INTERPLAY BETWEEN MCM HELICASE AND CHECKPOINT PROTEINS RESCUES REPLICATION FORK DEFECTS OF MCM10

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Mcm10 is essential in the initiation and elongation of DNA replication. It is implicated in the activation and stable assembly of various elongation factors such as the MCM2-7 helicase, Cdc45, and polymerase α primase (Polα) at the replication fork based on its physical interactions with these proteins. Second site suppressors of two temperature labile *mcm10* mutants have been identified and they have been shown to cluster in two regions of Mcm2 located at the interface of adjacent subunits of the hexameric MCM helicase. These dominant *mcm2* suppressors restore viability to the *mcm10* mutants without restoring the stability of Mcm10p, the interaction of Mcm10 with Mcm2, or the replication initiation defects of *mcm10*. Rather, they alleviate the elongation defect of *mcm10* in that they suppress the HU and MMS sensitivity and the fork pausing phenotype of *mcm10* as well as restore stability of Polα. This suppression requires the activity of genes involved in replication fork restart as well as key checkpoint regulators such as Rad53 and Mec1. Furthermore, stabilization of Polα is dependent on Mec1. These results suggest that at the restrictive temperature *mcm10* causes destabilization of the replication fork that result in degradation of Polα. This fork defect is alleviated by the altered activity of the MCM helicase as well as the coordinated action of checkpoint proteins that stabilize replication forks.

BIOGRAPHICAL SKETCH

Chanmi Lee was born on August 5th, 1979 in Seoul, Korea. When she was six years old, she followed her family to Chapel Hill, NC in the United States as her father started his ph. D. program in biochemistry. She had followed him to his laboratory and perhaps it was around this time when she began to picture herself working at such an atmosphere.

In 1998, Chanmi entered Ewha Womans University as an English literature major where she found that literature was not her calling. Two years later, while browsing through a chemistry textbook in the library, she was persuaded that chemical reactions explained the basis of life. She chose chemistry as her second major and spent the subsequent two and a half year period in frenzy to understand how movements of electrons create the properties and boundaries of everything in the world; that is, why rocks are hard, water flows, and humans exist.

In 2002, she joined the Molecular Biology and Genetics department at Cornell University to further her understanding of life. Soon she realized that cellular mechanisms are not defined by a simple set of rules, but are complex with many unknowns. Therefore, her quest to determine the basis of life will be followed for the years to come. During her time in Ithaca, Chanmi met Taeseung and they are now happily married.

This dissertation is dedicated to

my father, Nam Taek Lee,

my mother, Jeong-mi Lee,

my sister, Soe-young Lee,

my brother, Hyeongmin Lee,

and to my husband Taeseung Kang

for their love and support.

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My dad, mom, sister, and brother made it possible for me to be here. They were the ones that kept me going through difficult times by continuously encouraging me to pursue my academic interests and supporting me through their prayers and love. I thank my husband Taeseung for having made my life in Ithaca so much happier and for being with me through the good times and bad.

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CHAPTER 1

INTRODUCTION

DNA replication is the molecular basis for the preservation of life as the genome of an organism is duplicated for propagation into the next generation. Therefore, high fidelity of replication that prevents introduction of mutations is crucial. Cells maintain numerous proteins that function in supervising and repairing its genome. Coordination between the proteins within the replication machinery and proteins in DNA repair or checkpoint pathways are important in achieving this goal. In this thesis, I investigated the role of Mcm10 in maintaining replication fork stability using *Saccharomyces cerevisiae* as a model system and studied how different pathways, DNA replication, repair, and checkpoint, work together to ensure the integrity of the DNA replication fork.

Overview of Replication

In eukaryotes, initiation of replication is regulated to ensure that DNA is replicated only once per cell cycle and assembly of the proteins required for replication initiation occurs only during late M phase to G1 phase. A brief overview of the various stages of replication is shown in Figure 1.1 (Forsburg 2004). Replication occurs at specific regions of the DNA called origins. The origins are bound by the Origin Recognition Complex (ORC), composed of Orc1~6, throughout the cell cycle (Bell and Stillman 1992; Tanaka, Knapp et al. 1997). Between late M and G1 phase, Cdc6 and Cdt1 associate with the origins and subsequently facilitate the

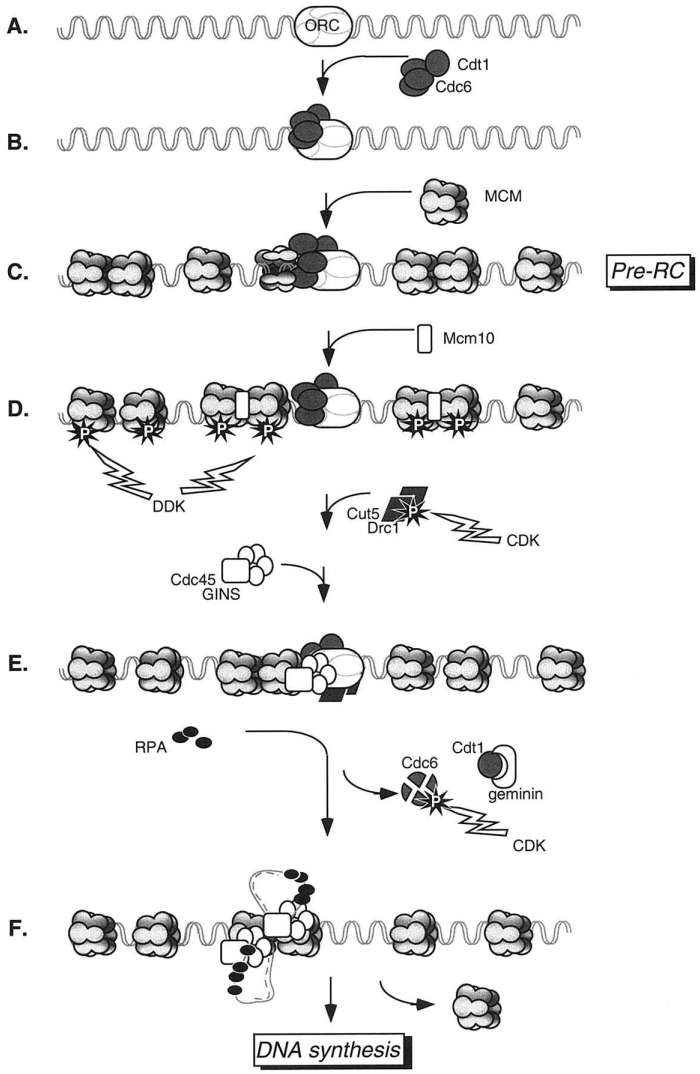


Figure 1.1 - Model of replication complex assembly during initiation and elongation (Forsburg 2004). (A) ORC binds to origins throughout the cell cycle. (B) Cdc6 and Cdc1 interact with the ORC. (C) MCM helicase is loaded at the origin to form the pre-RC. (D) Mcm10 is recruited and facilitates activation of the MCM helicase by the Cdc7/Dbf4 (DDK) complex. (E) Elongation factors, Cdc45 and GINS associate with the replication complex. (F) Replication commences with melting of the DNA.

recruitment of the Mcm2-7 helicase to form the pre-replication complex (pre-RC) (Coleman, Carpenter et al. 1996; Donovan, Harwood et al. 1997; Bell and Dutta 2002). Activation of the pre-RC to form the pre-initiation complex (pre-IC) occurs during the G1/S phase transition and requires several other proteins, which include the Cdc7p-Dbf4 kinase complex, Mcm10, Cdc45, and the GINS complex (Lei, Kawasaki et al. 1997; Zou, Mitchell et al. 1997; Zou and Stillman 1998; Forsburg 2004). Replication initiates as the complexes are released from replication origins with the synthesis of new DNA. Further assembly of replication initiation proteins at the origins are blocked until the next late M phase. Inhibition of replication complex assembly during S phase prevents re-initiation at origins. Once replication begins, the progression of the replication fork is carefully controlled to prevent unwanted fork stalling or collapse.

