2_{S164A}$, $mcm2_{S170A}$, or $mcm2_{AA}$ was compared. Serial 10-fold dilutions of the strains were spotted on YPD plates and grown at the indicated temperatures. **B.** The sensitivity of *MCM2* and $mcm2_{AA}$ strains to MMS and HU in liquid cultures is shown. The survival of the strains after a 4 hour exposure to the indicated concentration of drug relative to untreated cells is shown. **C.** Serial 10-fold dilutions of the indicated strains were spotted on YPD plates containing the indicated concentrations of caffeine and grown at 30°C for the indicated times. **D.** The growth of haploid *mcm2*Δ strains bearing CEN/ARS plasmids encoding *MCM2*, $mcm2_{S164A}$ and $mcm2_{S170A}$ under control of the *GAL10* promoter. Serial 10-fold dilutions of the strains were spotted on YP media containing 2 % (w/v) galactose and 5-FOA containing 2 % (w/v) galactose and grown at 30°C for 3 days.

3.3.4 Phosphomimetic mutations at S164 and S170

Substitution of an acidic residue in place of a phospho-acceptor residue will often mimic phosphorylation. I therefore asked whether substitution of glutamic acid for serine at positions 164 and/or 170 provided a phosphomimetic effect by examining the sensitivity to caffeine of cells bearing the mutations. Strains with the mutated alleles on *LEU2* plasmids were generated by plasmid shuffling. Cells bearing the single (*mcm2*$_{S164E}$, *mcm2*$_{S170E}$) or the double mutant (*mcm2*$_{EE}$) alleles grew to the same extent as cells containing wild type *MCM2* on YPD media at all temperatures tested (Figure 3-4A). More importantly, strains bearing the Glu substitutions at S164 and/or S170 were not sensitive caffeine, even at the highest concentration of caffeine supplied (20 mM: Figure 3-4B). *mcm2*$_{EE}$ cells were also resistant to MMS (data not shown). Thus, I concluded that the Glu substitutions at S164 and S170 of Mcm2 indicated that phosphorylation at these residues was required for Mcm2 to participate in the cell's response to DNA damage.

I next asked whether the putative phosphorylation of Mcm2 at S164 and S170 is the result of the activity of DDK *in vivo*. *CDC7* is an essential gene, but viability of a *cdc7* deletion strain can be restored by the *bob1* mutation in *mcm5*, albeit with a reduced growth rate[21]. The *cdc7*Δ*bob1* strain is sensitive to HU[21; 43]. If Mcm2 is the key target of DDK leading to the HU sensitivity of the *cdc7*Δ*bob1* strain, then the introduction of *mcm2*$_{EE}$ into the *cdc7*Δ*bob1* strain would suppress the HU sensitivity. Comparison of *cdc7*Δ*bob1* strains containing wild type *MCM2* or *mcm2*$_{EE}$ on plates containing HU indicates that *mcm2*$_{EE}$ does suppress the HU sensitivity of *cdc7*Δ*bob1* (Figure 3-4C). I also compared the growth of these strains on YPD plates containing caffeine and found that *mcm2*$_{EE}$ suppresses the caffeine sensitivity of a *cdc7*Δ*bob1* strain (Figure 3-4C). The difference in growth is dependent on the presence of the compounds; the *cdc7*Δ*bob1*

strains grow similarly on YPD regardless of whether the *mcm2* allele is wild type or the phosphomimetic mutant (Figure 3-4C). These findings suggest that phosphorylation of Mcm2 at S164 and S170 is performed by DDK *in vivo*, supporting the *in vitro* data mapping the DDK target sites.

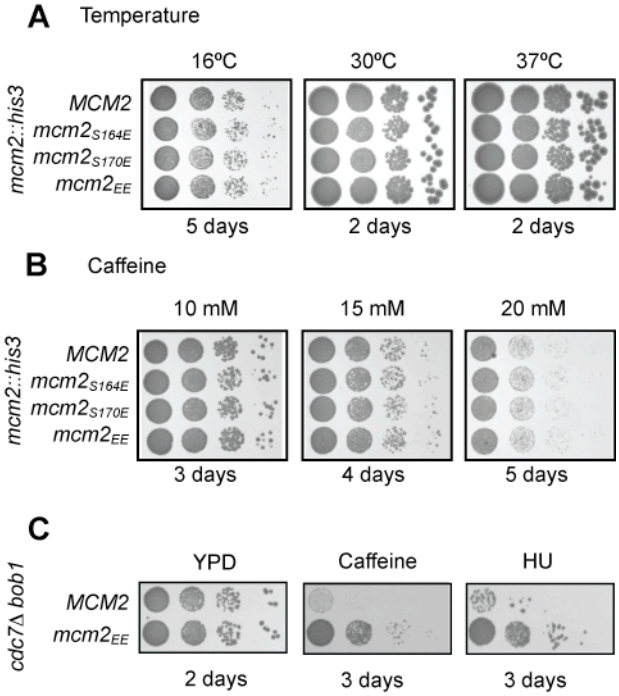


Figure 3-4 Mcm2 phosphomimetic mutants. A. The growth of haploid *mcm2Δ* strains bearing *CEN/ARS* plasmids encoding *MCM2*, $mcm2_{S164E}$, $mcm2_{S170E}$, or $mcm2_{EE}$ was compared. Serial 10-fold dilutions were spotted on YPD plates and grown for 3-5 days at the indicated temperatures. **B.** Serial 10-fold dilutions of the strains from part A were spotted on YPD media containing the indicated concentrations of caffeine and grown at 30°C for the indicated time. **C.** Ten-fold serial dilutions of haploid *cdc7Δbob1* strains with wild type *MCM2* or *mcm2::*$mcm2_{EE}$ ($mcm2_{EE}$) were spotted on YPD with and without caffeine (10 mM) or HU (100 mM) and incubated at 30°C for the indicated number of days.

3.3.5 Biochemical activities of the phosphomimetic Mcm2-7 complex

I sought to determine the mechanisms by which DDK phosphorylation of Mcm2 exerts its control. To do so, I examined the effect of phosphorylation of S164 and S170 on Mcm2 activity *in vitro* through use of the phosphomimetic mutations in Mcm2. Thus, I

purified Mcm2 protein containing glutamic acid substitutions at S164 and S170 ($Mcm2_{EE}$) and reconstituted it into Mcm2-7. Note that Mcm proteins are purified from *E. coli* expression strains and thus are not phosphorylated. The final step in the reconstitution is a gel filtration column that separates excess free subunits from the complex. Additionally, the gel filtration column allows me to verify that $Mcm2_{WT}$-7 and $Mcm2_{EE}$-7 elute as hexamers (Figure 3-5A). Notably, the peak elution for both complexes occurred around fraction 23, corresponding to a size of approximately 610 kDa, close to the predicted size for Mcm2-7 of 608 kDa. Analysis of the peak fractions by quantitative Western Blotting and densitometric measurement of the Coomassie Blue R250-stained gels indicated equal ratios of the Mcm proteins (within ~20 %). Thus, I concluded that Mcm2-7 complexes containing either wild type Mcm2 or $Mcm2_{EE}$ had formed.

I next measured DNA unwinding by $Mcm2_{WT}$-7 and $Mcm2_{EE}$-7 on short oligonucleotide forks whereby DNA unwinding is measured as displacement of a labeled oligonucleotide from its complementary strand. Using this assay, I detected robust unwinding by $Mcm2_{WT}$-7, which unwound up to 57 ± 4 % of the substrate (Figure 3-5B and C). In contrast, DNA unwinding by $Mcm2_{EE}$-7 was reduced to about 40-50% of wild type levels (Figure 3-5B and C). This result suggests that DDK-dependent modification of Mcm2 at S164 and S170 inhibits DNA unwinding by Mcm2-7. Consistent with this idea, treatment of wild type Mcm2-7 with DDK and ATP under conditions in which Mcm2 is the principal substrate for DDK (Figure 3-1B) also decreased DNA unwinding (Figure 3-5D). Interestingly, while DNA unwinding is reduced with $Mcm2_{EE}$-7, sufficient DNA unwinding must occur to support viability (Figure 3-4A).

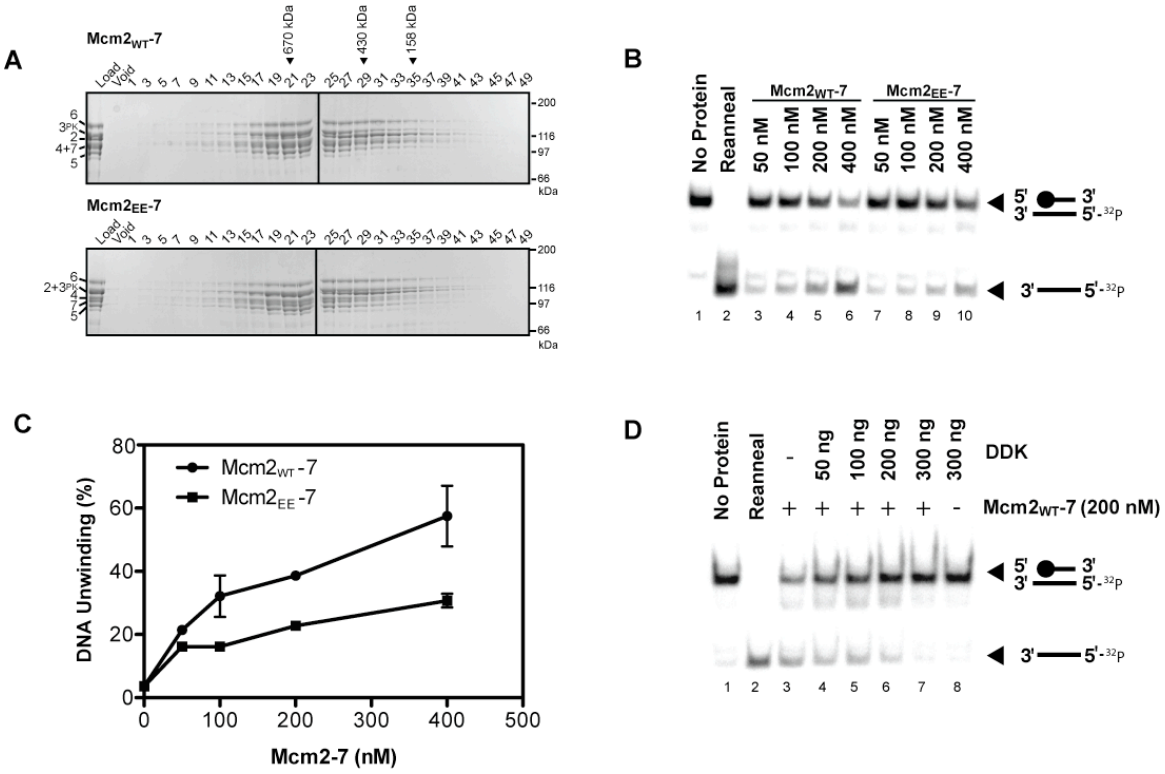


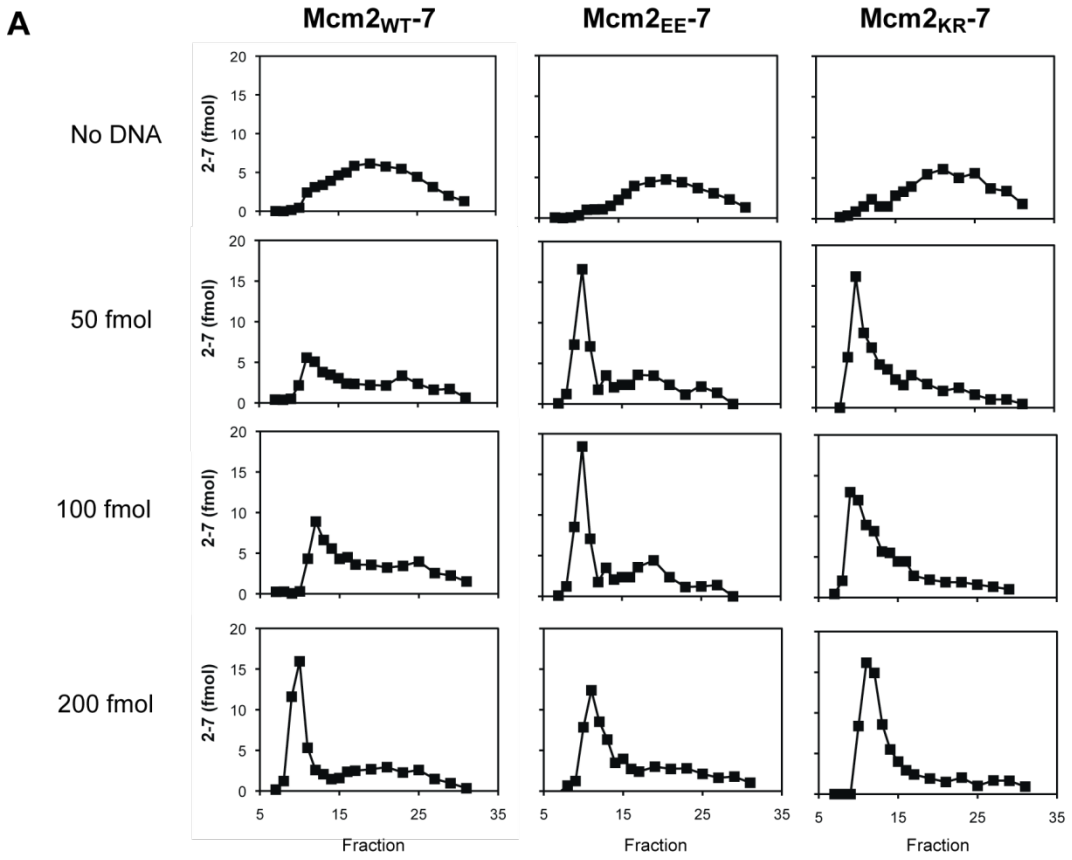
Figure 3-5 Reconstitution $Mcm2_{WT}$-7 and $Mcm2_{EE}$-7 complexes and biochemical activities. A. Mcm2-7 complexes containing wild type Mcm2 ("$Mcm2_{WT}$-7") or $Mcm2_{EE}$ ("$Mcm2_{EE}$-7") were reconstituted from individual subunits. Shown are Coomassie Blue-stained SDS polyacrylamide (6 %) gels of the fractions from the final step. The elution of molecular size standards from the column is indicated above the gel. The migration of molecular size markers and of Mcms through the gels is indicated on the left and right, respectively. **B.** DNA unwinding by $Mcm2_{WT}$-7 and $Mcm2_{EE}$-7 was examined. The peak fraction (fraction 23) from each reconstitution was assayed for DNA unwinding. **C.** The mean extent of DNA unwinding was calculated from three replicate experiments. Standard error of the mean of each point is shown. **D.** DNA unwinding by $Mcm2_{WT}$-7 in the presence of the indicated concentrations of DDK was measured. DDK was pre-incubated with ATP and $Mcm2_{WT}$-7 prior to measuring DNA unwinding.