MCM helicase

The MCM helicase is the central helicase presumed to function in replication (Tye 1999, Labib Diffley 2001). In eukaryotes, it is composed of six different but highly conserved subunits, Mcm2, 3, 4, 5, 6, and 7, while the archaeal MCM helicase is comprised of six identical subunits. The MCM proteins are highly conserved among different species and they belong to a subgroup of the AAA ATPase family which has the characteristic ATPase motifs, Walker A and B, as well as the arginine finger motif (Hickman and Dyda 2005). All 6 subunits of the eukaryotic MCM helicase are essential as they seem to play distinct roles (Kearsey and Labib 1998; Tye 1999) and the subunits associate *in vivo* to form a hexamer that contains one of each subunit (Forsburg 2004). Interaction between the subunits have been shown by yeast two-hybrid and co-immuniprecipitation assays (Lei, Kawasaki et al. 1996; Dalton and

Hopwood 1997; Bochman and Schwacha 2007). Different subcomplexes have been purified. Biochemical assays have shown that *in vitro*, Mcm4, 6, and 7 form the core complex with 3' to 5' helicase activity (Ishimi 1997, Kaplan 2003), while Mcm2, Mcm3 and Mcm5 interact to form subcomplexes of Mcm2,4,6,7, Mcm3,4,5,6,7, and Mcm2-7 (Kimura, Ohtomo et al. 1996; Thömmes, Kubota et al. 1997). Therefore, it is suggested that Mcm2, 3, and 5 functions as a regulator of Mcm4/6/7, similar to the classic F1 ATPase model (Lee and Hurwitz 2000; Tye and Sawyer 2000; Ishimi, Komamura-Kohno et al. 2001; Schwacha and Bell 2001). However, in regards to its role in replication, it has been shown in the *Xenopus* egg system that only the complete complex of Mcm2-7 can support DNA replication (Thömmes, Kubota et al. 1997).

Though the MCM helicase is believed to be the main replicative helicase, the exact actions of its helicase activity is not yet understood. The structure of the MCM helicase shows that the proteins assemble into a ring-shaped dodecamer, a dimer of hexamers (Forsburg 2004) with a central channel wide enough for passage of either single or double stranded DNA (Adachi, Usukura et al. 1997; Fletcher, Bishop et al. 2003). Such a complex can function as classical helicases that disrupt dsDNA interaction by encircling one strand of DNA and proceeding. On the other hand, based on its similarity to the F1 ATPase (Schwacha and Bell 2001) and X-rays studies that show specific DNA exit sites located within the helicase (Fletcher, Bishop et al. 2003), it is suggested that the helicase may not move around in the cell, but stays at one place and spools the DNA through its inner tunnel by rotation (Laskey and Madine 2003).

In addition to a lack of clarity in how the helicase functions, the lack of helicase activity displayed by the hexameric complex that comprises all six Mcm2-7 subunits in *in vitro* assays had puzzled scientists for many years. The helicase activity of the full Mcm2-7 hexamer was observed only when it was a part of a large assembly

of proteins that consists of Mcm2-7, GINS, and Cdc45 in Drosophila (Moyer, Lewis et al. 2006). However, in a recent study (Bochman and Schwacha 2008), it was discovered that the Mcm2-7 hexamer alone is able to display robust helicase activity and this depended on the salt properties of the buffer. In this study, Mcm2 and 5 are proposed to be salt-sensitive "gates" that allow loading of the protein onto the DNA. The "gate" must be opened to allow loading and closed for robust helicase activity.

Mcm10

Mcm10 is an essential gene known to be involved in various aspects of DNA replication. It is an abundant protein with approximately 40,000 copies per cell (Kawasaki 2000). However, its exact function is yet to be understood because Mcm10 seems to function in various processes in the cell. Mcm10 is required in both initiation and elongation steps of DNA replication and interacts with a wide range of replication factors such as ORC, DNA polymerases ε and δ (Kawasaki, Hiraga et al. 2000), Mcm2-7 (Merchant 1997), Cdc45 (Sawyer, Cheng et al. 2004), and polymerase α (Ricke and Bielinsky 2004). Mcm10 mainly localizes in the nucleus (Merchant 1997), but its interaction with DNA appears to depend on the cell cycle because Mcm10 binds to chromatin only during S phase (Ricke 2004).

Mcm10 is recruited to the pre-RC by interaction with the MCM helicase. Mcm10 stimulates phosphorylation and activation of the Mcm2-7 subunits by the Cdc7/Dbf4 kinase (Lee, Seo et al. 2003). It is required for loading of elongation factors such as Cdc45, GINS, and Polα. Mcm10 also functions in elongation of replication as it migrates with the replication fork (Aparicio, Weinstein et al. 1997; Takayama, Kamimura et al. 2003) (Tercero, Labib et al. 2000). Its presence is required for the stable association of other elongation factors (e.g. Polα and Cdc45) to the

replication fork. Depletion of Mcm10 results in instability of Polα and loss of Cdc45 association with the replication fork (Ricke and Bielinsky 2004). Ubiquitinated forms of Mcm10 have been found and it has been shown that the di-ubiquitinated form interacts with the proliferating cell nuclear antigen (PCNA) (Das-Bradoo, Ricke et al. 2006).Therefore, Mcm10, based on its broad range of interacting partners and requirement to keep critical fork components together, is important for the overall stability of the elongation complex.

DNA polymerases

In eukaryotes, polymerase ε (Polε) and δ (Polδ) are the main replicative polymerases, which seem to have distinct roles in leading and lagging strand synthesis (Garg and Burgers 2005). DNA synthesis at the lagging strand is carried out primarily by Polδ and synthesis of the leading strand involves both Polε and Polδ (Fukui, Yamauchi et al. 2004; Kunkel and Burgers 2008). DNA polymerases cannot synthesize DNA *de novo.* Therefore primases that make RNA primers are required for initiation of DNA synthesis. Polymerase α primase is composed of both DNA polymerase (Polα) and RNA polymerase activities (primase) that allows *de novo* DNA synthesis. The primase synthesizes the initial short RNA primer, which is immediately extended by the DNA polymerase to produce the short initiator DNA (iDNA) of about 30 bases (Waga and Stillman 1998). Polα carries out this function during initiation of both leading and lagging strand and throughout elongation of the lagging strand (Hubscher, Maga et al. 2002). However, Polα lacks proofreading activity which makes it potentially mutagenic (Niimi, Limsirichaikul et al. 2004). Therefore, the iDNA only functions as an initiator oligonucleotide, which must be removed, and further elongation is carried out by Polε on the leading strand or Polδ on the lagging strand.

Both Polε and Polδ are capable of high fidelity replication.

Replication of the lagging strand consists of more steps than on the leading strand because DNA synthesis only occurs in the 5’ to 3’ direction and in the lagging strand, this direction is opposite from the direction of the fork movement. New DNA on the lagging strand is synthesized discontinuously, creating Okazaki fragments, as the parental ssDNA loops out and becomes available. Multiple enzymes cooperate to synthesize the Okazaki fragments and linking them during the process of maturation. First, the DNA primase synthesizes a short RNA primer. Then the switch between Polα and the main lagging strand polymerase Polδ occurs, leading to the extension of the primer (Diede and Gottschiling 1999, Jin 2001 Waga and Stillman 1998, Mossi 2000). Finally, polδ, Fen1, and DNA ligase function together in removing the RNA primer, filling in the gap with DNA, and ligating the nick (Maga 2001, Ayyagari 2003, Garg 2004). This process must be very efficient to ensure maturation of all Okazaki fragments as a single nick left unprocessed may lead to double strand break (DSB) (Resnick and Martin 1976).

Replication associated DNA repair

The replication fork can run into various obstacles that hinder its progress. Maintaining the stability of theDNA replication complex is critical in order to prevent disassembly of the complex and replication fork collapse. Obstacles can be environmental or endogenous DNA-damaging agents that cause lesions in the DNA such as abasic sites, bulky adducts, and DNA strand breaks. Replication fork progression can also be blocked by topological stress, aberrant DNA structures, availability of the nucleotide pool, and protein complexes that bind tightly to the DNA. Active transcription of tRNA genes or rDNA by the RNA polymerases can also cause pausing or stalling of the replication fork (Deshpande and Newlon 1996; Lee, Johnson

et al. 1999; Weitao, Budd et al. 2003). Fork stalling or arrest can lead to exposure of single-strand DNA (ssDNA) gaps and double strand DNA breaks (DSBs) that may be lethal or cause cell cycle arrest (Sogo, Lopes et al. 2002; Weitao, Budd et al. 2003).

Nucleotide misincorporation, DNA nicks and gaps, fork slippage, aberrant fork structures, and fork collapse are events associated with replication that require the action of different repair pathways. Mismatch repair (MMR) pathway acts mainly during S phase to repair errors that escape the proofreading of polymerases (Jiricny 2006). Lesions that hinder the progression of the replication fork can be dealt with by translesion synthesis (TLS) polymerases that can replicate across DNA lesions. Gaps can be filled in by a template-switching mechanism that utilizes the information from the sister duplex (Lehmann, Niimi et al. 2007). TLS and template-switch are part of the post-replication repair (PRR) pathway that allows damage tolerance during replication. The pathways can be either error-prone or error-free depending on the different ubiquitination states of PCNA at lysine-164 as monoubiquitination at this residue is associated with error-prone repair and polyubiquitination is associated with error-free (Hoege, Pfander et al. 2002; Watts 2006).