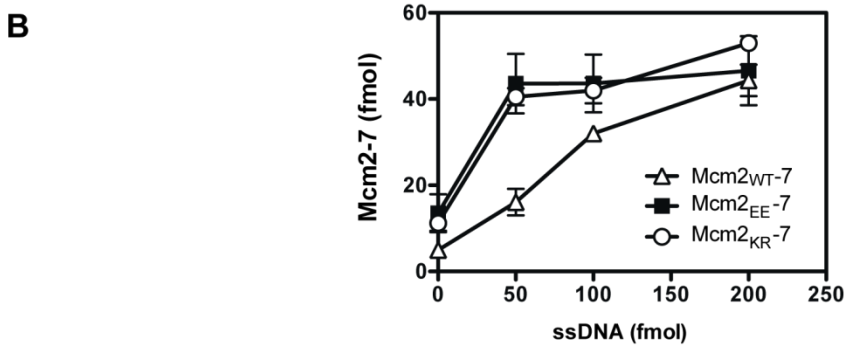
DNA unwinding requires that the helicase interact with DNA. Models for DNA unwinding by hexameric helicases include binding to single and/or double stranded DNA followed by translocation[3; 53; 54; 55; 56; 57; 58; 59; 60; 61; 62]. Thus, I examined DNA binding by

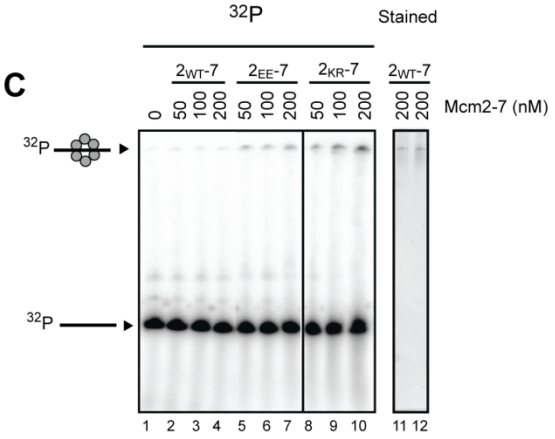
Mcm2-7, mutant and wild type, to determine whether DNA binding is also affected. I first used a gel filtration-based approach, described previously[7], in which binding was measured as the amount of Mcm2-7 that co-eluted with single stranded DNA (ssDNA). Radiolabeled $Mcm2_{WT}$-7 or $Mcm2_{EE}$-7 was incubated with increasing concentrations of M13mp19 circular ssDNA. Bound protein was separated from free protein via gel filtration and the amount of Mcm2-7 bound to DNA was determined from the amount of Mcm2-7 that co-eluted with ssDNA (Figure 3-6A). The results of three replicate experiments using wild type or mutant Mcm2-7 are shown in Figure 3-6B and indicate that $Mcm2_{EE}$-7 binds DNA with higher affinity than Mcm2-7. This effect is most apparent at the lowest concentration of ssDNA supplied (0.5 nM) where at least 3-fold more $Mcm2_{EE}$-7 bound to DNA than $Mcm2_{WT}$-7. The increased DNA binding observed with $Mcm2_{EE}$-7 is similar to my results with Mcm2-7 complexes containing ATP site mutations in Mcm2, including $Mcm2_{K549R}$, which is viable but strongly affects ATP hydrolysis by Mcm2[7]. For comparison, I also show ssDNA binding by $Mcm2_{K549R}$-7 (Figure 3-6A and B). These initial experiments indicated that Mcm2-7 complexes containing $Mcm2_{EE}$ or $Mcm2_{K549R}$ had higher affinity for ssDNA than complexes containing wild type Mcm2.

DNA binding was also measured by electrophoretic mobility shift assays (EMSA), which allowed me to examine a wider range of concentrations of protein. Increasing concentrations of $Mcm2_{WT}$-7, $Mcm2_{EE}$-7 or $Mcm2_{K549R}$-7 were incubated with 1 nM poly-dT_{60}. Binding of Mcm2-7 to DNA resulted in a shift in the migration of the DNA to a point near the top of the gel and coincided with the migration of Mcm2-7 as visualized by staining of the gel (Figure 3-6C). $Mcm2_{WT}$-7 shifted up to 30 fmol of the ssDNA supplied (Figure 3-6C and D). Although modest, this binding is similar to

previous reports using this approach[63] and yields a similar affinity as observed in the gel filtration experiments. In contrast, $Mcm2_{EE}$-7 shifted 8- to 10-fold more poly-dT_{60} (Figure 3-6C and D). $Mcm2_{K549R}$-7 shifted even more ssDNA at levels 15- to 18-fold over Mcm2-7 (Figure 3-6C and D). Overall, these results demonstrate that the phosphomimetic mutations in Mcm2 enhance ssDNA binding by Mcm2-7.


A
Mcm2WT-7
Mcm2EE-7
Mcm2KR-7
No DNA
50 fmol
100 fmol
200 fmol
2-7 (fmol)
Fraction


B
Mcm2-7 (fmol)
ssDNA (fmol)
Mcm2WT-7
Mcm2EE-7
Mcm2KR-7


C
32P
Stained
2WT-7
2EE-7
2KR-7
Mcm2-7 (nM)
1 2 3 4 5 6 7 8 9 10 11 12

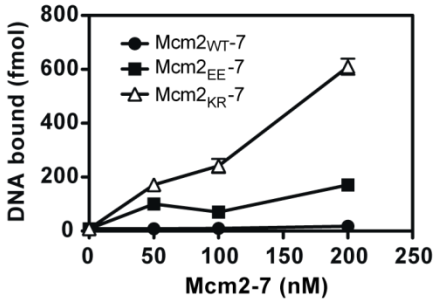

D
DNA bound (fmol)
Mcm2-7 (nM)
Mcm2WT-7
Mcm2EE-7
Mcm2KR-7

Figure 3-6 ssDNA binding by $Mcm2_{WT}$-7 and $Mcm2_{EE}$-7. A. DNA binding was measured using a gel filtration-based assay as described in "Materials and Methods." Binding of $Mcm2_{WT}$-7, $Mcm2_{EE}$-7 and $Mcm2_{KR}$-7 was measured in the presence of 5 mM ATP and the indicated concentrations of M13mp19 ssDNA. Shown are representative plots. The DNA-containing fractions were typically fractions 10-15. **B.** The amount of $Mcm2_{WT}$-7 (open triangle), $Mcm2_{EE}$-7 (filled square) and $Mcm2_{KR}$-7 (open circle) that associated with ssDNA for three replicate experiments at each DNA concentration from part A. **C.** EMSAs were also used to assess ssDNA binding. $Mcm2_{WT}$-7 ("2_{WT}-7"; lanes 2-4), $Mcm2_{EE}$-7 ("2_{EE}-7"; lanes 5-7), or $Mcm2_{KR}$-7 ("2_{KR}-7"; lanes 8-10) were mixed at the indicated concentrations with ^{32}P-labelled poly-d(T_{60}). Shown is a Phosphor screen image of a representative binding assay resolved on a 5 % native (TBE) polyacrylamide gel. The migration of free DNA and protein-DNA complex are indicated on the left. A portion of a gel containing free protein and protein-DNA complex was removed and stained with GelCode Blue (Pierce). The presence the Mcm subunits in the protein-containing band was verified by Western blotting (data not shown). **D.** The mean amount of DNA shifted was calculated from part B and plotted from three replicate experiments.

At first glance, my data seem contradictory. Mcm2-7 containing the $Mcm2_{EE}$ mutant binds DNA better than wild type, but unwinds DNA less well, an activity that requires DNA binding. Thus, I hypothesized that the increased affinity of $Mcm2_{EE}$-7 for ssDNA interfered with the ability of Mcm2-7 to translocate along the DNA for unwinding. To test directly whether the defect in DNA unwinding by $Mcm2_{EE}$-7 is the result of enhanced DNA binding, I inhibited DNA binding by $Mcm2_{EE}$-7 by addition of NaCl. DNA unwinding and DNA binding (measured by EMSA) by $Mcm2_{WT}$-7 or $Mcm2_{EE}$-7 were performed as described for Figure 3-6 except reactions were incubated with and without 100 mM NaCl. The addition of salt disrupted the $Mcm2_{EE}$-7-DNA interaction; $Mcm2_{EE}$-7 bound DNA at levels observed with $Mcm2_{WT}$-7 (Figure 3-7A). Moreover, when NaCl was added to $Mcm2_{EE}$-7 in a DNA unwinding assay, the amount of substrate unwound was increased to a level comparable to $Mcm2_{WT}$-7 (Figure 3-7B). These observations demonstrate that the defect in DNA unwinding by $Mcm2_{EE}$-7 is likely the result of enhanced DNA binding.

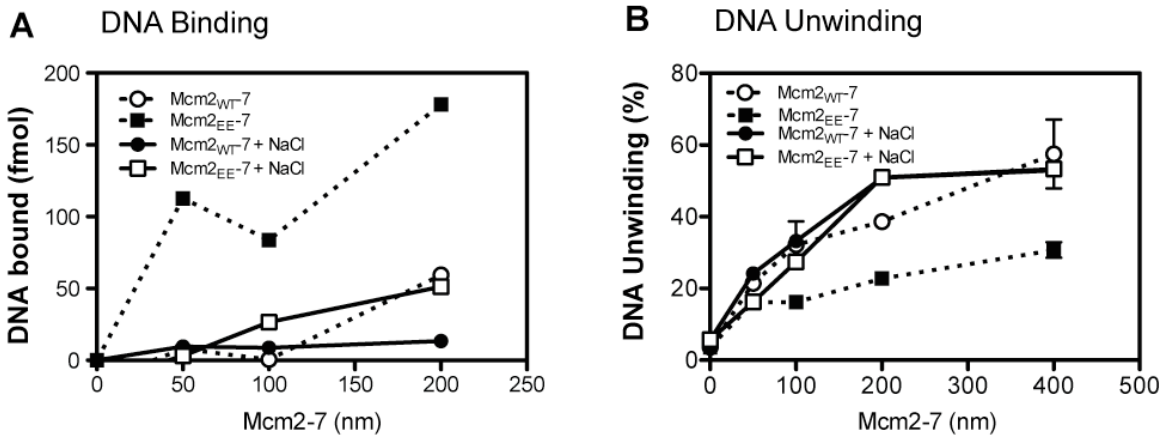


Figure 3-7 Disrupting DNA binding rescues the DNA unwinding defect of $Mcm2_{EE}$-7. A. DNA binding by the indicated concentrations of $Mcm2_{WT}$-7^{3PK} (filled circle) and $Mcm2_{EE}$-7^{3PK} (open square) in the presence of 100 mM NaCl was measured by EMSA. The amount of DNA shifted was calculated from a representative experiment as described in "Materials and Methods" and plotted. DNA binding by $Mcm2_{WT}$-7^{3PK} (open circle) and $Mcm2_{EE}$-7^{3PK} (filled square) in the absence of NaCl from Figure 3-4 in the main text is shown for a comparison. **B.** DNA unwinding by the indicated concentrations of $Mcm2_{WT}$-7^{3PK} (filled circle) and $Mcm2_{EE}$-7^{3PK} (empty square) in the presence of 100 mM NaCl was measured described in the main text. The amount of DNA unwinding was quantified from a representative experiment in plotted. DNA unwinding by $Mcm2_{WT}$-7^{3PK} (open circle) and $Mcm2_{EE}$-7^{3PK} (filled square) in the absence of NaCl from Figure 4 in the main text is shown for a comparison.

3.3.6 Phosphomimetic Mcm2 has reduced ATP hydrolysis

The increased ssDNA binding with both $Mcm2_{EE}$-7 and $Mcm2_{K549R}$-7 led me to predict that phosphorylation of Mcm2 affects ATP hydrolysis by Mcm2. The ATP sites of Mcm2-7 are found at subunit interfaces and residues from two subunits are required to form a functional ATP site[40]. Accordingly, ATP hydrolysis by Mcm2 is dependent on Mcm6 (Figure 3-8A; refs. 40; 64). The Mcm2/6 ATP site makes only a small contribution to the total ATP hydrolysis by Mcm2-7^{6} and even a large change in Mcm2 activity is difficult to detect in the intact complex. Thus I measured ATP hydrolysis by the Mcm2/6 pair rather than the intact Mcm2-7 complex. I mixed wild type Mcm2 or $Mcm2_{EE}$ with Mcm6 and measured the rate of ATP hydrolysis. Wild type Mcm2 mixed with Mcm6

hydrolyzed ATP at a rate of 4.8 ± 0.2 pmol ATP/min (Figure 3-8B). When $Mcm2_{EE}$ was mixed with Mcm6, ATP hydrolysis was significantly reduced to ~75 % of the wild type Mcm2/6 mixture (Figure 3-8B; 3.6 ± 0.6 pmol ATP/min; $p < 0.01$). For comparison, I also show ATP hydrolysis by $Mcm2_{K549R}$ (Figure 3-8B; 0.9 ± 0 pmol ATP/min).

Interestingly, similar to *mcm2*$_{AA}$ cells, cells bearing *mcm2*$_{K549R}$ are sensitive to caffeine (Figure 3-8C). Since $Mcm2_{K549R}$ has reduced ATP hydrolysis like $Mcm2_{EE}$, which does not result in sensitivity to caffeine, this suggests that there is a certain level of Mcm2 ATPase activity that is required in response to caffeine. Specifically, if the level is too low ($Mcm2_{K549R}$) or if the level is too high (unphosphorylated Mcm2 or $Mcm2_{AA}$) then cells cannot respond properly to caffeine. This idea predicts that a strain containing a *mcm2* allele with both the EE and K549R mutations would result in a greater sensitivity to caffeine than a strain bearing the *mcm2*$_{K549R}$ allele alone. Likewise, a strain with a *mcm2* allele with both the AA and K549R mutations would not be sensitive to caffeine. I generated plasmids expressing *mcm2*$_{K549R,EE}$ and *mcm2*$_{K549R,AA}$ under control of the *MCM6* promoter and used plasmid shuffling to introduce them into cells as the sole copy of *MCM2*. Cells containing each of the mutants grew similarly to wild type cells on YPD media (Figure 3-8C). However, when the same cells were spotted onto YPD media containing caffeine, the growth of the *mcm2*$_{K549R,EE}$ strain was markedly reduced relative to both wild type cells and cells containing the individual mutations (Figure 3-8C). The increased sensitivity of *mcm2*$_{K549R,EE}$ cells to caffeine suggests that phosphorylation of Mcm2 by DDK functions through modulation of Mcm2 ATPase activity. Furthermore, strains with the non-phosphorylatable version of the ATP site mutant (*mcm2*$_{K549R,AA}$) were not sensitive to caffeine and grew comparably to cells containing wild type *MCM2*.

Collectively, these data suggest that phosphorylation of Mcm2 by DDK controls Mcm2-7 activity through modulation of ATP hydrolysis by Mcm2.