DSBs can occur when forks arrest (Michel, Ehrlich et al. 1997) or collapse (Davis and Symington 2004) and these can be repaired by either homologous recombination (HR) or nonhomologous end-joining (NHEJ). Repair of DSBs are critical to cell viability as misrepair or failure to repair these damages can result in various genetic rearrangements or chromosome loss. While both HR and NHEJ function in the cell, which pathway is more efficiently utilized depends on the cell cycle and organism (Critchlow and Jackson 1998). NHEJ pathway repairs DSBs by binding the two ends of dsDNA and joining them to each other. The pathway can be both error-prone and error-free in repair of DSBs depending on whether re-ligation of the two DNA ends occurs precisely at the break site or extensive processing of the

DNA ends occur resulting in loss of genetic material (Moore and Haber 1996). The genes required for NHEJ in budding yeast have been identified by studying mutants defective in HR. These are *YKU70*, *YKU80*, *DNL4*, *LIF1*, *SIR2*, *SIR3*, *SIR4*, *RAD50*, *MRE11*, and *XRS2*. *RAD50*, *MRE11*, and *XRS2* function both in NHEJ and HR.

During S phase, HR is the preferred mechanism because of the availability of the sister chromatid (Aylon and Kupiec 2004; Ira, Pellicioli et al. 2004). HR is also an important mechanism for replication fork repair (Cox 2001; Courcelle and Hanawalt 2003). The main players in the HR pathway are members of the Rad52 epistasis group (Rad51, Rad52, Rad54, Rad55, Rad57, and Rad59), which were discovered by their requirement for recovery of the cells from ionizing radiation (Ajimura, Leem et al. 1993; Game 2000). While Rad52 is absolutely required for all HR processes, the requirement for the other members of this group can vary depending on the context of HR, whether it is in gene conversion, synthesis-dependent strand annealing (SDSA), amplification of telomeres in telomerase-deficient strains, or break-induced replication (BIR) (Ivanov, Sugawara et al. 1996; Le, Moore et al. 1999; Symington 2002). Generally, repair of DSBs by HR is accurate and conservative. However, loss of regulation in HR can be deleterious as hyperrecombination and accumulation of aberrant recombination intermediates can be lethal (Krejci, Van Komen et al. 2003).

A general model of DSB repair by HR is as follow (Figure 1.2). The double-stranded DNA ends are processed by the Mre11-Rad50-Xrs2 (MRX) complex, which mainly functions in initiation of 5’ to 3’ single-strand resection (Paull and Gellert 2000). Various DNA helicases and nucleases such as Sgs1, Srs2, Exo1, and Dna2 subsequently act on the ends to expose long ssDNA overhangs by resection that can invade the homologous regions in the sister chromatid (Ira, Pellicioli et al. 2004; Cotta-Ramusino, Fachinetti et al. 2005). The exposed ssDNA is usually first bound by

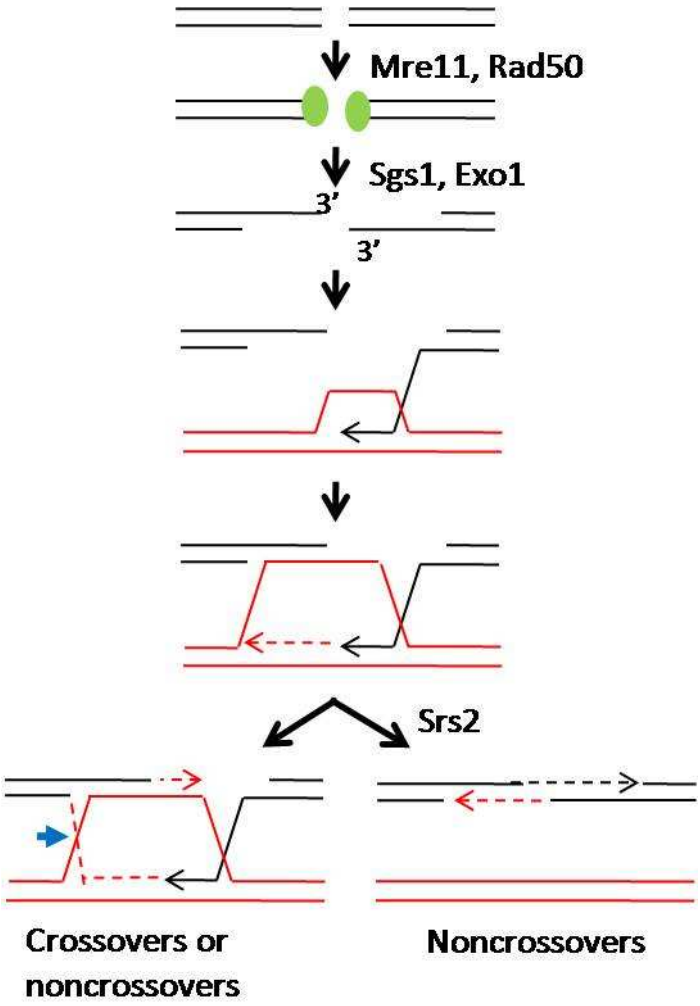


Figure 1.2 – Model of DSB repair by HR. MRX complex binds to the ends of DSBs to initiate processing and Sgs1 and Exo1 function in resection of the ends to produce 3' ssDNA. Rad51 filament facilitates strand invasion into homologous regions for DNA synthesis. A second strand invasion results in HJ formation. Resolution of HJs can produce either crossover products or noncrossover products. HJ can be disrupted by Sgs1(branch migration) or prevented by Srs2 (Rad51 removal) to produce noncrossovers as the main product.

Replication Protein-A (RP-A) that can function as a signal for checkpoint activation, but RP-A can be displaced by Rad51 through the action of the Rad52, Rad54, Rad55, and Rad57 (Sugawara, Wang et al. 2003; Sung, Krejci et al. 2003; Lisby, Barlow et al. 2004). Formation of Rad51 filament on the ssDNA facilitates strand invasion into dsDNA with sequence homology forming the D-loop (Paques and Haber 1999; Petukhova, Sung et al. 2000). In the synthesis-dependent strand annealing (SDSA) pathway where DNA synthesis from the invading 3' end occurs, the D-loop migrates without Holliday junction (HJ) formation. Alternatively, double HJs can form when both ends of the break participate, one in strand invasion and the other in second end capture (Szostak, Orr-Weaver et al. 1983). The HJs can then be resolved by two mechanisms; one is the classical method by the E. coli RuvA resolvase (reviewed in (Basto, Scaerou et al. 2004) and the other is utilizing DNA helicases of the RecQ family (Wu and Hickson 2003). The former can produce both noncrossovers and crossovers depending on which strands at the junction are cut while the latter produces noncrossovers exclusively through dissolution of HJs. Generally, noncrossovers are preferred over crossovers because crossovers can result in interchromosomal exchanges (Cromie and Leach 2000; Cheok, Bachrati et al. 2005).

DNA damage bypass and fork repair

Lesions that block the replication fork, single-strand gaps, or DSB can be repaired by exchange of genetic information between the damaged DNA and the undamaged complementary strand. HR is initiated by the damaged strand that allows repair and subsequent restarting of replication (Figure 1.3). In the events of fork collapse, replication forks can be re-established. Though the exact mechanisms of how forks are re-established are not well understood, most models depict recombination as the main method. The structure of collapsed replication forks that lead to DSBs

resemble that of broken chromosomes where only one end of the break is available to participate in strand invasion of a homologous region. As repair of broken chromosomes is carried out by break-induced replication (BIR), this may be an attractive model of how replication can restart at collapsed forks (Kraus, Leung et al. 2001). This pathway can be either Rad51-dependent or independent and requires the function of proteins in HR and DSB repair, such as Srs2, Rad52 and Mre11 (Ira and Haber 2002).

In the damage bypass mechanism, the replication fork is re-established beyond the damaged sites. In the post-replication repair (PRR) pathway, replication resumes by re-priming downstream of the lesion (Figure 1.3). This leaves a single-strand gap that can be repaired by SDSA later on (Barbour and Xiao 2003; Gangavarapu, Prakash et al. 2007). Another bypass mechanism involves template switching where the nascent strand of the damaged template is displaced to pair with the other nascent strand (Branzei and Foiani 2007). It has been proposed that hemicatenanes formed by sister chromatid junctions could mediate such pairing of nascent strands (Lopes, Cotta-Ramusino et al. 2003; Liberi, Maffioletti et al. 2005). Lastly, the fork can progress past DNA lesions by recruiting translesion polymerases to simply synthesize across the damaged site. This process can be error-free or mutagenic depending on the nature of the DNA damage and the choice of translesion polymerase used (McCulloch, Kokoska et al. 2004).