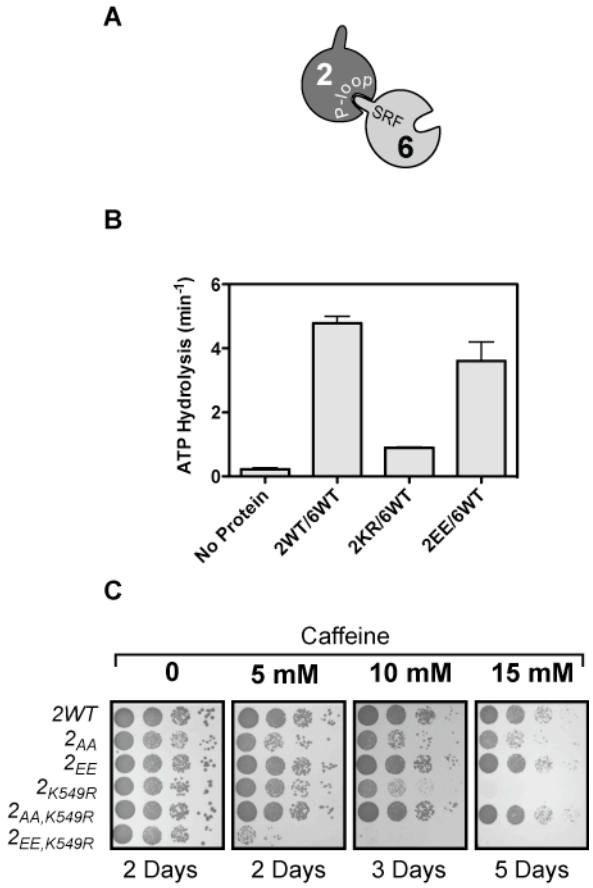


Figure 3-8 Phosphomimetic substitutions of Mcm2 reduce ATP hydrolysis. A. A schematic showing the Mcm2/6 subunit interface. The phosphate binding loop (P-loop) of Mcm2 and the SRF motif of Mcm6, required for ATP hydrolysis by Mcm2 are shown. **B.** ATP hydrolysis by Mcm2, $Mcm2_{EE}$ and $Mcm2_{K549R}$ in the presence of Mcm6 was measured. The rate of ATP hydrolysis by each pair was calculated and plotted with the standard error of the mean. Each complex hydrolyzed ATP above background (no protein) and the rates of hydrolysis were significantly different from one another ($p < 0.01$; $n = 3$). **C**. Haploid strains deleted at *mcm2* and containing *CEN/ARS* plasmids bearing wild type *MCM2*, $mcm2_{AA}$, $mcm2_{EE}$, $mcm2_{AA,K549R}$ or $mcm2_{EE,K549R}$ were serially diluted 10-fold and then spotted on YPD and YPD containing the indicated concentrations of caffeine and grown for the indicated times at 30°C.

3.4 DISCUSSION

3.4.1 Phosphorylation of Mcm2 by DDK and cell viability

Here, I show that cells in which *mcm2* is lacking the DDK phosphorylation sites, S164 and/or S170, are viable when expressed at endogenous levels. It was previously suggested that mutation of S170 to alanine affected viability[27]. However, it should be noted that $mcm2_{S170A}$ was expressed from the strong *GAL10* promoter in that study. When I utilised *GAL10* to express the *mcm2* alleles, I also observed lethality with $mcm2_{S170A}$, suggesting that the differing observations were not due to strain backgrounds but rather to overexpression of $mcm2_{S170A}$.

The finding that that the N-terminal region of Mcm2 can be deleted without affecting cell growth[9] agrees with my observation that mutation of the DDK sites in Mcm2 does not affect viability. Consistent with the idea that phosphorylation of Mcm2 is not required for initiation of DNA replication, a recent study from Sheu and Stillman indicated that phosphorylation of Mcm4 by DDK is sufficient for replication initiation in budding yeast[9]. Moreover, *in vitro* phosphorylation of origin-bound Mcm2-7 by DDK results in only low levels of Mcm2 phosphorylation, but higher levels of Mcm4 phosphorylation[26]. Together these observations suggest that DDK phosphorylation of Mcm2 is not essential for DNA replication initiation. Rather, I propose that phosphorylation of Mcm2 is important in response to DNA damage. This provides a possible explanation for the lethality of $mcm2_{S170A}$ when it is expressed from the *GAL10* promoter. I suspect that overexpression of *MCM2* induces genomic instability.

3.4.2 Regulation of Mcm2-7 activities by DDK phosphorylation of Mcm2

I propose that DDK-dependent phosphorylation of Mcm2 at S164 and/or S170 controls Mcm2-7 activity through its effects on ATP hydrolysis by Mcm2. I have defined a link between phosphorylation of Mcm2 by DDK and ATP hydrolysis by Mcm2. Specifically, phosphomimetic substitutions of glutamic acids at S164 and S170 reduce ATP hydrolysis by Mcm2. The N-terminus of Mcm2, where the DDK sites are located, is predicted to be unstructured and is in a separate domain from the sequences required for ATP hydrolysis. Thus, DDK phosphorylation of Mcm2 likely affects ATP hydrolysis allosterically.

Interestingly, both the non-phosphorylatable Mcm2 mutant (*mcm2*$_{AA}$), which is predicted to have higher ATPase activity, and a mutant severely affected in ATP hydrolysis (*mcm2*$_{K549R}$) are sensitive to caffeine. This suggests that there is a specific range of ATP hydrolysis by Mcm2 that is required for cells to survive in the presence of caffeine. In the absence of phosphorylation, ATP hydrolysis is too high, resulting in improper regulation of DNA binding and unwinding by Mcm2-7. If ATP hydrolysis is too low (*mcm2*$_{K549R}$) then Mcm2 cannot perform its essential function in response to caffeine. The lack of the caffeine sensitivity of cells containing *mcm2*$_{K549R,AA}$ also supports this idea.

ATP sites require residues from two Mcm subunits to function and are found at the interface of subunits[40]. The location of the ATP sites at the subunit interfaces is thought to facilitate communication of the nucleotide binding status between subunits. The proposed communication between Mcm subunits may account for the enhanced DNA binding of Mcm2$_{EE}$-7 and Mcm2$_{K549R}$-7. Mcm2/6 is thought to communicate its nucleotide binding status to Mcm2/5. The Mcm2/5 interface has been proposed to

function as a "gate" that opens to allow passage of DNA into the interior of the helicase ring and then closes for DNA unwinding[5]. Opening and closing of the gate is mediated by ATP binding and hydrolysis by adjacent Mcm subunits including Mcm2/6[65]. I did not specifically assay for function of the gate, but I note that enhanced DNA binding was observed with both linear oligonucleotides and circular ssDNA using Mcm2 mutants that affect ATP hydrolysis.

3.4.3 The potential functions of DDK phosphorylation of Mcm2 in the cell

I hypothesize, based on my data, that DDK likely targets Mcm2 in response to DNA damage. Mcm2 was already known to be a substrate for DDK in cells. This was demonstrated by the Tye lab in Western blots of 2D gels of cell lysates in which acidic spots were dependent on Cdc7[23]. It should be noted that these experiments included drug treatments to synchronize cells and thus the cells were under stress.

Caffeine is a purine analog that inhibits kinases of the PI3K-like family. These kinases include the checkpoint kinases, ATM and ATR (Tel1 and Mec1 in budding yeast) as well as TOR pathway kinases[50; 51; 52]. Inhibition of the checkpoint kinases by caffeine is thought to lead to an accumulation of DNA damage[49]. The sensitivity of strains containing $mcm2_{AA}$ to MMS suggests a role for DDK-modification of Mcm2 in response to DNA damage. Thus, DDK phosphorylation of Mcm2 may not occur in response to inhibition of PI3K-like kinases *per se* but rather in response to DNA damage generated by inhibiting the checkpoint kinases. I suggest that DDK phosphorylation of Mcm2 may stabilize binding of Mcm2-7 to chromatin when the cell responds to DNA damage. Mcm2-7 is more abundant on chromatin than is expected for one complex at each replication fork[1]. In mammalian and *Xenopus* cells, the "excess" Mcm2-7 is not required

for normal cell growth but is important when cells are placed under replicative stress[66; 67; 68]. Decreased DNA unwinding may also be required in response to DNA damage to either slow growth of cells and/or to allow time for repair of damaged DNA.

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CHAPTER 4 PHOSPHORYLATION OF *SACCHAROMYCES CEREVISIAE* MCM2 BY CDC7/DBF4 IS INVOLVED IN THE CELL'S RESPONSE TO DNA DAMAGE

4.1 Introduction

Phosphorylation of replication proteins by various kinases is one mechanism by which cells control the progression of DNA replication. In Chapter 3, I showed that phosphorylation of minichromosome maintenance protein (Mcm) 2 by the Dbf4-dependent kinase (DDK) comprised of the Cdc7 catalytic subunit and the Dbf4 regulatory is not essential for the normal growth of budding yeast. In the present chapter, I explore the biological function of phosphorylation of Mcm2 by DDK. Mcm2-7 is the replicative helicase in eukaryotic cells and subunits of Mcm2-7 are modified by DDK, including Mcm2, Mcm4 and Mcm6[1; 2; 3; 4; 5; 6; 7; 8; 9]. Phosphorylation of Mcm4 at the time of replication initiation is likely the essential function of DDK in the budding yeast, *Saccharomyces cerevisiae*[10]. DDK also functions in the S phase checkpoint[11; 12; 13; 14; 15; 16; 17]. A yeast strain lacking *cdc7* but supported for growth by phosphomimetic substitutions of the DDK target site in Mcm4 is sensitive to replicative stress[10]. Thus, phosphorylation of another target(s) by DDK other than Mcm4 is required in the S phase checkpoint.

Checkpoints throughout the cell cycle survey for DNA damage and halt cell cycle progression in the presence of DNA damage and/or cell stress. If the DNA damage occurs during the S phase, the cell will slow replication fork progression and inhibit the initiation of late-firing origins to allow time to repair the damage (reviewed in ref. 18). The checkpoint kinase, Rad53 is a key player in the S phase checkpoint in budding yeast and its activation through autophosphorylation is a hallmark of the activation of the S phase checkpoint[19]. After the DNA damage has been dealt with, the S phase checkpoint is

attenuated in part through the inactivation of Rad53 by its dephosphorylation[20]. DNA replication resumes once the S phase checkpoint is inactivated. DDK appears to have roles in most, if not all, of the stages of the S phase checkpoint[12; 13; 17; 21; 22; 23; 24; 25; 26; 27; 28; 29]. Studies in budding yeast and fission yeast show that DDK regulates replication fork stability[12] and may also be involved in replication fork restart during recovery from the S phase checkpoint[16; 30]. The activities of DDK may also contribute to the activation of the S phase checkpoint in budding yeast and human cells[17; 21; 22; 23].

The initiation and checkpoint functions of DDK are related. In response to DNA damage, the Dbf4 subunit of DDK is a substrate for Rad53[13; 24; 25; 26; 27; 28; 29]. Modification of Dbf4 by Rad53 causes the dissociation of DDK from origins[13; 26; 27]. Rad53 also has a role modifying DDK activity in replication initiation independent of the S phase checkpoint[31]. This function of Rad53 appears to positively regulate the activity of DDK through stabilization of the Dbf4 subunit[31].

In Chapter 3, I established that a version of *Saccharomyces cerevisiae* Mcm2 in which the DDK phosphosites (S164 and S170) are mutated to alanines ($mcm2_{AA}$) is viable under normal growth conditions. However, in the presence of caffeine, an inhibitor of kinases involved in cell stress and cell growth[32; 33; 34; 35; 36], the growth of cells containing $mcm2_{AA}$ is impaired. Furthermore, the growth of $mcm2_{AA}$-containing cells is impaired in the presence of the DNA damaging agent, methyl methanesulfonate (MMS).

In the present chapter, I examined the cellular function of DDK-dependent phosphorylation of Mcm2 through genetic studies and phenotypic analyses of strains bearing mutations in the DDK phosphorylation sites of Mcm2 ($mcm2_{AA}$). Strains

containing *mcm2$_{AA}$* progress normally through the cell cycle, indicating that phosphorylation of Mcm2 is not required for initiation. However, cells containing *mcm2$_{AA}$* have a lengthened S phase in the presence of caffeine, indicating an S phase-specific growth defect in these cells. I examined the genetic interactions of *mcm2$_{AA}$* using synthetic genetic array (SGA) analysis and identified 8 synthetic sick or lethal (SSL) interactions. Additionally, a checkpoint-defective mutant of *rad53* (*mec2-1*) is synthetically lethal with *mcm2$_{AA}$*. The *mec2-1* allele and many of these gene deletions increase DNA damage in the cell. This suggests that the *mcm2$_{AA}$* strain cannot tolerate increased levels of endogenous DNA damage in the cell. I also looked for suppressors of the caffeine sensitivity of the *mcm2$_{AA}$* strain using SGA analysis and identified 16 deletions that suppressed the growth defect of the *mcm2$_{AA}$* strain in the presence of caffeine. In general, deletion of these genes contribute to replication fork slowing or suppress DNA damage. DDK phosphorylation of Mcm2 is also required for the response to specific types of DNA damage since strains with *mcm2$_{AA}$* are sensitive to agents that cause DNA base damage such as MMS and 5-fluorouracil (5-FU), but is not sensitive to phleomycin which induces double strand breaks. Taken together, these studies are consistent with a model in which DDK-dependent phosphorylation of Mcm2 is required for the response to DNA base damage during the S phase checkpoint.

4.2 Materials and methods

4.2.1 Materials

Caffeine, hydroxyurea and methyl methane sulfonate were purchased from Sigma Aldrich (99 % purity) and molecular biology enzymes from New England BioLabs. Geneticin (G418) was purchased from either United States Biological or Santa Cruz Biotechnology and phleomycin was purchased from Santa Cruz Biotechnology. 5-FU was purchased from Nutritional Biochemical Corp. Primers were purchased from Integrated DNA Technologies. YPD is 1 % (w/v) Yeast Extract (Difco), 2 % (w/v) Bacto Peptone (Difco) and 2 % Dextrose (Difco).

4.2.2 Strains and manipulations

All strains used in these studies are described in Table 4-1. Unless otherwise stated, the parental strain is BY4743 (*MAT***a/α** *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ/ura3Δ MET15/met15Δ0 LYS2/lys2Δ0*; ref. 37). The diploid Magic Marker collection (*MAT***a/α** *ura3Δ0/ura3Δ0 leu2Δ0/leu2Δ0 his3Δ1/his3Δ1 lys2Δ0/LYS+ met15Δ0/MET15+ can1Δ::LEU2+-MFA1pr-HIS3/CAN1+ XXX/xxx*Δ) was purchased from Open Biosystems[38]. For growth assays on plates, 10-fold serial dilutions of a saturated culture were spotted on the indicated media and grown at 30°C. Liquid growth assays were performed as described in Chapter 3.

Table 4-1. ***Saccharomyces cerevisiae*** **strains used in this study.**

Strain	Relevant Phenotype	Reference/Source
MDY139	*MAT***a** *mcm2::mcm2$_{AA}$*	This study
MDY166	*MAT***α** *ura3Δ0 leu2Δ0 his3Δ1 mcm2::mcm2$_{AA}$*	Chapter 3
MDY159	*MAT***α** *mcm2::mcm2$_{EE}$*	Chapter 3
MDY167	*MAT***α** *MCM2-URA3*	Chapter 3
MDY169	*MAT***α** *mcm2$_{AA}$-URA3*	Chapter 3
MDY191	*MAT***α** *mcm2$_{EE}$-URA3*	Chapter 3
mec2-1	*MAT***a** *ade2-1 can1-100, his3-11,-15, leu2-3,-112, trp1-1, GAL, psi+, dbf4::DBF4-Myc18, LEU2, MEC2::mec2-1(rad53-11)*	39

4.2.3 Cell cycle arrest and fluorescence-activated cell sorting

The yeast strains BY4741 (*MAT***a** *his3Δ1 leu2Δ0 ura3Δ met15Δ0*) and MDY139 were grown in YPD to an OD_{600} of 0.5. Fifteen millilitres of each culture was treated with hydroxyurea (200 mM) for 4 hours to arrest cells in the S phase. For arrests in the G1 phase, the α-factor mating pheromone (Sigma Genosys Canada) was added to a final concentration of 10 μg per ml of culture. After arrest, 1 ml of sample was removed from each strain, pelleted and fixed by resuspension in 1 ml 70 % ethanol. The remaining cells were washed twice with water, resuspended in pre-warmed (30°C) YPD or YPD containing 10 mM caffeine and grown at 30°C with shaking. One millilitre of culture was removed at 15 minute time intervals, up to 60 minutes, pelleted and fixed in 1 ml 70 % ethanol. For each fixed sample, 7 x 10^6 cells were pelleted, washed twice with water and then treated with 200 ug/ml RNase A (Sigma) in 50 mM Tris-HCl (pH 8) for ~ 3 hours at 37°C. Cells were again pelleted and resuspended in 0.5 ml of 50 mM Tris-HCl (pH 7.5) containing 2 mg/ml Proteinase K (BioShop) and incubated for 30 minutes at 50°C. After the Proteinase K treatment, cells were pelleted and resuspended in 100 μl 200 mM Tris-HCl (pH 7.5), 200 mM NaCl and 78 mM magnesium chloride. A 5000-fold dilution of Sytox Green DNA stain (Molecular Probes) in 50 mM Tris-HCl (pH 7.5) was prepared

and 750 µl was added to each 100 µl sample prior to analysis. Cell sorting was performed on a FACSCalibur (BD Biosciences) by the London Regional Flow Cytometry Facility.

4.2.4 SGA Analysis

SGA analysis was performed as described previously[40], except for the following modifications. Manipulation of the gene arrays was performed manually using a 3.18-mm 48-pinner tool following the manufacturer's directions (V&P scientific, San Diego, CA). An initial screen was first performed using MDY169 (*MAT***α** *mcm2$_{AA}$-URA3*) or MDY167 (*MAT***α** *MCM2-URA3)* as the query strain that was mated with the haploid yeast Magic Marker deletion collection generated from the diploid strain collection[38]. Media always lacked uracil beyond the sporulation step to ensure maintenance of the *URA3* gene. After selection of haploid cells that contained a gene deletion ($G418^R$) and *mcm2$_{AA}$* or *MCM2* gene linked to *URA3* (Ura^+) the plates were scanned on a HP scanjet 3970. Colony size was then quantified with the Array Analysis tool in ImageQuant TL (GE Healthcare). A strain was determined to be slow growing if the colony size was as small or smaller than the mean colony size of 10 deletion strains that have reduced growth as annotated in the *Sacccharomyces cerevisiae* genome database (SGD; www.yeastgenome.org). For positive SSL interactions, the colony sizes of the same spots in the *MCM2-URA3* screen were examined. The colony sizes at these spots was never below the mean colony size mentioned above, indicating that linking *MCM2* to the *URA3* gene was not responsible for the synthetic effects observed. A false positive rate (growth on either *MCM2* or *mcm2$_{AA}$* plates when the gene deletion is reported as lethal in the SGD) was roughly 5 % and is similar to false positive rates reported previously[40]. From this initial screen, I compiled a list of 234 genes that had SSL interactions with *mcm2$_{AA}$*. An initial screen to

examine for suppression of the caffeine sensitivity of *mcm2$_{AA}$* was also performed by transferring the haploid *mcm2$_{AA}$ xxx*Δ cells to YPD media containing 15 mM caffeine. The plates were incubated at 30°C for 4 days, imaged and then quantified. A colony was determined to be a suppressor when its size was larger than the colony size of the *mcm2$_{AA}$* strain. The deletions of 369 genes suppressed the growth defect of *mcm2$_{AA}$* in caffeine in the initial screen.

Next, the SSL and suppressor deletions identified from each screen were retested from the mating step in triplicate. After imaging and quantification as described above, an SSL interaction with *mcm2$_{AA}$* with a gene deletion was determined if a spot containing the haploid cells was below the threshold in at least two of the three replicates. Suppression of the *mcm2$_{AA}$* growth defect on caffeine-containing media was determined by growth of a colony on YPD containing 15 mM caffeine in at least two of the three replicates. To ensure the quality of each plate, three genes that are known to be lethal as haploids (*EBP2*, *DAD1* and *CDC27*) and three viable gene deletions strains (*srn2*Δ, *prx1*Δ and *nde2*Δ) were included on each plate. For the caffeine suppressor screens, *mcm2$_{AA}$* alone, and three gene deletions known to cause caffeine sensitivity (*ard1*Δ, *rom2*Δ and *rtg2*Δ) were included on each plate. Similarly, gene deletions that are not sensitive to caffeine (*rrf1*Δ, *sro7*Δ and *rpl37B*Δ) were included on each plate. Forty-one SSL interactions and 86 caffeine suppressors were identified in this step.

Next, the *mcm2$_{AA}$* strain was re-mated with each of the gene deletions isolates in the SSL and suppressor screens, diploids selected, sporulated and tetrads were dissected. SSL interactions were verified if Ura+ and G418^{R} spore progeny were never recovered or grew slowly relative to the corresponding single mutation. The number of spores

analyzed in each dissection is indicated Table 4-1. For the caffeine suppressors, 10-fold serial dilutions of a saturated culture of a viable *mcm2$_{AA}$ xxx*Δ (G418^{R} and Ura^{+}) spore progeny was spotted onto YPD with and without 15 mM caffeine. A spore progeny containing the corresponding single mutants (*xxx*Δ or *mcm2$_{AA}$*) were also spotted on the same plate.

4.3 Results

4.3.1 The cell cycle of mcm2$_{AA}$ cells

DDK functions in both replication initiation[3; 10] and the S phase checkpoint[12; 27]. Thus, I sought to determine if phosphorylation of Mcm2 affects the cell cycle progression of normally growing cells. Comparing cell cycle profiles of strains can determine subtle defects in cell cycle progression and/or the distributions of cells between the phases of the cell cycle. I first compared the cell cycle profiles of *mcm2$_{AA}$* cells to wild type cells by fluorescence-activated cell sorting (FACS) analysis (Figure 4-1). FACS analysis sorts cells according to their DNA content and counts them. The cell cycle profiles of the *MCM2* and *mcm2$_{AA}$* strains growing asynchronously in rich media were similar, indicating that phosphorylation of Mcm2 by DDK does not affect the distribution of cells in a given phase of the cell cycle (Figure 4-1A). I next arrested the *MCM* and *mcm2$_{AA}$* strains in S phase with hydroxyurea and then examined their growth after released into rich media (Figure 4-1B). It should be noted that the *mcm2$_{AA}$* strain is not sensitive to HU after a short exposure (Chapter 3). Similar to the results with asynchronously growing populations, the progression of the cell cycle of the *mcm2$_{AA}$* strain was similar to the *MCM2* strain (Figure 4-1B). Next, I looked for growth defects in *mcm2$_{AA}$* cells arrested in G1 with alpha factor and released into YPD media. Both *MCM2* and *mcm2$_{AA}$* progressed

through the cell cycle similarly and completed the cell cycle in ~45-60 minutes. Together, these data demonstrate that phosphorylation of Mcm2 by DDK is not necessary for cell cycle progression under normal growth conditions. Lastly, I examined the progression of the *MCM2* and $mcm2_{AA}$ strains through the cell cycle after alpha factor arrest and release into YPD media containing 10 mM caffeine. The *MCM2* strain completed cell cycle progress in 90 minutes in caffeine (Figure 4-1D). In contrast, the $mcm2_{AA}$ strain progressed slowly through S phase and did not fully complete the cell cycle, even by 120 minutes post-release (Figure 4-1D). These data suggest a growth defect in the S phase of the $mcm2_{AA}$ strain in the presence of caffeine.

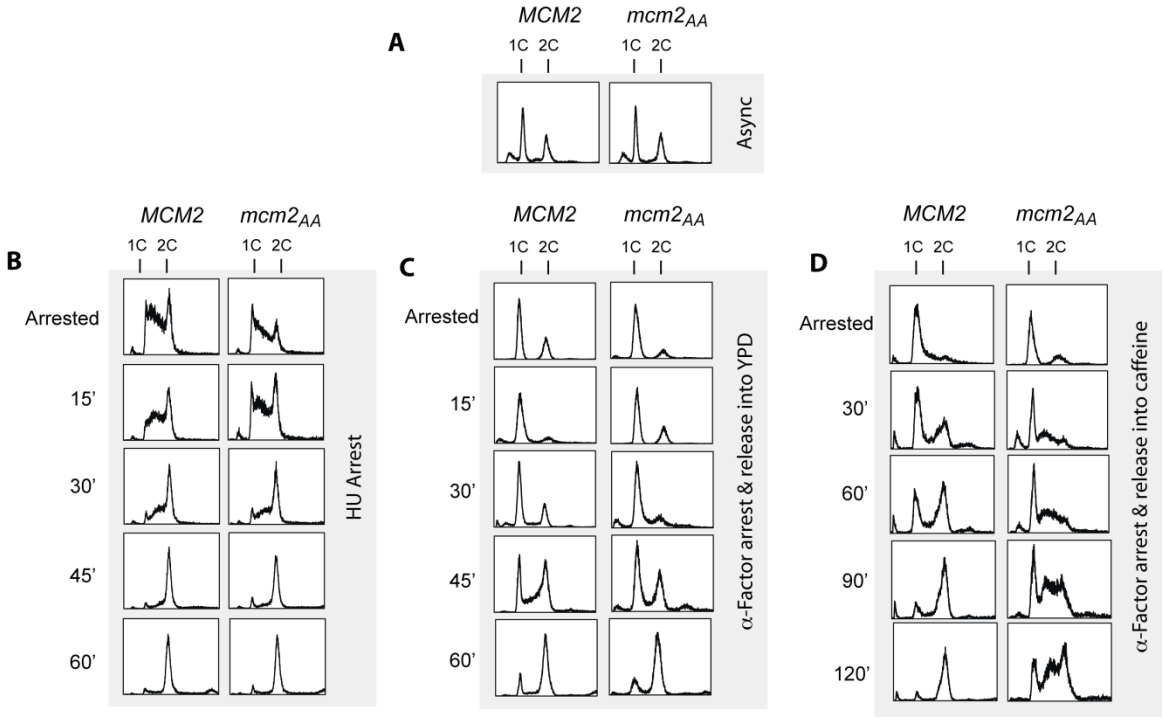

Figure 4-1 Analysis of the cell cycle of *MCM2* and *mcm2$_{AA}$* strains. A. The DNA content of asynchronously growing *MCM2* or *mcm2$_{AA}$* strains was analyzed by FACS analysis as described in "Materials and Methods". "1C" and "2C" indicate 1 or 2 chromosomal copies of DNA, respectively. **B.** The *MCM2* or *mcm2$_{AA}$* strains were treated with hydroxyurea for 4 hours ("arrested") and the DNA content determined by FACS analysis. Cells were washed and resuspended in YPD media. An aliquot of cells was removed at 15 minute intervals over 1 hour and the DNA content measured by FACS analysis. **C.** *MCM2* and *mcm2$_{AA}$* cells were treated with α-factor mating pheromone for 3 hours at 30°C, washed and resuspended in YPD. The DNA content of an aliquot of fixed cells was analyzed by FACS analysis at 15 minute intervals over 1 hours. **D.** *MCM2* and *mcm2$_{AA}$*-containing cells were treated with α-factor mating pheromone for 3 hours at 30°C, washed and resuspended in YPD containing 10 mM caffeine. The DNA content of an aliquot of fixed cells was analyzed by FACS analysis at 30 minute intervals over 2 hours.

4.3.2 Synthetic sick and lethal interactions with mcm2$_{AA}$

It is widely accepted that DDK functions in the S phase checkpoint[13; 26; 27; 28; 29; 41; 42; 43]. Rad53 is a central player in the S phase checkpoint (Reviewed in ref. 44) in budding yeast and also has a role in DDK function during replication initiation[31]. To ask whether phosphorylation of Mcm2 is important for Rad53's checkpoint function, I tested whether

or not $mcm2_{AA}$ interacts with a mutant version of *rad53* (*mec2-1*) that is defective in the checkpoint function of Rad53, but not Rad53's essential function in replication initiation. I mated cells containing either a wild type copy of *MCM2* or $mcm2_{AA}$ linked to the *URA3* gene with the *mec2-1* strain, sporulated the diploids and examined the phenotypes of the spore progeny. I recovered spores that contained both *MCM2* and the *mec2-1* mutation in the expected 1 to 4 ratios (5 of 19). However, I did not recover any spore progeny that contained $mcm2_{AA}$ and *mec2-1* (0 of 22), indicating that $mcm2_{AA}$ is synthetically lethal with *mec2-1*.

I next examined the genetic interactions of $mcm2_{AA}$ with the viable yeast haploid deletion collection. I employed synthetic genetic array (SGA) analysis using the $mcm2_{AA}$ strain as a query strain and mated it with the non-essential haploid deletion collection[40]. Synthetic sick or lethal (SSL) interactions were determined by the absence or reduced growth of a yeast colony on the plates containing $mcm2_{AA}$. I detected 39 SSL interactions with $mcm2_{AA}$ in at least 3 of the 4 replicates. To eliminate the possibility of identifying false positive interactions using the SGA technique, each SSL interaction was verified by sporulation and tetrad dissection followed by screening for Ura^+ ($mcm2_{AA}$) and $G418^R$ (deletion) spore progeny. Synthetic lethal interactions were verified by the absence of spore colonies that were both Ura^+ and $G418^R$ and synthetic slow interactions were examined by the relative growth of a double mutant to the corresponding single mutant strains. After manual verification, 8 deletions showed SSL interactions with $mcm2_{AA}$ and are listed in Table 4-2 (note that *mec2-1* is also listed in Table 4-2). I classified these genes based on their gene ontology (GO) terms in SGD. The genes represent diverse cellular functions, including the cell's response to replicative stress and DNA damage.

These data are consistent with a role for phosphorylation of Mcm2 by DDK in the cell's response to DNA damage.

Table 4-2. SSL interactions with *mcm2$_{AA}$*.