Helicases in DSB and fork repair

DNA helicases are directional enzymes that can either translocate 3’ to 5’ or 5’ to 3’ on the DNA to unwind the double strands in an ATP-dependent manner. Cells

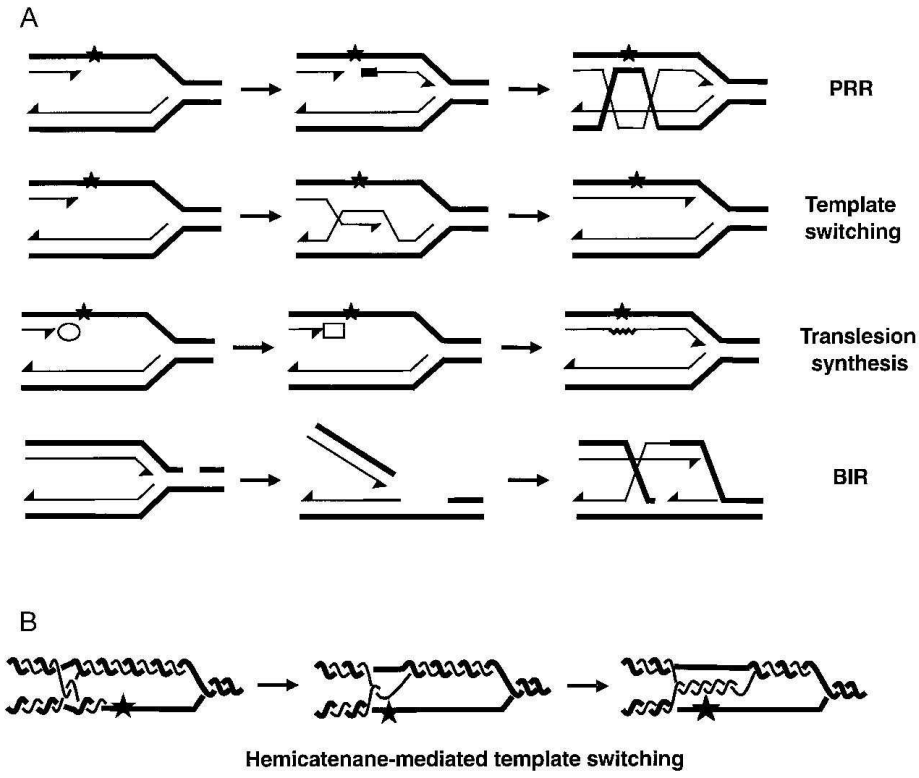


Figure 1.3 – Models of how replication forks can bypass DNA damage. (Foiani, Kumar et al. 2006) Asterisk indicates the DNA lesion. Oval indicates error-free polymerase and rectangle indicated error-prone polymerase. (A) DNA lesions can be bypassed without repair through the PRR, template switching, and translesion synthesis pathways. Collapsed replication forks resembling broken chromosomes can be bypassed by BIR as strand invasion of the intact dsDNA by the broken end sets up the replication fork. (B) Template switching can be mediated by hemicatenanes that form from sister chromatid junctions.

have many different types of helicases that function in various aspects of nucleic acid metabolism. As many as 15 in yeast and 25 in human cells have been identified (Narendra Tuteja 2004). Any process that requires separation of the two complementary DNA strands to transiently break the hydrogen bonds between the bases requires DNA helicases. During general DNA replication, the MCM helicase associates with and travels ahead of DNA polymerase to generate ssDNA that serves as templates for replication. During recombination-dependent repair of stalled replication forks, other helicases such as Dna2, Srs2, and Sgs1 appear to function.

RecQ helicases are DNA helicases that are important for genome stability. In humans, mutations in RecQ helicases have been linked to various diseases. Humans have 5 RecQ homologs and mutations in three of the the RecQ homologs BLM, WRN, and RECQL4 cause cancer predisposition syndromes (Hanada and Hickson 2007). RecQ was initially identified in *E. coli* and was implicated in degradation of the nascent lagging strand at replication block sites to facilitate RecA binding (Courcelle and Hanawalt 1999; Courcelle and Hanawalt 2003). Sgs1 is the budding yeast RecQ helicase. Deletion of Sgs1 is synthetically lethal with another DNA helicase Srs2 (Gangloff, Soustelle et al. 2000) and either of the Mms4-Mus81 endonuclease (Kaliraman, Mullen et al. 2001). Synthetic lethality with Srs2 or Mms4-Mus81 is suppressed by deletions of genes in the HR pathway (Gangloff, Soustelle et al. 2000), which had suggested that the lethality is due to hyperrecombination. However, Sgs1's role in recombination is complex as it also functions in promoting recombination during repair of DSBs. Sgs1 is involved in the synthesis-dependent strand annealing (SDSA) pathway that utilizes HR to repair DSBs. This pathway can lead to both crossover and noncrossover products and Sgs1 function in suppressing crossover products (Ira, Malkova et al. 2003; Mimitou and Symington 2008). Therefore, Sgs1 seems to regulate HR by suppressing aberrant hyper-recombination and promoting

required recombination activities. Other important functions of Sgs1 are at the replication fork. Loss of Sgs1 leads to instability of arrested forks as replication factors fail to remain associated with the fork (Cobb, Bjergbaek et al. 2003; Bjergbaek, Cobb et al. 2005). It is also implicated in processing of Holliday junctions (Ira, Malkova et al. 2003) and activation of checkpoint pathway by direct interaction with Rad53 (Bjergbaek, Cobb et al. 2005).

The Srs2 helicase was identified as a hyper-recombinant mutant (Rong, Palladino et al. 1991) that functions in the Rad6-dependent DNA damage tolerance pathway (Barbour and Xiao 2003). The involvement of Srs2 in this pathway is based on the observation that *srs2* suppresses *rad6* mutants (Lawrence and Christensen 1979). Like Sgs1, Srs2 also functions in regulating HR to prevent potentially deleterious recombination products. Srs2 is suggested to prevent recombination intermediates by disrupting Rad51 filaments (Krejci, Van Komen et al. 2003). One of the mechanism by which its anti-recombinase action is utilized is in channeling repair of lesions away from HR and into the post-replication repair (PRR) pathway. However, as in the case of Sgs1, Srs2 also function in DSB repair by HR, especially to promote noncrossover products by way of SDSA (Ira, Malkova et al. 2003). The anti-recombinase activity of Srs2 may function in inhibiting invasion by the second end of the break that will lead to crossover products. It also functions in the checkpoint response as it is phosphorylated during the S-phase checkpoint response and is required for full activation of Rad53 (Liberi, Chiolo et al. 2000).

Checkpoint pathway

Checkpoint proteins function in monitoring the cell cycle to ensure important events such as replication and chromosome segregation are completed correctly

(Hartwell and Weinert 1989; Nyberg, Michelson et al. 2002). It is a biochemical mechanism that prevents cell cycle transition until each stage is fully finished. In the occurrence of DNA damage, activation of the checkpoint proteins delays the cell cycle to ensure repair and recovery (Sandell and Zakian 1993). First, onset of mitosis is delayed to allow the cells sufficient time for completion of replication and/or repair. Second, firing of late replication origins is inhibited to prevent new replication fork from forming. Thirdly, existing replication forks are stabilized from collapsing and stalled forks are repaired so that replication can resume. It is suggested that the cell cycle restarts once the damage is removed, the checkpoint proteins are inactivated (Pellicioli, Lucca et al. 1999). However, in the events of irreparable damage, the cells do not arrest indefinitely, but can resume progression through the cell cycle, a process known as adaptation (Sandell and Zakian 1993; Toczyski, Galgoczy et al. 1997).