Gene[a]	ORF	Function	GO[b]	Phenotype of single mutant[c]	*N*[d]
mec2-1	YPL153C	Checkpoint Kinase	1	MMS, HU	19
CHK1	YKL150W	Checkpoint Kinase	1,2,5		18
CTF4	YDR359C	Sister Chromatin Cohesion	1,2	HU, MMS	21
SOD1	YLR079W	Response to oxygen radicals	1,6,8	HU, MMS,	23
BUD23	YDR159W	Bud site selection	2,3,4	RG	16
PEP3	YOR061W	E3-ligase	3,8	RG	17
SKN1	YDR451C	Sphingolipid biosynthesis	7,8		20
IMG1	YPR139C	Respiration and mitochondrial genome maintenance	9	RG	21
VMA13	YDR512C	Subunit of Vacuolar ATPase	10	CS, OS	21

4.3.3 Suppression of the caffeine sensitivity of the mcm2$_{AA}$ strain

In Chapter 3, I observed that the *mcm2$_{AA}$* strain is sensitive to caffeine on plate media. This observation provided a tool that was easily adaptable to the SGA technology and allowed me to query for suppressor mutations. To do so, I transferred the viable *mcm2$_{AA}$ xxx*Δ haploid colonies from the SSL analysis to YPD containing caffeine and examined them for increased growth relative to cells containing *mcm2$_{AA}$* alone. Eighty one gene deletions suppressed the caffeine sensitivity of *mcm2$_{AA}$*. To ensure that I had identified *bona fide* suppressors, haploid *mcm2$_{AA}$ xxx*Δ cells were generated by tetrad

[a] Genes are grouped by their GO terms as annotated in the *Saccharomyces cerevisiae* database.
[b] Gene ontology: (1) Response to cell stress/chemical stimuli (2) cell cycle, (3) transport, (4) RNA metabolic process, (5) signalling process/protein modification process, (6) transcription, (7) carbohydrate metabolic process, (8) cell wall, membrane, & vesicle mediated transport, (9) mitochondrial organization, and (10) other.
[c] Relevant phenotype as annotated in the *Saccharomyces cerevisiae* database. CS, sensitivity to caffeine; HU, sensitivity to hydroxyurea; MMS, sensitivity to methyl methanesulfonate; OS, sensitivity to oxidative stress; RG, respiratory growth absent or decreased.
[d] Number of spore progeny checked.

dissection and the growth of serial dilutions of a *mcm2$_{AA}$ xxx*Δ spore colony was examined on caffeine relative to the single mutations (Figure 4-2). Sixteen of the genes were identified as suppressors after this verification step (Figure 4-2). I then classified these genes into known biological functions based on gene ontology annotations in the SGD as well as their reported functions in the literature (Table 4-3). In general, these genes are involved in the cell's response to DNA damage. These observations prompted me to more closely examine the types of cell stresses to which the *mcm2$_{AA}$* strain is sensitive.

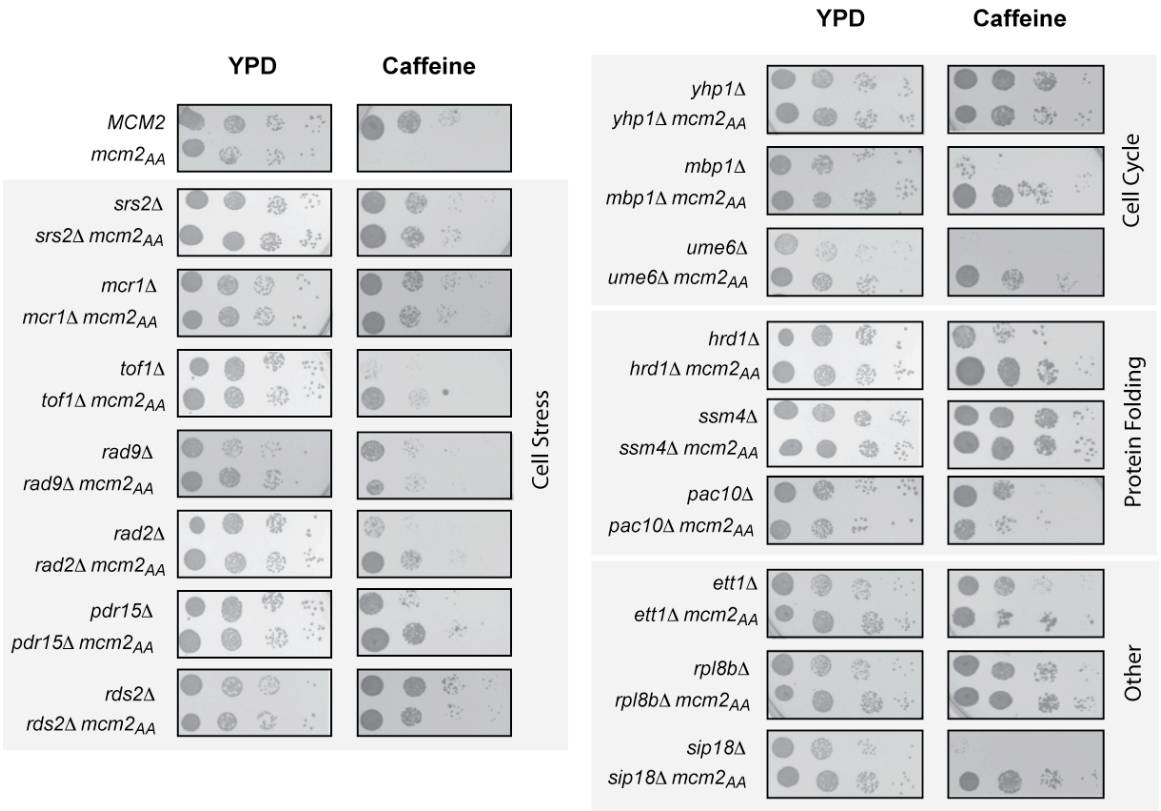

Figure 4-2 Suppressors of the sensitivity of the *mcm2$_{AA}$* strain to caffeine. Ten-fold serial dilutions of the indicated haploid *mcm2$_{AA}$ xxx*Δ or *xxx*Δ strain were spotted on YPD media and YPD media containing 15 mM caffeine and grown at 30°C. YPD plates were incubated for 2 days and YPD containing caffeine plates were incubated for 4 days. The *MCM2* and *mcm2$_{AA}$* strains were included on each plate for comparison.

Table 4-3. Summary of phenotypes associated with suppressors of the caffeine sensitivity of the *mcm2$_{AA}$* strain.

Gene[a]	ORF	Function	GO[b]	Annotated phenotype of *xxx*Δ[c]	*mcm2$_{AA}$ xxx*Δ suppression[d]		
					MMS	HU	5-FU
SRS2	YCR009C	DNA repair and meiosis	1	HU	+		+
MCR1	YBR274W	Oxidative stress response	1		+		+
TOF1	YCL038C	Subunit of fork pausing complex	1,2,5	HU, MMS	+	+	+
RAD9	YLR263W	Transmission of checkpoint signal	1,2,4,5 6	HU, MMS, OS,RG	-		+
RAD2	YNL072W	DNA repair	1,4,6	MMS	+	+	+
PDR15	YGR133W	Pleiotropic drug transporter	1,3		+	+	
RDS2	YNL097C	Transcription factor	1,7	RG	+	-	+
YHP1	YGR143W	Transcription factor	2,4,6		+	+	N.D.
MBP1	YDR248C	Transcription factor	2,4,6	RG	+	+	+
UME6	YNR059W	Transcription factor	2,4,5,6	CS, HU,	+		+
HRD1	YPL089C	Protein folding	8		+		+
SSM4	YIL030C	Protein folding	8		+	+	+
PAC10	YDR286C	Protein folding	8	HU		+	+
SIP18	YNL072W	Osmotic stress	9		+		+
RPL8B	YHR202W	Ribosomal protein	9		+	+	+
ETT1	YDR248C	Translational termination	9			-	+

4.3.4 Growth of mcm2$_{AA}$ strain in agents that disrupt cell wall integrity

Caffeine also affects the cell wall integrity pathway by inhibiting the TorC1 in budding yeast[32]. I grew the *mcm2$_{AA}$* strain in the presence of Calcofluor white and ethanol to ensure that the sensitivity of the *mcm2$_{AA}$* strain to caffeine is due to inhibiting the

[a] Genes are grouped by their GO terms as annotated in the *Saccharomyces cerevisiae* database. Horizontal lines separate different classes.
[b] Gene ontology: (1) Response to cell stress/chemical stimuli (2) cell cycle, (3) transport, (4) RNA metabolic process, (5) signalling process/protein modification process, (6) transcription, (7) carbohydrate metabolic process, (8) ER-mediated degradation & protein-folding and (9) other.
[c] Relevant phenotype as annotated in the *Saccharomyces cerevisiae* database. CS, sensitivity to caffeine; HU, sensitivity to hydroxyurea; MMS, sensitivity to methyl methanesulfonate; OS, sensitivity to oxidative stress; RG, respiratory growth absent or decreased.
[d] "+" indicates enhanced growth relative to *mcm2$_{AA}$* or xxxΔ cells on the same plate; "-" indicates *mcm2$_{AA}$ xxx*Δ cells have decreased growth of the double mutant relative to the single mutations; and "N.D." indicates phenotype not determined. Growth of all the strains was comparable on YPD plates.

checkpoint pathway and not the cell wall integrity pathway. Calcofluor white and ethanol disrupt the cell wall integrity[45; 46]. Both the *MCM2* strain and *mcm2$_{AA}$* strain grew to the same extent in the presence of these agents indicating the cell wall integrity pathway is unaltered in *mcm2$_{AA}$*-containing cells (Figure 4-3).

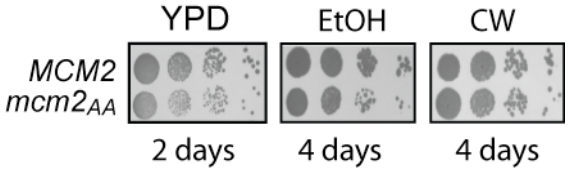

Figure 4-3 The growth of the *mcm2$_{AA}$* strain in cell wall destabilizing agents. The growth of *MCM* and *mcm2$_{AA}$* strains on YPD media containing 6 % ethanol ("EtOH") or 10 µg/ml calcofluor white ("CW").

4.3.5 Growth of mcm2$_{AA}$ and mcm2$_{EE}$ cells in the presence of replicative stress

To further define what types of cell stresses affect the growth of the *mcm2$_{AA}$* strain, I examined the growth of the *mcm2$_{AA}$* strain on media containing various agents that cause replicative stress (Figure 4-4A & B). Cells that contain *mcm2$_{AA}$* had reduced growth relative to *MCM2* cells on YPD plates containing MMS or 5-fluorouracil (5-FU; Figure 4-4A), but not on plates containing phleomycin (Figure 4-4B). Each of these agents has different effects on budding yeast cells. MMS damages DNA by methylating guanines and adenines[47]. The effects of 5-fluorouracil in yeast cells are two-fold: it inhibits the pyrimidine biosynthesis pathway and misincorporates uracil into nascent DNA[48]. Phleomycin, similar to the structurally-related bleomycin, causes double strand DNA breaks[49]. The *mcm2$_{AA}$* strain is also sensitive to hydroxyurea, an inhibitor of ribonucleotide reductase, which interferes with the integrity of DNA replication forks and induces an S phase checkpoint[26; 50].

I noted in Chapter 3 that a version of *MCM2* in which S164 and S170 are mutated to glutamic acids (*mcm2*$_{EE}$) provides phosphomimetic functions in the presence of caffeine. If caffeine inhibits the checkpoint resulting in the accumulation of DNA damage, then *mcm2*$_{EE}$ should provide phosphomimetic functions in the presence of agents that DNA damage directly. To examine whether this phosphomimetic phenotype applied to the growth of *mcm2*$_{EE}$ cells in the presence of DNA base damage, I examined the growth of *mcm2*$_{EE}$ cells in the presence of HU, MMS and 5-FU. As predicted, the *mcm2*$_{EE}$ strain grew similar to wild type cells in the presence of each of these agents (Figure 4-4C).

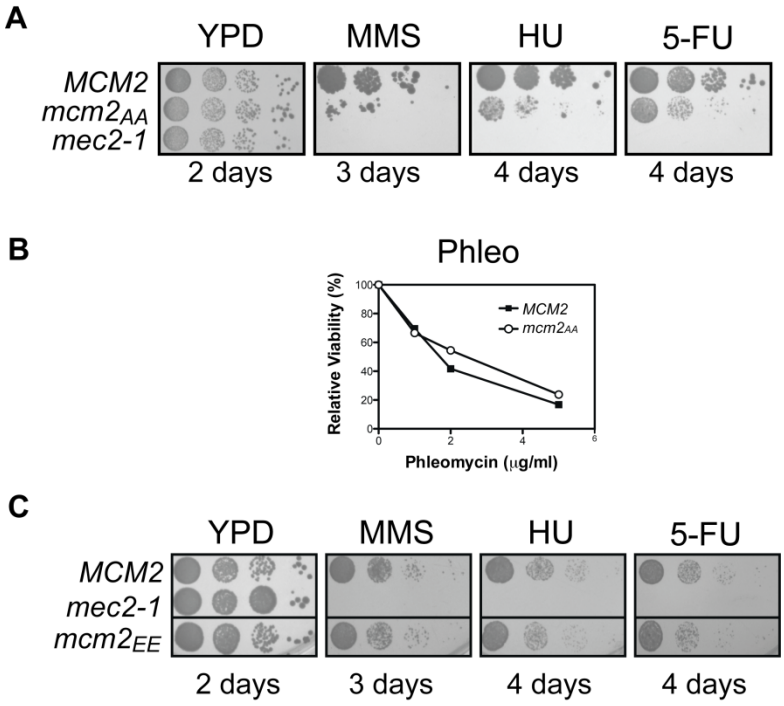

Figure 4-4 Growth of *mcm2*$_{AA}$ and *mcm2*$_{EE}$ cells under replicative stress. A. Ten-fold serial dilutions of *MCM2* and *mcm2*$_{AA}$ cells were spotted onto YPD media and YPD containing 200 mM hydroxyurea ("HU"), 0.03 % methyl methane sulfonate ("MMS") or 400 μM 5-fluorouracil ("5-FU") and grown at 30°C for the time indicated below each image. The *mec2-1* strain was included on the plates containing HU, MMS and 5-FU as a quality control. **B.** Growth of *MCM2* and *mcm2*$_{AA}$ strains in the presence of phelomycin in liquid culture. The survival of the strains after a 4 hour exposure to the indicated concentration of phelomycin relative to untreated cells is shown. **C.** Ten-fold serial

dilutions of *MCM2* or $mcm2_{EE}$ cells were spotted on YPD media and YPD media containing 200 mM hydroxyurea ("HU"), 0.03 % methyl methane sulfonate ("MMS") or 400 μM 5-fluorouracil ("5-FU") and grown at 30°C for the time indicated below each image. The *MCM2*, *mec2-1* and $mcm2_{EE}$ strains shown were spotted on the same plate.

4.3.6 Suppressors of the sensitivity of $mcm2_{AA}$ in the presence of MMS, 5-FU and HU

It now seemed likely that the effects of caffeine on the $mcm2_{AA}$ strain were due to the inhibition of checkpoint kinases resulting in increased DNA damage. I predicted that gene deletions that suppress the sensitivity of $mcm2_{AA}$ to caffeine will also suppress the sensitivity of the $mcm2_{AA}$ strain to agents that damage DNA. I tested the growth of 10-fold serial dilutions of the $mcm2_{AA}$ *xxx*Δ strains from Figure 4-2 on YPD plates containing 0.03 % MMS, 200 mM HU and 400 μM 5-FU and examined their growth relative to the *MCM2* and $mcm2_{AA}$ strains and was done for Figure 4-2. Table 4-3 summarizes the results of visually comparing the growth of each $mcm2_{AA}$ *xxx*Δ strain to the strains containing the single mutations ($mcm2_{AA}$ or *xxx*Δ). As predicted, deletion of most of these genes suppressed the growth defects of the $mcm2_{AA}$ strain in the presence of MMS (13 of 16) and 5-FU (14 of 16; Table 4-3). However, fewer gene deletions (8 of 16) suppressed the sensitivity of $mcm2_{AA}$ to HU (Table 4-3). Taken together, these data support the role of phosphorylation of Mcm2 by DDK in the cell's response to DNA damage.

4.4 Discussion

4.4.1 Phosphorylation of Mcm2 by DDK is not required for replication initiation

DDK functions in both initiation and in the S phase checkpoint[1; 2; 3; 4; 5; 6; 7; 8; 9; 13; 26; 27; 28; 29]. I previously showed that a version of budding yeast *mcm2* in which the serines targeted by DDK (S164 and S170) are mutated to alanines ($mcm2_{AA}$) does not affect the growth of *S. cerevisiae* cells when expressed at endogenous levels (Chapter 3). In

agreement with this observation, the N-terminal region of Mcm2 containing serine 164 and 170 can be deleted and support growth in budding yeast[10]. To further confirm that phosphorylation of Mcm2 is not required for initiation, I examined the cell cycle of the *mcm2$_{AA}$* strain by FACS analysis. Phosphorylation of Mcm4 is the essential function of DDK and is required for initiation[10]. Shue and Stillman (2006) generated a strain containing a mutant version of *mcm4* in which the region containing the serines targeted by DDK is deleted. This strain had a large population of cells with a 2C DNA content and the cells were slow to enter the S phase relative to wild type cells, indicating a defect in initiation[8]. Conversely, here I show that the DNA content of asynchronously growing *mcm2$_{AA}$*-containing cells and the progression of the *mcm2$_{AA}$* strain through the cell cycle are similar to wild type cells in rich media (Figure 4-1). This supports the idea that phosphorylation of Mcm2 is not required for replication initiation under normal growth conditions. In the presence of caffeine however, the progression of the *mcm2$_{AA}$* strain is altered. The *mcm2$_{AA}$*-containing cells enter S phase at a similar time as wild type cells but then progress through the S phase slowly compared to wild type cells. This observation indicates that caffeine exerts its effects on the *mcm2$_{AA}$* strain during the S phase.

4.4.2 Genetic interactions with mcm2$_{AA}$

The effects of caffeine on yeast cells are pleiotrophic, inhibiting the TorC1 kinase in the TOR signalling pathway which affects cell growth and cell wall integrity[32; 33; 34] as well as inhibiting kinases involved in the DNA checkpoint such as Tel1/Mec1[35; 36]. Here, the *mcm2$_{AA}$* strain was not sensitive to agents that destabilize the cell wall, thus, the effects of caffeine on the growth of *mcm2$_{AA}$*-containing cells are likely due to the inhibition of the S phase checkpoint and not due to inhibition of the cell wall integrity

pathway. In general, the 16 gene deletions that suppress the sensitivity of the *mcm2$_{AA}$* strain to caffeine have functions in the cell's response to DNA damage. I previously proposed a model in which phosphorylation of Mcm2 by DDK is involved in slowing the progression of replication forks in the presence of DNA damage (Chapter 3). In this model, the Mcm2$_{AA}$ mutant is defective in slowing replication forks in the presence of DNA damage. Consistent with my model, gene deletions that result in replication fork slowing in budding yeast suppress the sensitivity of the *mcm2$_{AA}$* strain to caffeine; Tof1 promotes replication fork progression and deletion of *tof1* slows replication forks[51]. Additionally, deletion of *SRS2*, *RAD2* or *UME6* induces homologous recombination, which has been shown to slow replication forks[52; 53; 54; 55; 56; 57; 58; 59; 60; 61; 62].

Another potential mechanism for suppressing the sensitivity of *mcm2$_{AA}$*-containing cells to caffeine involves reducing the amount of endogenous DNA damage in the *mcm2$_{AA}$*-containing cells. Three suppressor mutations (*rad9*Δ*, rds2*Δ and *mbp1*Δ) identified here are defective in respiratory growth (as annotated in SGD). Interestingly, respiration by the mitochondria is thought to be a major source of reactive oxygen species (ROS), which damage DNA[63; 64]. Yeast that are grown on limiting media exchange between respiration and glycolysis in a cyclical manner and replicate only during the glycolytic phase[65]. Genomic instability increases if replication is forced to occur during respiration[65]. ROS damage DNA, thus mutations that interfere with respiration and decrease the amount of ROS in the cell decrease the amount of spontaneous DNA damage that occurs in S phase. This damage is likely sufficient to arrest cell growth in the presence of caffeine. Protection against ROS can also occur by protein misfolding. When proteins are misfolded, the protein misfolding response is induced and has a protective

effective against ROS in cells[66]. Here, we find deletions of *hrd1*, *ssm4* and *pac10* suppress the caffeine sensitivity of the $mcm2_{AA}$ strain. Hrd1, Ssm4 and Pac10 are required for targeting misfolded proteins for degradation[67; 68]. It should also be noted that caffeine suppresses signalling in the TOR pathway. Inhibition of TOR signalling by mutation has been previously shown to increase the amount of respiration and production of ROS in yeast[69]. Additionally, inhibition of ATM in human cells (Tel1 in yeast) increases ROS in the cell[70; 71]. Thus, caffeine not only inhibits the signalling to repair DNA damage via the S phase checkpoint by inhibiting the Mec1/Tel1 kinases, but also likely increases the amount of ROS in the cell contributing to the damage of DNA.

I identified a common function among many of the gene deletions upon examination of the SSL interactions with $mcm2_{AA}$: they contribute to increased DNA damage. Rad53 is the central player in the S phase checkpoint and loss of Rad53 contributes to increased DNA damage (reviewed in ref. 44). Furthermore, the checkpoint effector kinase, Chk1 is synthetic lethal with $mcm2_{AA}$. Chk1 is activated by Mec1/Tel1 in response to DNA damage and is necessary for DNA repair[72; 73; 74]. Ctf4 participates directly in homologous recombination repair to repair DNA damage[75] and furthermore, a genetic network mapping study finds that *CTF4* belongs to a network of genes required for the response to oxidative stress[76]. *SOD1* and *PEP3* are also involved in the oxidative stress response[76] and deletion of *vma13* also increases the sensitivity of cells to oxidative DNA damage[77; 78]. Interestingly, in a proteomics study that identified several targets of S phase checkpoint kinases in yeast cells identified Skn1, Sod1 and Vma13 indicating their activities may be regulated by the S phase checkpoint[79]. Collectively, the SSL interactions with $mcm2_{AA}$ suggest that the $mcm2_{AA}$ strain cannot tolerate gene mutations that result in

increased levels of endogenous DNA damage and therefore, that these two phosphosites in Mcm2 are critical for the cell's response to DNA damage.

4.4.3 The requirement for phosphorylation of Mcm2 by DDK in response to DNA base damage

Checkpoint activation is elicited by multiple types of cellular stress (reviewed in ref. 18). Here I find that DDK-dependent phosphorylation of Mcm2 is important for the response to base damage but not for the response to double strand breaks; $mcm2_{AA}$-containing cells are sensitive to agents that cause point mutations or nucleotide misincorporations (MMS and 5-FU) but not to an agent that causes double strand breaks (phleomycin). In budding yeast, Mec1 and Tel1 do not directly bind to the DNA damage but instead recognize the complexes that assemble at the sites of DNA damage or recognize the complexes that form as part of the DNA repair processes (reviewed in ref. 80). Mec1 binds to complexes that are typically generated in base excision repair, nucleotide excision repair and mismatch repair whereas Tel1 binds to complexes that recognize double-strand breaks[80; 81; 82]. Although the functions of Mec1 and Tel1 in the checkpoint partially overlap, each likely has distinct downstream consequences as part of checkpoint activation[19; 80; 83]. I speculate that phosphorylation of Mcm2 by DDK requires the Mec1-mediated response since the $mcm2_{AA}$ strain is sensitive to agents that cause point mutations. The $mcm2_{AA}$ strain is also sensitive to HU. The primary effect of HU on budding yeast cells is the inhibition replication fork progression by inhibiting ribonucelotide reductase (RNR)[26]. RNR catalyzes the rate limiting step in the production of deoxyribonucleotides and inhibition of RNR by HU lowers dNTP levels in the cell[84; 85; 86]. However, insufficient RNR activity in fission yeast has been shown to increase the rate

of spontaneous mutations by more than 10-fold[87]. I surmise the sensitivity of the *mcm2$_{AA}$* strain to HU is due to the secondary effect of increased mutations caused by HU. This idea is supported by the fact that the growth of the *mcm2$_{AA}$* strain is reduced only after long-term exposure to HU on plate media and not after short-term exposures in liquid culture (Chapter 3).

4.4.4 The growth of mcm2$_{AA}$ xxxΔ cells in replicative stress

Gene deletions that suppressed the caffeine sensitivity of *mcm2$_{AA}$*-containing cells also largely suppressed the MMS and 5-FU sensitivity of the *mcm2$_{AA}$* strain (13 and 14 of 16, respectively; Table 4-3). However, deletion of *rad9* in combination with *mcm2$_{AA}$* enhanced the sensitivity of cells to MMS on plates and in liquid media (Table 4-3 and data not shown). In budding yeast, Rad9 has important roles in both DNA mismatch repair[88] and in activating Rad53[89]. These dual roles may reflect the differences in phenotypes observed when *mcm2$_{AA}$ rad9*Δ cells are subjected to caffeine versus MMS. Noteworthy also are the differences in sensitivity of *mcm2$_{AA}$ xxx*Δ cells to hydroxyurea. Fewer of gene deletions suppress the sensitivity of the *mcm2$_{AA}$* strain to HU (8 of 16; Table 4-3). Interestingly, studies in budding yeast of the genetic networks that are required in response to HU-induced stress and MMS-induced stress indicate that although common mechanisms exist for the cell to deal with both stresses, unique gene networks are also utilized to deal with either MMS or HU[90; 91]. I speculate that the differences in the number of suppressors of the sensitivity of the *mcm2$_{AA}$* strain to HU and MMS arise from how budding yeast deal with these different types of stresses.

4.4.5 The role of Mcm2 phosphorylation by DDK in budding yeast

Mutations of genes involved in the S phase checkpoint can result in the accumulation of ssDNA in budding yeast and increased DNA damage[92; 93; 94]. When the S phase checkpoint is activated, slowing of DNA unwinding by Mcm2-7 is likely required to prevent the inadvertent accumulation of ssDNA and to allow the cell time to repair damaged DNA. DNA unwinding by a Mcm2-7 complex containing the DDK-specific phosphomimetic version of the Mcm2 subunit ($Mcm2_{EE}$-7) is reduced (Chapter 3). I propose a model in which phosphorylation of budding yeast Mcm2 by DDK slows DNA unwinding, and thus replication fork progression, during the S phase checkpoint in response to DNA base damage.

4.5 References

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CHAPTER 5 CONCLUSIONS AND IMPLICATIONS: MCM2 AS AN ACTIVE REGULATOR OF MCM2-7 IN THE CELL'S REPONSE TO DNA DAMAGE

5.1 Why study the regulation of Mcm2-7?

DNA replication is an essential process for all living organisms. Study of the regulation of DNA replication is critical for understanding how perturbations in the process can result in proliferative diseases, such as cancer. Replicative helicases, the complexes responsible for unwinding DNA in cells during DNA replication, exist in different oligomeric forms, varying from monomers to dodecamers. Furthermore, they vary in their subunit compositions (reviewed in ref. 1). Many helicases function as oligomers of identical subunits[1; 2; 3; 4; 5; 6]. In contrast, the replicative helicase in eukaryotes is comprised of six distinct subunits, Mcm2 through 7, each of which is proposed to possess unique functions[7; 8]. Studies of the functions of each subunit are essential to understand the mechanisms of DNA unwinding within eukaryotic cells. A further level of complexity is added when examining the modification of individual Mcm subunits by kinases. One of the kinases that modulates the activity of Mcm2-7 is the Dbf4-dependent kinase (DDK), comprised of the Cdc7 catalytic subunit and the Dbf4 regulatory subunit[9; 10; 11; 12; 13; 14; 15; 16; 17]. Further justification for studying the regulation of Mcm2-7 by DDK is highlighted in a number of studies demonstrating the potential for targeting DDK in anti-cancer therapeutics[18].

In this thesis, I have defined the Mcm2 subunit as an active regulator of DNA unwinding and binding by the Mcm2-7 complex. Specifically, Mcm2 utilizes ATP binding and hydrolysis to regulate DNA unwinding and binding by Mcm complexes. I have also demonstrated that DDK regulates ATP hydrolysis by Mcm2. DNA unwinding

is inhibited in a version of the Mcm2-7 complex with a DDK-specific phosphomimetic mutant of the Mcm2 subunit. This is the first report that modification of a subunit of Mcm2-7 by DDK affects the DNA unwinding activity of the Mcm2-7 helicase. Lastly, I have defined the biological relevance of Mcm2's regulatory function in budding yeast; phosphorylation of Mcm2 by DDK is important for the cell's response to DNA damage.

5.2 Mcm2 is an active regulator of Mcm complexes

A subcomplex of Mcms comprised of Mcm4, Mcm6 and Mcm7 that was originally isolated from HeLa cells unwinds DNA[19]. Before DNA unwinding by Mcm2-7 was observed by Schwacha and Bochman[20], Mcm4/6/7 served as a model for DNA unwinding for many years and has generated many insights into the mechanism of how Mcm complexes unwind DNA[21; 22; 23; 24]. Mcm2, Mcm3 and Mcm5 inhibit DNA unwinding by Mcm4/6/7[19; 25; 26; 27; 28]. This observation has led to the suggestion that Mcm4, Mcm6 and Mcm7 form the catalytic core of Mcm2-7 and that Mcm2, Mcm3 and Mcm5 function as regulators within Mcm2-7 (Figure 5-1). The function(s) that each subunit has in the cell is essential since each subunit is essential (reviewed in ref. 29).

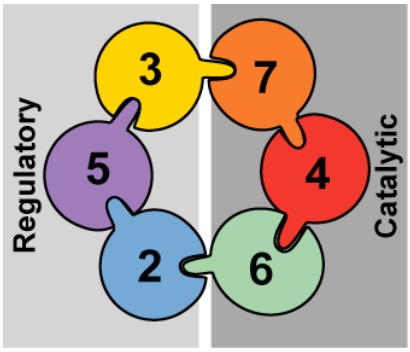

Figure 5-1 Model of the function of Mcm subunits within Mcm2-7. One half of the Mcm2-7 complex is comprised of the Mcm2, Mcm3 and Mcm5 subunits which are thought to function as regulators of DNA binding and unwinding by Mcm2-7. The other half of the complex is comprised of the Mcm4, Mcm6 and Mcm7 subunits which are proposed to directly catalyze DNA unwinding. Adapted from ref. 7.