In *S. cerevisiae*, the S-phase checkpoint pathway is activated by a series of phosphorylation cascade that is mediated mainly by Mec1, Rad53, and Dun1 proteins (Foiani, Pellicioli et al. 2000). A summary of the proteins involved in the S-phase checkpoint pathway is shown in Figure 1.4. The nature of the signal that activates the checkpoint pathway is still unclear, however, the central player and the one most widely studied is RP-A, which binds to ssDNA (Sogo, Lopes et al. 2002; Zou and Elledge 2003; Byun, Pacek et al. 2005). While RP-A coated ssDNA is present at normal replication forks, the extent of ssDNA increases when forks stall (Sogo et al 2005) and during DNA repair. Multiple lines of evidence support this. It has been shown that certain RP-A mutants have checkpoint defects and exhibit faster adaptation to DNA damage (Longhese, Neecke et al. 1996; Pellicioli, Lucca et al. 1999). Also, in Xenopus egg extracts, RP-A is required for recruitment of ATR (Mec1) to ssDNA (You, Kong et al. 2002; Lee, Kumagai et al. 2003). Lastly, RP-A is sufficient for binding of ATR to ssDNA in vitro (Zou and Elledge 2003). Therefore, exposure of

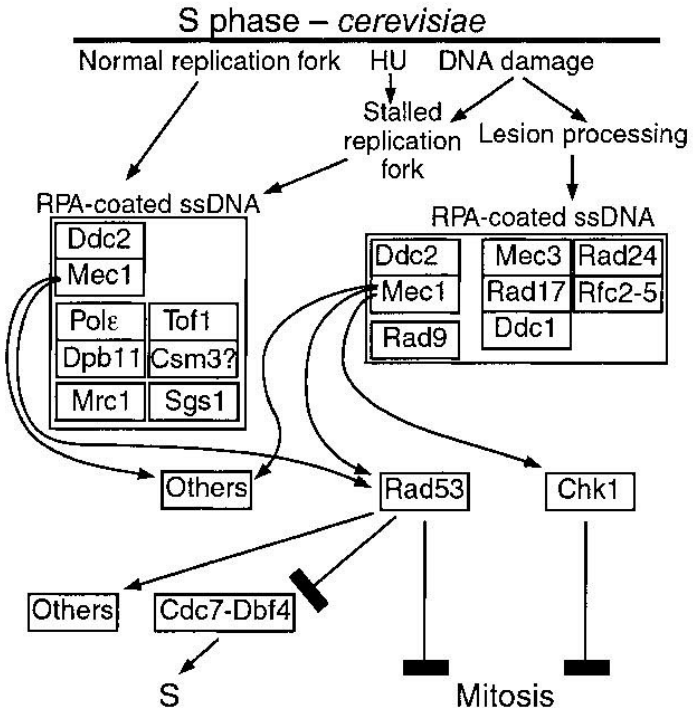


Figure 1.4 - Overview of S phase checkpoint pathways in budding yeast (Foiani, Kumar et al. 2006). Two main types of replication stress that the checkpoint pathways respond to are replication fork stalling and DNA lesions. Mec1 and Rad53 are the sensor and effector kinases, respectively, that function in response to the signals for checkpoint activation, which is RP-A-coated ssDNA.

ssDNA as the signal for checkpoint activation suggests that the checkpoint is activated not by the DNA lesion itself, but rather by its effect on hindering replication fork progression. That is, uncoupling of the MCM helicase from the polymerase activity by DNA lesions or depletion of the nucleotide pool that only inhibit polymerase progression can generate long regions of ssDNA (Byun, Pacek et al. 2005).

In budding yeast, Mec1, an ortholog of the human ATR, and Ddc2 (ATR-interacting partner: ATRIP) are recruited to sites of DNA damage by interaction with RP-A coated ssDNA (Rouse and Jackson 2002). This interaction activates the kinase activities of Mec1 and Ddc2. Mec1 has multiple phosphorylation targets, but an important target is Rad53 (Foiani, Pellicioli et al. 2000). Activation of Rad53 slows down S phase and prevents firing of the late origins (Paulovich and Hartwell 1995; Santocanale and Diffley 1998). Another consequence of Rad53 activation, which is considered to be critical for the S-phase checkpoint response, is stabilization of the replication fork. When wild-type cells are treated with hydroxyurea which depletes the deoxynucleotide triphosphate (dNTP) pool and causes replication forks to stall, forks resume elongation once HU is removed. However, in *rad53* mutants, the forks are unable to restart elongation (Desany, Alcasabas et al. 1998). Electron microscopy of *rad53* mutants revealed that stalled forks are rapidly broken down and the cells accumulate long regions of ssDNA (Sogo, Lopes et al. 2002). This suggests that Rad53 functions in stabilizing the replication fork. Phosphorylation of other proteins in DNA replication and repair by Rad53 (Brush, Morrow et al. 1996; Pellicioli, Lucca et al. 1999) may contribute to fork stabilization. Though Mec1 and Rad53 are necessary for a broad range of activities, their essential function is to phosphorylate and down regulate Sml1 which is an inhibitor of the ribonucleotide reductase (RNR). The lethality of *MEC1* and *RAD53* deletion in yeast comes from unrestrained Sml1 function that inhibits RNR and lowers the pool of deoxyribonucleotides (dNTP)

required for DNA replication (Zhao, Chabes et al. 2001).

Mrc1 was identified as a mediator of the checkpoint response due to replication fork defects (Alcasabas, Osborn et al. 2001). Mec1 is the upstream kinase that hyperphosphorylates Mrc1 and Rad53 is the downstream kinase. Apart from its function in mediating checkpoint activation, Mrc1, in a complex with Tof1, plays an important role in directly stabilizing replication forks as it interacts with and travels with the fork. Loss of Mrc1 leads to slow S phase even in normal conditions (Szyjka, Viggiani et al. 2005; Tourrière, Versini et al. 2005; Hodgson, Calzada et al. 2007) and in *mrc1* or *tof1* strains, when the cells are exposed to HU, DNA synthesis uncouples from the movement of the fork complex (Katou, Kanoh et al. 2003). Recovery of fork progression after HU removal, when nucleotide production is resumed, also requires both proteins (Tourrière, Versini et al. 2005). Tof1 has additional roles in stabilizing paused complexes. It is required for programmed pausing of replication forks at rDNA, which is a mechanism that ensures that DNA replication does not collide with the active transcription of the rDNA region (Calzada, Hodgson et al. 2005; Tourrière, Versini et al. 2005; Mohanty, Bairwa et al. 2006).

The accumulation of aberrant DNA structures in checkpoint defective cells underscores the importance of checkpoint proteins in stabilizing replication forks. In the absence of checkpoint proteins, replisome components dissociate from stalled replication forks, leading to unusual fork structures or fork collapse. These aberrant DNA structures that result from checkpoint defects have been visualized by 2D gels (Lopes, Cotta-Ramusino et al. 2001) and electron microscopy (Sogo, Lopes et al. 2002). 2D gel analysis has shown that wild-type cells are able to maintain replication intermediates such as bubble structures that rise from initiation at origins and Y structures that are progressing replication forks for several hours of HU treatment. These replication forks are stable as they resume activity once HU is removed.

However, in *rad53*, the bubble structures are unstable as their intensity decreases and a novel "cone shaped" signal appears. The cone signal is suggested to be a combination of aberrantly processed replication forks such as regressed forks, a marked feature of fork instability. These persisted even after HU removal, indicating that the forks do not resume progression properly (Lopes, Cotta-Ramusino et al. 2001). Electron microscopy has been used to visualize the X shaped molecules where the nascent DNA strand pair due to replication fork regression (Sogo, Lopes et al. 2002).

Mcm helicase and Polα **as targets of checkpoint**

Replication proteins can be direct targets of the DNA damage checkpoint pathway. As a crucial member of the replication complex, the regulation of the MCM helicase by the DNA damage checkpoint can directly impact the replication complex. Preventing MCM helicase disassembly from stalled replication forks is important because replication licensing does not allow MCM helicases to reassemble once replication initiates. Uncontrolled helicase activity can also be deleterious because this will produce extensive ssDNA accumulation. On the other hand short stretches of ssDNA due to the helicase activity can be utilized to activate the checkpoint (Byun, Pacek et al. 2005). A study by Cortez and colleagues provides evidences that the MCM helicase is a direct target of checkpoints in mammalian cells (Cortez, Glick et al. 2004). They show that subunits of the MCM helicase directly interact with the checkpoint proteins, Mcm3 being phosphorylated by ATM and Mcm2 being phosphorylated by ATR. Also, Mcm7 interacts directly with the ATR-interacting partner (ATRIP) and decrease in Mcm7 level leads to intra S-phase checkpoint defect.

Polα is another central replication protein that may be a direct checkpoint target. The polymerase switching between Polα to Polδ is unique to eukaryotic replication because in bacteria, there is no switching between different DNA

polymerases and the main DNA polymerase directly utilizes the RNA primer to synthesize long DNA strands (Johnson and O'Donnell 2005). It is suggested that the reason why Polδ does not directly synthesize from RNA primers is because Polα may be required for monitoring simultaneous replication initiation from multiple origins in eukaryotes. Polα may also be targets of S phase stress response that arrest DNA replication as production of RNA-DNA primers is suggested to be required to arrest replication until DNA damage is repaired (Pellicioli, Lucca et al. 1999; Michael, Ott et al. 2000). In Xenopus egg extracts, production of primers by Polα contributes to checkpoint activation (Byun, Pacek et al. 2005).