The results I presented in Chapter 2 define a regulatory role for Mcm2 in the Mcm2-7 complex. Prior to my work, Mcm2 was thought to inhibit the Mcm4/6/7 complex passively, simply through interaction of Mcm2 with Mcm4/6/7[28; 30]. Here, I provided evidence that ATP binding and hydrolysis by Mcm2 is required to regulate the DNA binding and unwinding activities of Mcm complexes (Chapter 2). Consistent with this role, recent studies show that recombinant budding yeast Mcm2-7 complexes containing the lethal lysine to alanine mutation in the P-loop of either the Mcm2 or the Mcm3 subunits unwind DNA *in vitro*[31; 32]. Similar mutations in the Mcm4, Mcm5, Mcm6, or Mcm7 subunits do not support DNA unwinding in Mcm2-7 complexes and have a greater effect on ATP hydrolysis by Mcm2-7. These data are consistent with Mcm2 and Mcm3 having regulatory roles and Mcm4, 6 and 7 functioning in the catalysis of DNA unwinding. In my work, mutation of the P-loop of Mcm5 did not alter its ability to inhibit DNA unwinding by Mcm4/6/7, however, further studies are required to elucidate the nature of DNA unwinding inhibition by Mcm3 and Mcm5. The functions of each subunit may change when other factors associate with Mcm2-7, such as Cdc45 and

GINS. The formation the CMG complex by association of Cdc45 and GINS with Mcm2-7 stimulates the helicase activity of *Drosophila* Mcm2-7[33]. In this study, Ilves *et al.* made 6 different CMG complexes in which each Mcm subunit contained a lysine to alanine substitution in the P-loop analogous to those used in the studies with yeast Mcms[33]. In stark contrast to the findings with yeast Mcms, the lysine to alanine substitutions in the Mcm2 or Mcm3 subunits resulted in CMG complexes that unwound DNA poorly whereas similar substitutions in Mcm4, Mcm6 or Mcm7 unwound DNA similarly to wild type CMG. A CMG complex containing a mutant version of Mcm5 did not unwind DNA which is consistent with the findings using budding yeast Mcm2-7[33]. The differences between *Drosophila* CMG complexes and yeast Mcm2-7 complexes may be attributed to differences in activity of Mcm2-7 when associated with Cdc45 and GINS and/or that Mcm2-7 behaves differently in *Drosophila* and budding yeast. Future studies with yeast CMG will help in addressing this question.

5.3 Allosteric regulation using nucleotide

My studies have determined that Mcm2 uses ATP hydrolysis to allosterically regulate the DNA binding and unwinding activity of Mcm2-7. An example of using ATP binding and hydrolysis to allosterically regulate DNA binding and unwinding can also be observed in the monomeric PcrA helicase of *E. coli*[34]. Mutations at an arginine residue in PcrA (R260) resulted in a mutant protein that hydrolyzes ATP and binds DNA similar to the wild type protein but was unable to unwind DNA[34]. ATP is also used to allosterically regulate the dimeric Rep DNA helicase complex from *E. coli*[35; 36; 37]. In the Rep dimer the nucleotide binding status of one subunit is allosterically communicated to another subunit to regulate ssDNA association[35; 36; 37]. Hexameric helicases also uses ATP to allosterically

regulate DNA unwinding and binding including the AAA+ superfamily-member RuvB which resolves Holliday junctions formed during recombinational repair and homolgous recombination at stalled replication forks in *E. coli*[38; 39].

5.4 Mcm2 communicates its nucleotide-binding status to other subunits

The mechanisms by which helicases bind and hydrolyze ATP have given insights into the potential mechanisms by which ATP binding and hydrolysis may function within complexes of Mcms. In the crystal structure of the BPV E1, the subunits vary in their nucleotide-binding status sequentially around the helicase ring[4]. When nucleotide binds to one subunit, *trans*-interacting residues of a neighbouring subunit are pulled toward the bound nucleotide causing conformational changes in the *trans*-subunit (Figure 5-2; ref. 4). SV40 LTag and the archael SsoMCM complex function similarly[40; 41].

Interactions in *trans* are important at the subunit interface of Mcm proteins as well. The arginine 708 of the SRF motif of Mcm6 is predicted to act in *trans* with the nucleotide bound to Mcm2[7]. Consistent with this idea, I showed in Chapter 2 that substitution of this arginine with an alanine ($Mcm6_{R708A}$) has the same effect on ATP hydrolysis in the Mcm2/6 pair as does mutating lysine 549 to alanine in the Mcm2 P-loop. The $mcm6_{R708A}$ mutation is lethal in budding yeast[31] and is similar to the lethality of the $mcm2_{K549A}$ mutation I observe (Figure 2-9). These observations support the conclusion that interactions in *trans* between Mcm2 and Mcm6 are required for Mcm2's regulatory function.

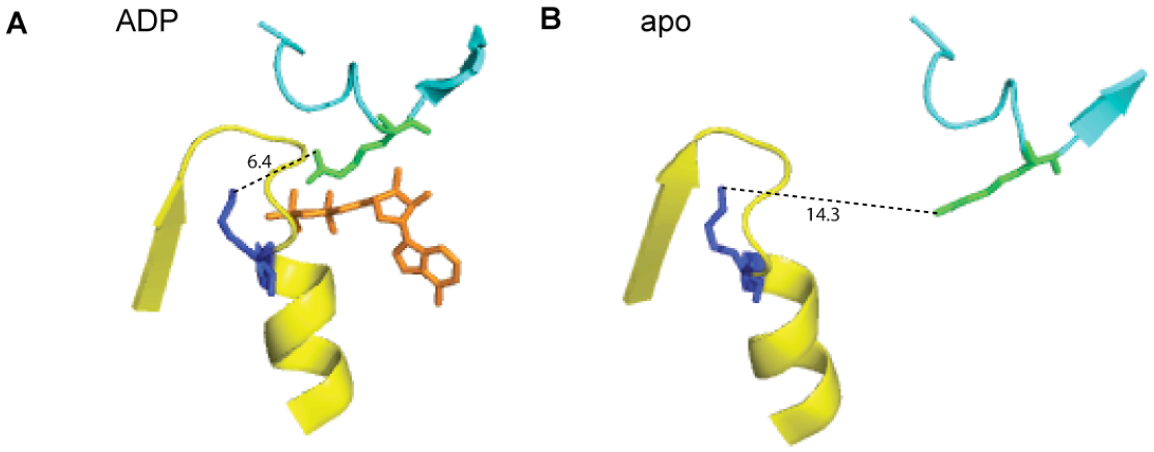

Figure 5-2 Structural changes of the ATP sites of BVP E1 upon nucleotide binding. A view the subunits interfaces of the BVP E1 crystal structure (PDB ID=2GXA) and in the **(A)** ADP-bound form and **(B)** nucleotide-free form ("Apo"). In yellow is the P-loop motif of one subunit and in cyan the SRF motif of a neighbouring subunit. Highlighted in blue is the conserved lysine (K439) in the P-loop and in green the arginine (R538) of the SRF motif. Distances (in angstroms) between K439 and R538 are shown by the dashed line.

5.5 The roles of nucleotide hydrolysis in regulating DNA binding and unwinding in helicases

The conformational changes associated with nucleotide binding and hydrolysis are critical for DNA binding and unwinding in helicases. In the crystal structure of SV40 LTag, the *trans*-interacting arginine (R538) of the SRF motif is located at the base of the pre-sensor 1 (PS1) β-hairpin (Figure 5-3A; refs. 4; 41). Mutation of the PS1 hairpin in SV40 LTag, as well as in SsoMCM and BVP E1, interfere with DNA binding and unwinding[42; 43; 44]. However, DNA unwinding is affected more strongly than DNA binding demonstrating that mutations of the PS1 β-hairpin affect translocation of the

helicase along DNA[42; 43; 44]. The position of this hairpin with respect to DNA is dependent on whether ATP, ADP or no nucleotide occupies a subunit's ATP site.

I theorize that the ATP-binding status of Mcm2 is communicated to the Mcm6 subunit to regulate DNA unwinding and binding by the Mcm2-7 complex. Thus Mcm2 is not directly involved in DNA binding by Mcm2-7 *per se* but indirectly through altering the conformation of a neighbouring subunit (such as Mcm6). Mutation at the ATP site of Mcm2 results in a conformation of Mcm2-7 that favours DNA binding, but decreases DNA unwinding (Figure 2-8, 3-5 and 3-6). Genetic and biochemical studies of the interactions between the ATP sites and β-hairpins of Mcm2-7 will aid in addressing this question.

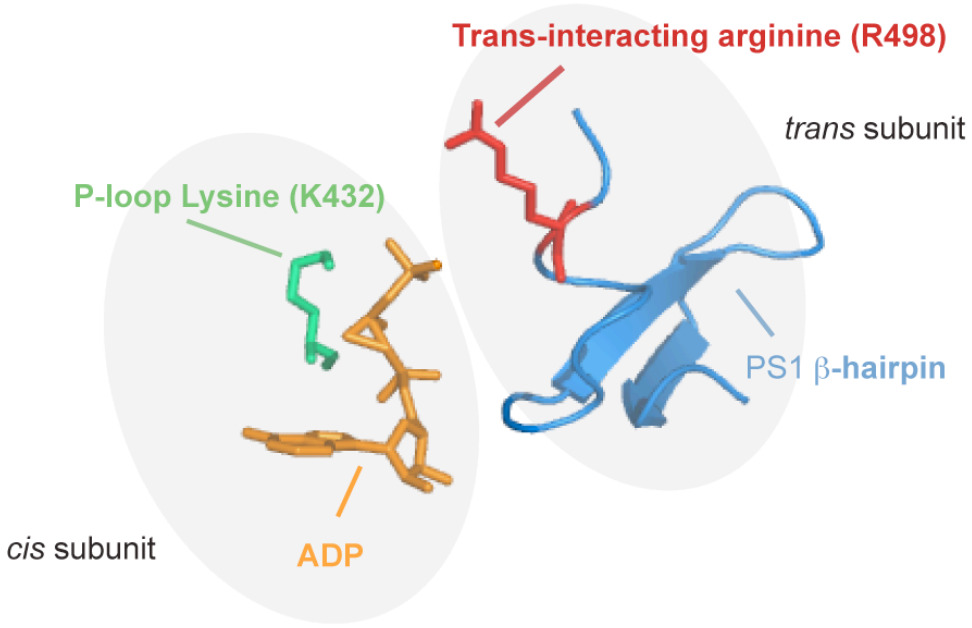

Figure 5-3 *Trans*-interacting residues in SV40 LTag. A. A view of the structure of the β-hairpin (residues 495-523) of one subunit of SV40 LTag (*trans* subunit) and a residue of the phosphate binding loop of a neighbouring (*cis*) subunit (PDB ID=1SVM). Highlighted in red is a *trans*-interacting arginine residue (R498) at the base of the β-hairpin (blue), in green is a strictly conserved *cis*-interacting lysine (K432) of the phosphate binding loop and in orange the bound nucleotide (ADP). The grey background is a representation of the space occupied by the *cis* and *trans* subunits.

5.6 When is Mcm2 required to regulate DNA binding by Mcm2-7?

DNA binding by Mcm2-7 is important during at least two stages of DNA replication: initiation and elongation. During initiation, Mcm2-7 first binds to replication origins. During elongation, Mcm2-7 at the replication fork must remain associated with DNA for DNA unwinding. Here, I propose a model in which ATP hydrolysis by Mcm2 is required to regulate translocation of the helicase along DNA (Figure 5-4A). In this model, wild type levels of ATP hydrolysis at the Mcm2/6 interface support translocation of the

helicase. If ATP hydrolysis by the Mcm2 subunit is inhibited then Mcm2-7 binds DNA too tightly and translocation by Mcm2-7 is inhibited.

An alternative model is possible in which DNA binding by Mcm2-7 is regulated by ATP hydrolysis by Mcm2 during initiation (Figure 5-4B). I support the model in which ATP hydrolysis by Mcm2 is required to regulate translocation of Mcm2-7 based on a number of observation presented in this thesis and from other works. First, complexes of Mcm2-7 that contain ATP site mutants of Mcm2 unwind DNA poorly but bind DNA tightly. This suggests that the mutant Mcm2-7 complex is not defective in binding DNA but is defective in translocating along the DNA (Figures 3-5 and 3-6). If initiation were defective I would anticipate a reduced affinity of Mcm2-7 for DNA. Second, an initiation defect would likely result in growth defects; however, cells containing an ATP site mutant of Mcm2 that is severely compromised for ATP hydrolysis ($mcm2_{K549R}$) grow like wild type cells (Figure 2-9). Third, work from Chapters 3 and 4 demonstrates that DDK-dependent phosphorylation of Mcm2 regulates ATP hydrolysis. Under normal growth conditions, cells harbouring a *mcm2* allele that cannot be modified by DDK ($mcm2_{AA}$) grow normally and progress through the cell cycle similar to wild type cells when growth is unperturbed (Figures 3-3 and 4-1). In the studies of Sheu and Stillman (2006), a mutant version of *mcm4* that is defective in initiation had a large 2C DNA-content in asynchronously growing cells and were slow to enter S phase relative to wild type cells[16]. In contrast, $mcm2_{AA}$-containing cells had wild type-like distributions of 1C and 2C DNA and entered S phase at a similar time as wild type cells. Lastly, the essential function of DDK in yeast is likely the phosphorylation of the Mcm4 subunit during initiation[45], thus

DDK-dependent modification of Mcm2, and the consequent regulation of its ATP hydrolysis activity, is most likely not required for initiation.

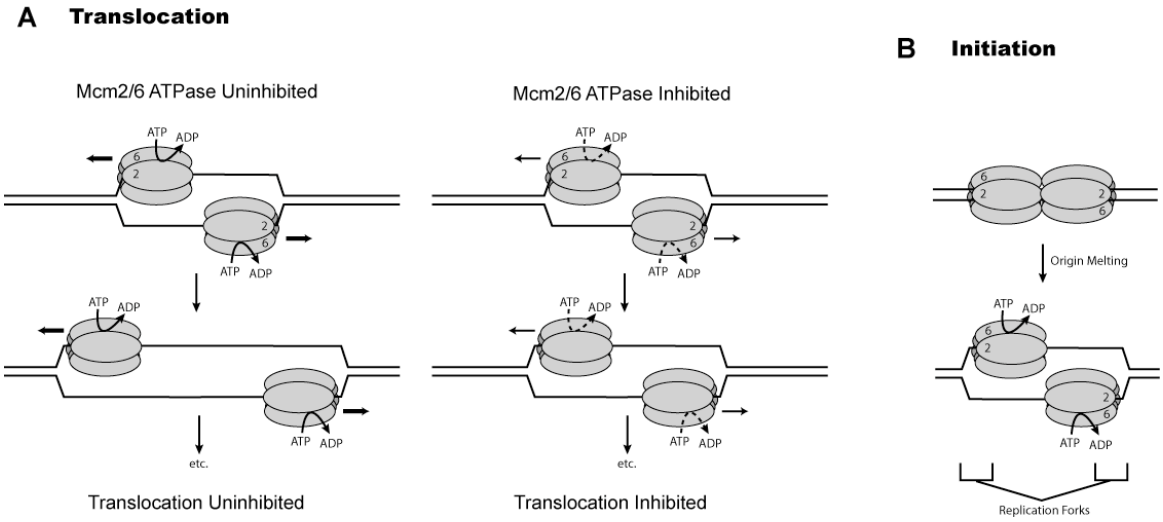

Figure 5-4 Models of ATP hydrolysis by Mcm2 regulating DNA binding and unwinding by Mcm2-7. A. A model predicting the requirement of ATP hydrolysis by Mcm2 in regulating translocation of the Mcm2-7 helicase. Depicted is the movement of two Mcm2-7 hexamers at initiated replication forks. Wild type levels of ATP hydrolysis at the Mcm2/6 subunit interface support translocation of the helicase ("Mcm2/6 ATPase Uninhibited"). Mutations at the Mcm2/6 ATP site that decrease ATP hydrolysis interfere with translocation ("Mcm2/6 ATPase Inhibited"). **B.** A model depicting the involvement of ATP hydrolysis by Mcm2 in regulating the DNA binding activity of Mcm2-7 during initiation. "Origin melting" refers to the production of ssDNA from dsDNA during initiation generating the replication bubble. Bi-directional replication forks are shown. ATP hydrolysis at the Mcm2/6 subunit interface is shown but ATP hydrolysis at the other subunit interfaces is omitted for simplicity. Only DNA unwinding by a single hexamer in the steric exclusion model is shown for clarity of the figure, but the other models of DNA unwinding depicted in Figure 1-5 are possible.

5.7 When is ATP hydrolysis by Mcm2 regulated by Mcm2 phosphorylation?

I demonstrated phosphorylation of Mcm2 by DDK is required in the cell's response to DNA damage in Chapters 3 and 4 of this thesis. Environmental toxins, radiation and even replication itself can damage the genome[46]. The cell has established intricate mechanisms to deal with DNA damage. If DNA damage occurs during the S

phase, the S phase checkpoint in cells will halt DNA replication to allow the cell time to deal with the damage[47; 48]. Phosphorylation of Mcm2 by DDK provides one possible mechanism by which the advancement of replication forks can be slowed to allow time for repair in the event of DNA damage. Consistent with this hypothesis, cells that presumably cannot slow DNA unwinding (*mcm2$_{AA}$*) are sensitive to DNA damage (Figure 4-3). DDK is involved in the S phase checkpoint[49; 50; 51; 52; 53; 54; 55] and likely has at least two functions in the cell's response to DNA damage. First, Rad53-mediated phosphorylation of Dbf4 inhibits the firing of late origins by removing DDK from origins[49; 50; 51]. Second, DDK's kinase activity is likely required during the S phase checkpoint and DDK is found at replication forks during DNA damage to perform unknown functions[52; 53; 54; 55]. My results support an active role for DDK in the S phase checkpoint with respect to Mcm2 phosphorylation; the *mcm2$_{EE}$* phosphomimetic mutant suppresses growth defects of cells deleted at *cdc7* (but maintained by *mcm5-bob1*) in caffeine and HU (Figures 3-4). Together, these data are consistent with my model in which phosphorylation of Mcm2 by DDK is required to slow DNA unwinding and contributes to replication fork stability.

5.8 Putting it all together: a model for how phosphorylation of Mcm2 is required to slow replication forks

Phosphorylation can alter a protein by affecting its activity. Phosphorylation of Mcm2 alters its ability to hydrolyze ATP and regulates the DNA binding and unwinding by Mcm2-7 complex (Figures 3-5, 3-6 and 3-7). I proposed a model in Figure 5-4A in which ATP binding and hydrolysis by Mcm2 is required to regulate translocation of Mcm2-7. Expanding on this model, an unphosphorylated Mcm2 subunit within Mcm2-7

in cells growing normally would support DNA unwinding because its ATP hydrolysis activity is uninhibited (Figure 5-5A). Upon phosphorylation of Mcm2 by DDK in cells under replicative stress, ATP hydrolysis is inhibited and DNA unwinding is decreased due to the tighter association of the phosphorylated Mcm2-7 complex with ssDNA (Figure 5-5B). In budding yeast, uncontrolled replication fork progression in the presence of replicative stress caused by mutant checkpoint proteins generates unprotected ssDNA which is deleterious to the cell and increases the rate of mutagenesis[56; 57]. Similar to these checkpoint mutants, Mcm2-7 may accumulate unwanted ssDNA in the presence of replicative stress when DNA unwinding cannot be slowed, such as with the $mcm2_{AA}$ allele. Consistent with this idea, a recent study reports that depletion of Cdc7 in human cells increases the replication fork speed in Chk1-depleted cells (Chk1 in human cells is the functional homolog of Rad53 in yeast) suggesting that Cdc7 is required to slow the replication fork[58]. Additionally, intact Mcm2-7, Cdc45, GINS and polymerase complexes are found at stalled replication forks thus, DNA unwinding by Mcm2-7 must be inhibited at stalled replication forks[59]. It is not simply disassembly of the replisome that causes fork pausing.

The screen for suppressor mutations of the $mcm2_{AA}$ allele presented in Chapter 4 also provides evidence that phosphorylation of Mcm2 by DDK is required to slow replication forks. I postulate that $mcm2_{AA}$ cells are sensitive to replicative stress because DNA unwinding by Mcm2-7 in these cells cannot be slowed in response to genotoxic assaults. I therefore anticipated that intergenic mutations that slow replication forks may suppress the sensitivity of $mcm2_{AA}$ cells to replicative stress. Indeed, this is what I observed. Many of the gene deletions I identified as suppressors of the caffeine, MMS, 5-

FU and HU sensitivities of the *mcm2*$_{AA}$ strain can be attributed to slower moving replication forks. However, future studies of replication fork progression in *mcm2*$_{EE}$ cells are warranted to test this idea directly.

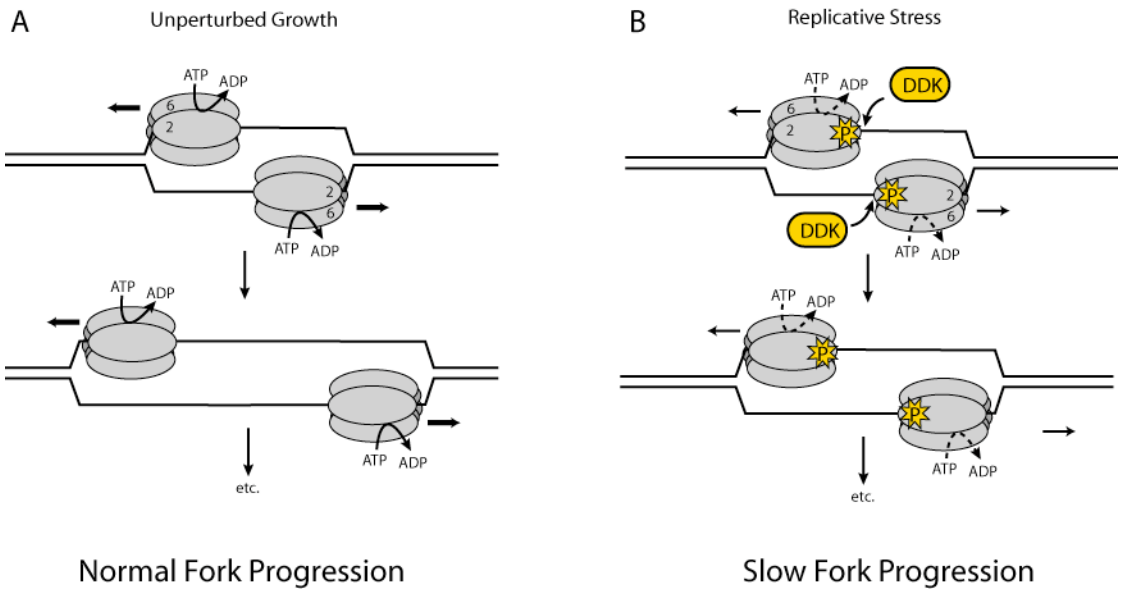

Figure 5-5 Model of phosphorylation of Mcm2 in regulating DNA unwinding by Mcm2-7 during the cell's response to DNA damage. A. Shown for comparison is the model from Figure 5-4C that would occur in cells that are undergoing unperturbed DNA replication ("Unperturbed Growth"). **B.** Depicted is a model for inhibition of replication fork progression upon phosphorylation of the Mcm2 subunit of Mcm2-7. In this model, DDK phosphorylates the Mcm2 subunit when DNA damage occurs. Consequently ATP hydrolysis by Mcm2 is reduced (dashed arrows) and DNA unwinding, and hence replication fork progression, is slowed.

5.9 How is DNA unwinding resumed if Mcm2 is phosphorylated?

The responses induced by the DNA damage checkpoint must be attenuated for growth of a cell to resume. In budding yeast, recovery from the DNA damage checkpoint is mediated in part through dephosphorylation of Rad53 by the protein phosphatases Ptc2 and Ptc3[60; 61; 62]. Alternatively, if the cell cannot repair the damage or encounters too much DNA damage, they can undergo adaptation to the checkpoint, which also involves

the inactivation of Rad53[63]. The adaptation response involves the cell ignoring the DNA damage and progressing through the cell cycle to repair the DNA damage in a subsequent cell cycle or continue to proliferate with the mutation. A feature common in both the recovery and adaptation responses is the resumption of DNA replication. If phosphorylation of the Mcm2 subunit leads to slower DNA unwinding by Mcm2-7 during replicative stress it implies that to resume DNA unwinding at a normal rate, Mcm2 must be dephosphorylated by a phosphatase. It is important to note that cells harbouring *mcm2$_{EE}$* grow at the same rate as wild type cells under all conditions I have tested (Figure 3-4). These observations are paradoxical with the fact that DNA unwinding by Mcm2$_{EE}$-7 is inhibited (Figure 3-5). The concentration of Mcms in cells is ~20-fold in excess to the number required for bidirectional replication forks (reviewed in ref. 29). In human cells and *Xenopus* egg extracts, dormant origins that are not used for DNA replication during normal cell growth can be licensed by the excess Mcm subunits in the presence of replicative stress[64; 65; 66]. These dormant origins fire near regions where replication forks have stalled[65]. In *mcm2$_{EE}$* cells, no overt phenotype such as the slowing of the S phase would be observed if licensing and firing these dormant origins negated the effects of a slow-moving replication forks. However, further studies on replication fork progression and origin utilization in *mcm2$_{EE}$* cells are needed to confirm this hypothesis.

5.10 Validation of the *in vivo* Mcm2 target residues of DDK

My evidence that DDK specifically targets serines 164 and 170 in budding yeast Mcm2 is two-fold. First, phosphomimetic substitutions of these sites (*mcm2$_{EE}$*) can bypass a growth defect of cells lacking yeast DDK (but supported for growth by the *mcm5-bob1* mutation) in the presence of caffeine and HU. Second, the *E. coli*-purified

$Mcm2_{AA}$ protein is not a substrate for DDK *in vitro* (Figure 3-1). It is formally possible that in cells these sites are modified by other kinases downstream of DDK during the cell's response to replicative stress. However, this seems unlikely for two reasons. First, studies of Mcms from a variety of species demonstrate that DDK directly modifies Mcm subunits, including Mcm2[10; 14; 15; 16; 17; 45; 67; 68; 69; 70; 71; 72; 73]. Second, to my knowledge, DDK has not been shown to activate other kinases that would subsequently modify Mcm subunits. The use of phospho-specific antibodies directed at phospho-S164 and -S170 of Mcm2 would be extremely useful in validating my findings in cells.

5.11 Phosphorylation of Mcm2 by DDK and the S phase checkpoint

Rad53 physically interacts with Cdc7 and Rad53 affects DDK activity[53; 74; 75; 76; 77]. It is perhaps not surprising then that I also detect a genetic interaction between $mcm2_{AA}$ and a checkpoint-defective version of *rad53* (*mec2-1*). Cells deleted at *chk1* are also synthetic lethal with $mcm2_{AA}$ (Table 4-2). Chk1 is an effector kinase of the DNA damage checkpoint and its activities work in parallel with Rad53 to pause replication forks[78; 79]. Although DDK's active role in the checkpoint has been alluded to in other studies[52; 53; 54; 55], it is unclear if or how Rad53 may influence DDK's function in this role. Based on the physical interactions between DDK and Rad53 and genetic studies with *cdc7, dbf4*, *rad53* and *chk1* mutants[52; 53; 54; 55; 74; 75; 76; 77], it seems likely that Rad53 and Chk1 may act in parallel pathways to activate DDK in the S phase checkpoint. Figure 5-6 presents a summary model of the induction of phosphorylation of Mcm2 during the S phase checkpoint in response to DNA base damage. Phosphorylation of the Mcm2 by DDK subunit inhibits its ATPase activity which, in turn, inhibits DNA unwinding by Mcm2-7. I

propose slowing DNA unwinding by Mcm2 is required to slow replication fork progression and allows the cell time to repair damaged DNA.

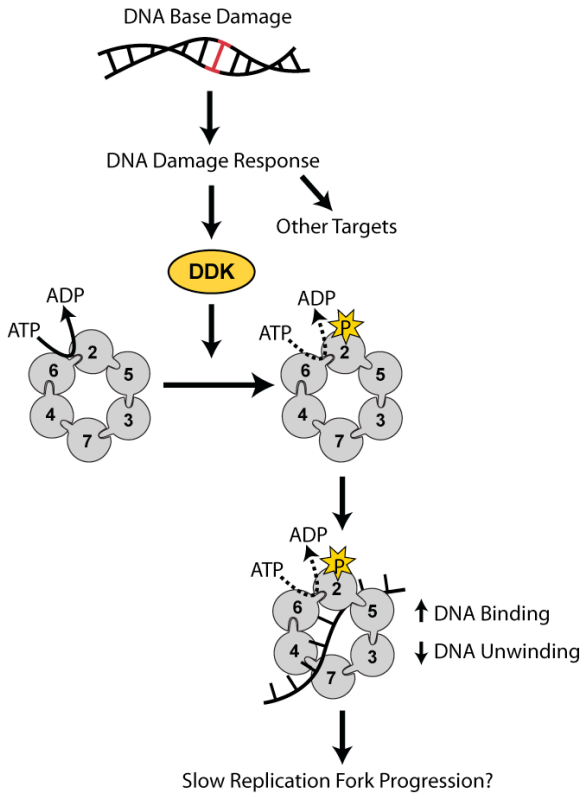

Figure 5-6 A summary model of the induction of DDK-dependent phosphorylation of Mcm2 and its downstream consequences. Shown is a flowchart representing the events I propose induce phosphorylation of Mcm2 by DDK and the consequences of this phosphorylation event on the activity of Mcm2-7.

5.12 Conclusions

Overall, the studies presented in this thesis have implications in the understanding of the fundamental cellular process of DNA replication. I have demonstrated for the first time that the Mcm2 subunit of Mcm2-7 is an active regulatory subunit that requires ATP hydrolysis for its function. Furthermore, I have shown that nucleotide-dependent regulation by Mcm2 is controlled by phosphorylation of Mcm2 by the Cdc7/Dbf4 kinase.

This provides a mechanism by which the activity of Mcm2 can be controlled and also addresses the question of the function of phosphorylation of Mcm2 by DDK in budding yeast. My work also shows that phosphorylation of Mcm2 is required for the cell's response to DNA base damage. Future studies to determine if this function of DDK is conserved in human cells are warranted and have implications in targeting DDK as an anti-cancer therapeutics.

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