Mcm10 is known to interact with both the MCM helicase and Polα (Merchant, Kawasaki et al. 1997; Ricke and Bielinsky 2004). It is likely to be an important component of the replication fork as one of the phenotypes of *mcm10* mutants is the pausing of replication forks. My goal is to understand the essential function of *MCM10.* What causes the lethality of the *mcm10* temperature sensitive mutant and how do mutations in *MCM2* suppress *mcm10* temperature sensitivity? In chapter 3, I will show the *mcm10* phenotypes that are commonly suppressed by *mcm2* and the factors that display synthetic effects with *mcm10.* In chapter 4, I will describe the factors required for efficient suppression of *mcm10* ts by *mcm2*, determined by genetic analysis. Because Mcm10 appears to play an important role in replication fork stability, understanding how a mutation in the MCM helicase can suppress the defects of Mcm10 will provide further insights into how the fork components interact together to maintain stability of the replication fork.

CHAPTER 2

MATERIALS AND METHODS

Strains and plasmids.

Strains used in this study are listed in Table 1. All strains were isogenic derivatives of W303-1A, unless otherwise indicated. Strains carrying various deletions were made by crossing strain *mcm10-1 mcm2* to the appropriate deletion strain from this lab and selecting desired segregants by their conditional phenotypes and/or auxotrophy and by sequencing. Genotypes were confirmed by PCR, sequencing, or by plasmid complementation where applicable. Plasmids used in this study are listed in Table 2. Plasmids used for yeast two-hybrid analysis were constructed by the Gateway system (Invitrogen, San Diego).

Suppressor screen.

Suppressor screen for random suppressor mutations of *mcm10-1* were carried out as described (Liachko and Tye 2005). Plasmid-based mutagenesis of *MCM2* was subsequently carried out to screen for non-cold-sensitive suppressor mutations. *MCM2* was cloned into a plasmid and mutagenized in *E. coli* using xl1-red competent cells. Mutagenized plasmids were obtained from *E. coli*, transformed into *mcm10-1* and plated at 37°C to select for suppressors.

Protein-protein interactions.

Wild-type W303 strain with the pSH18-34 reporter plasmid was transformed with pGAD2F and pBTM116 constructs for two-hybid assay (Fields and Song 1989).

Table 1 – Strains Used in this Study

Strains		**Source**
Isogenic to W303		
W303-1A	*MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1*	R. Rothstein
W303-1B	*MATα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1*	R. Rothstein
BTY100	*W303 MATa mcm10-1*	This Lab
BTY101	*W303 MATα mcm10-1*	This Lab
BTY103	*W303 MATa mcm10-43*	This Lab
BTY102	*W303 MATα mcm10-43*	This Lab
ILY230	*MATa 13myc-MCM10 TRP1*	This Lab
ILY232	*MATa 13myc-mcm10-43 TRP1*	This Lab
SSY84	*MATa 13myc-mcm10-1 HIS3MX*	This Lab
CLY88	*MATa 13myc-mcm10-43 TRP1 mcm2-G400D*	This Study
CLY90	*MATa 13myc-mcm10-1 HIS3MX mcm2-G400D*	This Study
CLY91	*W303 MATa mcm2-P399L*	This Lab
CLY92	*W303 MATa mcm2-G400D*	This Lab
CLY93	*W303 MATa mcm2-D472G*	This Lab
CLY94	*W303 MATa mcm2-R617H*	This Lab
ILY215	*W303 MATa mcm2-S619F*	This Lab
CLY95	*W303 MATa mcm2-P399L mcm10-1*	This Study
CLY96	*W303 MATa mcm2-G400D mcm10-1*	This Study
CLY97	*W303 MATa mcm2-D472G mcm10-1*	This Study
CLY98	*W303 MATa mcm2-R617H mcm10-1*	This Study
ILY245	*W303 MATa mcm2-S619F mcm10-1*	This Study
XL10	*W303 MATa mrc1::HIS3 sml1::URA3*	This Lab
XL336	*W303 MATa tof1::URA3*	This Lab
XL16	*W303 MATa rad53::URA3 sml11::HIS3*	This Lab
XL18	*W303 MATa mec1::LEU2 sml1::URA3*	This Lab
XL161	*W303 MATα rad9::URA3*	This Lab
XL232	*W303 MATa sgs1::URA3*	This Lab
XL299	*W303 MATα dnl4::URA3*	This Lab
XL49	*W303 MATα rad51::HIS3*	This Lab
XL158	*W303 MATα srs2::HIS3*	This Lab
XL324	*W303 MATa mrc1AQ::HIS3*	This Lab
CLY89	*W303 MATα ddc1::KanMX*	This Study
CLY99	*W303 MATa exo1::URA3*	This Study
CLY84	*W303 MATα mre11::LEU2*	This Lab
CLY144	*W303 MATa rad52::TRP1*	This Lab
CLY100	*W303 MATa mcm10-1 mrc1::HIS3 sml1::URA3*	This Study
CLY101	*W303 MATa mcm10-1 tof1::URA3*	This Study
CLY102	*W303 MATa mcm10-1 rad53::URA3 sml11::HIS3*	This Study
CLY103	*W303 MATa mcm10-1 mec1::LEU2 sml1::URA3*	This Study
CLY104	*W303 MATa mcm10-1 rad9::URA3*	This Study
CLY105	*W303 MATa mcm10-1 sgs1::URA3*	This Study
CLY106	*W303 MATa mcm10-1 dnl4::URA3*	This Study

Table 1 – (Continued)

CLY107	*W303 MATa mcm10-1 rad51::HIS3*	This Study
CLY108	*W303 MATa mcm10-1 exo1::URA3*	This Study
CLY109	*W303 MATa mcm10-1 mrc1AQ::HIS3*	This Study
CLY110	*W303 MATa mcm10-1 ddc1::KanMX*	This Study
CLY85	*W303 MATα mcm10-1 mre11::LEU2*	This Study
CLY145	*W303 MATa mcm10-1 rad52::TRP1*	
CLY111	*W303 MATa mcm2-G400D mrc1::HIS3 sml1::URA3*	This Study
CLY112	*W303 MATa mcm2-G400D tof1::URA3*	This Study
CLY113	*W303 MATa mcm2-G400D rad53::URA3 sml11::HIS3*	This Study
CLY114	*W303 MATa mcm2-G400D mec1::LEU2 sml1::URA3*	This Study
CLY115	*W303 MATa mcm2-G400D rad9::URA3*	This Study
CLY116	*W303 MATa mcm2-G400D sgs1::URA3*	This Study
CLY117	*W303 MATa mcm2-G400D dnl4::URA3*	This Study
CLY118	*W303 MATa mcm2-G400D rad51::HIS3*	This Study
CLY119	*W303 MATa mcm2-G400D exo1::URA3*	This Study
CLY120	*W303 MATa mcm2-G400D srs2::HIS3*	This Study
CLY121	*W303 MATa mcm2-G400D mrc1AQ::HIS3*	This Study
CLY122	*W303 MATa mcm2-G400D ddc1::KanMX*	This Study
CLY86	*W303 MATα mcm2-G400D mre11::LEU2*	This Study
CLY146	*W303 MATa mcm2-G400D rad52::TRP1*	
CLY123	*W303 MATa mcm2-G400D mcm10-1 mrc1::HIS3 sml1::URA3*	This Study
CLY124	*W303 MATa mcm2-G400D mcm10-1 tof1::URA3*	This Study
CLY125	*W303 MATa mcm2-G400D mcm10-1 rad53::URA3 sml11::HIS3*	This Study
CLY126	*W303 MATa mcm2-G400D mcm10-1 mec1::LEU2 sml1::URA3*	This Study
CLY127	*W303 MATa mcm2-G400D mcm10-1 rad9::URA3*	This Study
CLY128	*W303 MATa mcm2-G400D mcm10-1 sgs1::URA3*	This Study
CLY129	*W303 MATa mcm2-G400D mcm10-1 dnl4::URA3*	This Study
CLY130	*W303 MATa mcm2-G400D mcm10-1 rad51::HIS3*	This Study
CLY131	*W303 MATa mcm2-G400D mcm10-1 exo1::URA3*	This Study
CLY132	*W303 MATa mcm2-G400D mcm10-1 srs2::HIS3*	This Study
CLY133	*W303 MATa mcm2-G400D mcm10-1 mrc1AQ::HIS3*	This Study
CLY134	*W303 MATa mcm2-G400D mcm10-1 ddc1::KanMX*	This Study
CLY87	*W303 MATa mcm2-G400D mcm10-1 mre11::LEU2*	This Study
CLY147	*W303 MATa mcm2-G400D mcm10-1 rad52::TRP1*	
CLY135	*W303 MATα 3xHA-Cdc17 HIS3 13myc-MCM10 TRP1*	This Study
CLY144	*W303 MATα 3xHA-Cdc17 HIS3 mcm10-1*	This Study
CLY145	*W303 MATα 3xHA-Cdc17 HIS3 mcm10-1 mcm2-G400D*	This Study
CLY136	*W303 MATa 3xHA-Cdc17 HIS3 13myc-mcm10-43 TRP1*	This Study
CLY137	*W303 MATα 3xHA-Cdc17 HIS3 13myc-mcm10-43 TRP1 mcm2-G400D*	This Study

Table 1 – (Continued)

CLY138	*W303 MATa 3xHA-Cdc17 HIS3 mec1 13myc-MCM10*	This Study
CLY139	*W303 MATa 3xHA-Cdc17 HIS3 mec1 13myc-mcm10-43 TRP1 mcm2-G400D*	This Study
CLY140	*W303 MATa 3xHA-Rad53 KanMX*	This Study
CLY141	*W303 MATa 3xHA-Rad53 KanMX mcm10-1*	This Study
CLY142	*W303 MATa 3xHA-Rad53 KanMX mcm2-G400D*	This Study
CLY143	*W303 MATa 3xHA-Rad53 KanMX mcm10-1 mcm2-G400D*	This Study

Table 2 – Plasmids Used in this Study

Plasmid Name	**Description**	**Source**
pRS315	YCP *LEU2*	New England Biolabs
pRS315MCM10	YCP *LEU2 MCM10*	This Lab
pRS315mcm2-G400D	YCP *LEU2 mcm2-G400D*	This Lab
pRS316MCM10	YCP *URA3 MCM10*	This Lab
pGAD2F	2μ *LEU2 GAD4-AD*	S. Fields
pBTM116	2μ *TRP1 LEXA-DBD*	S. Fields
pSH18-34	*URA3* LacZ with LEXA binding sites	S. Fields
pGADgw	pGAD2F with Gateway Cassette	This Lab
pBTMgw	pBTM116 with Gateway Cassette	This Lab
pGBKgw	pGBKT7 with Gateway Cassette amp^r	This Lab
pBTMMCM10	pBTMgw *MCM10*	This Lab
pBTMmcm10-1	pBTMgw *mcm10-1*	This Lab
pBTMmcm10-43	pBTMgw *mcm10-43*	This Lab
pBTMMCM2	pBTMgw *MCM2*	This Lab
pBTMmcm2-G400D	pBTMgw *mcm2-G400D*	This Lab
pBTMmcm2-S619F	pBTMgw *mcm2-S619F*	This Lab
pGADMCM10	pGADgw *MCM10*	This Lab
pGADmcm10-1	pGADgw *mcm10-1*	This Lab
pGADMCM2	pGADgw *MCM2*	This Lab
pGADmcm2-G400D	pGADgw *mcm2-G400D*	This Lab
pGADmcm2-S619F	pGADgw *mcm2-S619F*	This Lab
YCp1	*LEU2 CENV ARS1*	This Lab
YCp121	*LEU2 CENV ARS121*	This Lab

Transformants were selected on appropriate dropout plates. Interactions were assessed by the appearance of blue colonies on plates containing X-gal (Sigma). Relevant strains were inoculated for saturated cultures and spotted onto X-gal plates and photographed after 2–4 days of growth at 30°C.

Western blotting analysis.

Mcm10 in wild-type, *mcm10-1*, or *mcm10-43* strain were tagged with 13xMyc and introduced into *mcm2-G400D* or *mcm2-S619F*. Cdc17 was tagged with 3xHA at the C-terminus. The strains were grown to log phase at 30°C and subsequently shifted to 37°C. Samples were collected at various time points for western blot analysis. Proteins were extracted either by treating the cells briefly with mild alkali and then boiling in SDS-PAGE sample buffer as described in (Kushnirov 2000) or by glass bead lysis in the presence of protease inhibitors. Extraction of phosphorylated Rad53 also contained phosphatase inihibitors. The mild alkali treatment (0.2M NaOH) method produced protein extraction yield similar to that of glass bead lysis. Mouse anti-Myc (Santa Cruz) and mouse anti-HA (Roche) antibodies were used to probe for the appropriate Myc-tagged and HA-tagged proteins. Goat anti-mouse horse-radish peroxidase-conjugated secondary antibodies were obtained from Bio-Rad. Blots were visualized by chemiluminescence reagents (Promega).

Plasmid stability assays.

MCM assays were carried out as described in (Donato, Chung et al. 2006). Wild-type and mutant strains were transformed with a plasmid that contains an origin of replication, a centromere, and the *LEU2* selectable marker. Assessment of plasmid loss rate in the mutants was done as described.

Two-dimensional DNA gel electrophoresis.

Two-dimensional DNA gel electophoresis was performed according to the neutral–neutral method (Brewer and Fangman 1987). Cells were broken by vortexing with glass beads in lysis buffer (17% glycerol, 50 mM MOPS, 150 mM potassium acetate, 2 mM magnesium chloride, 500 µM spermidine, and 150 µM spermine; pH7.2). Spheroplasts were collected by centrifugation for 10 minutes at 8000 rpm (4°C) and resuspended in G2 buffer (Qiagen). RNase A (Qiagen) and proteinase K (Invitrogen) were added to the buffer to 200 µg/ml and 400 µg/ml final concentration respectively and the solution was incubated at 37°C for 4 hours with gentle shaking every 30 minutes. The solution was centrifuged for 5 minutes at 5000 rpm (4°C) and the supernatant was passed through Genomic-Tip 100/G column (Qiagen) to purify DNA. Wash and elution was performed according to Qiagen Genomic-Tip 100/G manual. DNA was precipitated from the eluate with isopropanol and resuspended in 500µl distilled water.

Different restriction enzymes were used to visualize replication intermediates at different DNA loci. At the *ARS1* region, purified DNA was digested to completion with *Nco*I to produce a 5kb fragment. To detect fork pausing at the Sup53 tRNA gene, the DNA was digested with BamHI and EcoRI that produced a 3.5kb DNA fragment. The region encompassing the rDNA locus was digested with BglII to produce a 4.6kb fragment. To enrich the sample for replicating DNA, digested DNA was passed through BND cellulose (Sigma-Aldrich) columns as described in (Dijkwel, Vaughn et al. 1991).

Probes were made as followed. *ARS1* probes were made by amplifying a 1.5 kb region centered at *ARS1* by PCR. *SUP53* probes were made by amplifying a 1 kb region centered at *SUP53*. rDNA probes were made by amplifying a 1.1 kb region from the pNOY102 plasmid obtained from Dr. Nomura's lab that carries the rDNA

locus. The sequence of the forward primer used to amplify the rDNA region is 5'-ACA GAT GTG CCG CCC CAG CCA AAC TCC-3' and the sequence of the reverse primer is 5'-CCT GGA TAT GGA TTC TTC ACG GTA ACG-3' (Weitao, Budd et al. 2003). The probes were radiolabeled with [α-^{32}P] dATP using the Prime-It II Random primer labeling kit from Stratagene. 1D and 2D gel electrophoresis of the prepared DNA was carried out and replication intermediates were visualized by southern blot.

Florescence-Activated Cell Sorting (FACS)

FACS analysis was carried out to visualize cell cycle progression. 1ml of log phase cells were spun down and fixed overnight by resuspending the cell pellets in cold 70% EtOH. After fixation, the cells were dried and resuspended in 1ml 50 mM Sodium Citrate. The samples were sonicated briefly (3 times for 3 seconds) at setting 4 on the VirSonic Ultrasonic Cell Disrupter 100 (SP Industries). 2 µl of 100 mg/ml RNaseA (Qiagen) was added and the samples were incubated at 37°C for 1 hour. Next, 25 µl of 20 mg/ml Proteinase K (Invitrogen) was added and the samples were incubated at 42°C for 1 hour. 1µl of 1 mM SYTOX Green (Invitrogen Molecular Probes) was added to each sample before analysis at the Flow Cytometry Core Laboratory at Cornell University.

CHAPTER 3

REPLICATION FORK DEFECTS OF MCM10 ARE RESCUED BY MUTATIONS IN THE MCM HELICASE

Introduction

In eukaryotes, DNA replication is highly regulated to ensure the integrity of the genome. Initiation of replication is regulated to ensure that the genome is replicated exactly once per cell cycle. Once replication begins, progression of the replication fork is carefully controlled to prevent unwanted fork stalling or collapse. Mcm10 is a protein that functions in both aspects of replication, being required for initiation of replication at origins and stability of the elongation fork.

Pre-replication complexes (pre-RCs) composed of ORC, Cdc6, Cdt1, and the MCM helicase are assembled at replication origins during late mitosis and early G1 phase (Bell and Dutta 2002). Mcm10 is an essential protein that is recruited to the pre-RCs by interaction with the MCM helicase and is known to be involved in various aspects of the replication process. It is required at the onset of S-phase for activation of the helicase by the Cdc7p-Dbf4p kinase complex and successful transition of the initiation complex to the elongation complex (Lei, Kawasaki et al. 1997; Zou, Mitchell et al. 1997; Zou and Stillman 1998). Mcm10 also interacts with a wide range of replication factors such as ORC, DNA polymerases epsilon and delta (Kawasaki, Hiraga et al. 2000), Cdc45 (Sawyer, Cheng et al. 2004), and Polα (Ricke and Bielinsky 2004) and is required for loading of the elongation factors Cdc45, GINS, and Polα.

After initiation, Mcm10 migrates with the replication fork (Aparicio,

Weinstein et al. 1997; Tercero, Labib et al. 2000; Takayama, Kamimura et al. 2003) and is required for the stable association of Polα and Cdc45 with the replication fork. Depletion of Mcm10 at the restrictive temperature results in the instability of Polα, loss of Cdc45 association with the replication fork (Ricke and Bielinsky 2004; Sawyer, Cheng et al. 2004), and incomplete DNA replication. Even at the permissive temperature, *mcm10* cells display a prolonged S phase and replication forks that pause at unfired origins (Merchant, Kawasaki et al. 1997; Araki, Kawasaki et al. 2003). Therefore, Mcm10 is important for the overall stability of the elongation complex, but its essential function remains unknown.

Mutations in *MCM2*, a subunit of the MCM helicase, that suppress the temperature sensitivity of *mcm10* mutants were identified (Liachko and Tye 2005). In this study, I investigated the mechanism by which suppression occurs. The *mcm10-1* and *mcm10-43* mutants both produce unstable forms of the Mcm10 protein that is degraded at the restrictive temperature. The *mcm2* suppressors are allele non-specific suppressors in that they suppress the temperature sensitivity of both *mcm10-1* and *mcm10-43*. In order to determine the mechanism by which *mcm2* mutants suppress *mcm10* temperature sensitivity, I analyzed which of the *mcm10* mutant phenotypes are suppressed by the *mcm2* suppressors. I mainly focused on the mechanism by which *mcm2-G400D*, and in some cases *mcm2-S619F*, suppress *mcm10*.

I found that the *mcm10* phenotypes suppressed by *mcm2* are related to the replication fork defects of *mcm10*. Therefore, the cause of lethality at the restrictive temperature seems to be during replication elongation as the integrity of the fork becomes compromised. Such an event will lead to DNA damages such as gaps and breaks that must be repaired. Persistent occurrence of these damages due to the intrinsic instability of the replication fork can be overwhelming and cause cell death. The *mcm10* mutant displayed synthetic growth defects or lethality with DNA repair

genes involved in DSB and replication fork repair, suggesting DSB and aberrant replication fork structures occur frequently in *mcm10*. Mutations in *MCM2* suppress most of these phenotypes suggesting that suppression of *mcm10* by *mcm2* occurs by bypassing the function of Mcm10 at the replication fork

Results

Mutations in MCM2 suppress the temperature sensitivity of *mcm10-1* and *mcm10-43*

Two temperature sensitive (ts) mutants of *MCM10*, *mcm10-1* (P269L) and *mcm10-43* (C320Y), share many of the same phenotypes (Homesley, Lei et al. 2000). Both mutants show reduced replication initiation activity and fork pausing at unfired replication origins at the permissive temperature, but arrest in S phase at the restrictive temperature. Both protein products are heat labile (Ricke and Bielinsky 2004; Sawyer, Cheng et al. 2004) suggesting that lability may be the cause of these phenotypes. To determine the essential role of Mcm10 that was compromised at the restrictive temperature, several suppressor screens for *mcm10* temperature sensitivity were carried out (Liachko and Tye 2005). In the first screen, spontaneous ts suppressors that simultaneously acquired cold sensitivity (cs) were isolated. Six cs suppressors were cloned and sequenced. They were all missense mutations in conserved regions of the *MCM2* gene. They all lie in *MCM2* at two positions, R617 or S619 , and they were all dominant suppressors (Liachko and Tye 2005). To identify other mutations in *MCM2* that suppress the ts phenotype of *mcm10*, a plasmid carrying *MCM2* was randomly mutagenized. The mutagenized plasmid was transformed into *mcm10* cells and the transformation reaction was plated at 37°C for identification of dominant suppressors.

The resultant suppressor alleles were sequenced and integrated into the genome of both wild-type and *mcm10* cells. In all, 10 dominant suppressors were analyzed. All converged in three positions of *MCM2* at P399, G400 and R401 with one exception that lies at D472 and the rest at R617 and S619 (Fig. 3.2A). *mcm2-G400D, S619F, and S619Y* were isolated more than once independently. The *mcm2* mutations are all allele-nonspecific suppressors as they suppress both *mcm10-1* and *mcm10-43* (Fig. 3.1). As both *mcm10-1* and *mcm10-43* express unstable form of the protein that degrades at the restrictive temperature, suppression by the *mcm2* mutants is likely to involve either restoration of Mcm10 stability or compensatory changes such as increased affinity between the proteins or bypass of function.

All of the *mcm2* suppressor mutations except one are located in two small regions of Mcm2 that are conserved throughout archaeal and eukaryotic MCM helicase (Fig. 3.2A). In particular, the residues G400 and R617 in eukaryotic *MCM2* are highly conserved in all eukaryotic MCM2-7 subunits and the archaeal MCM protein (Figure 3.3). Based on a recent study of the archaeal MCM helicase crystal structure from *Sulfolobus Sulfataricus* (Brewster, Wang et al. 2008), these two regions are at the interface of adjacent subunits of the MCM helicase with the residue corresponding to ScG400 of one subunit juxtaposed to the residue corresponding to ScR617 of the neighboring subunit (Fig. 3.2B). The positions of the mutated residues suggest that suppression of *mcm10* temperature sensitivity by the different *mcm2* mutations may occur through a common mechanism and may involve altered interaction between the subunits at that particular interface.

mcm2 suppressors do not restore Mcm10-1 protein-protein interactions or stability

Interaction of Mcm10 with various Mcm2-7 subunits and interaction among

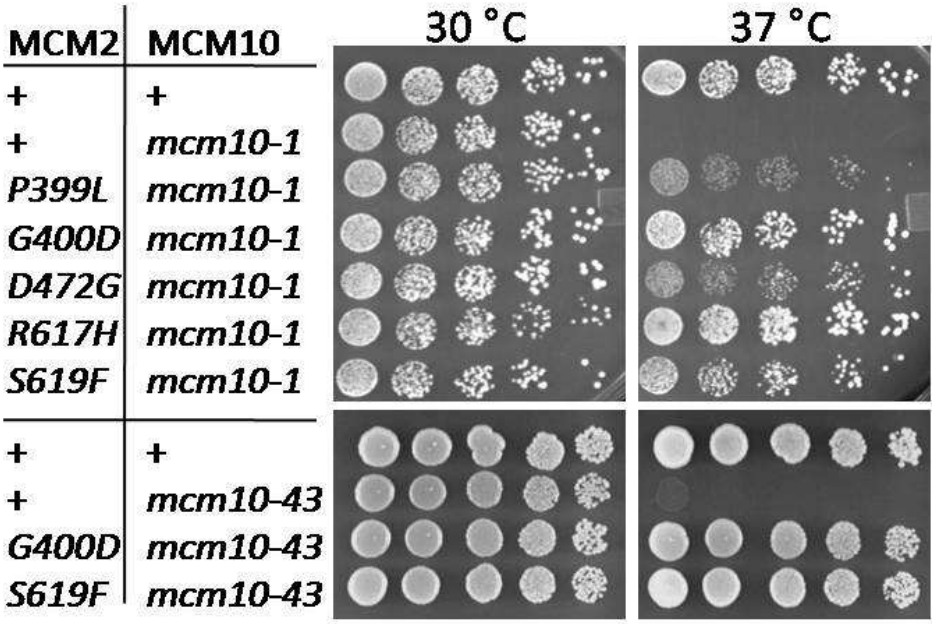
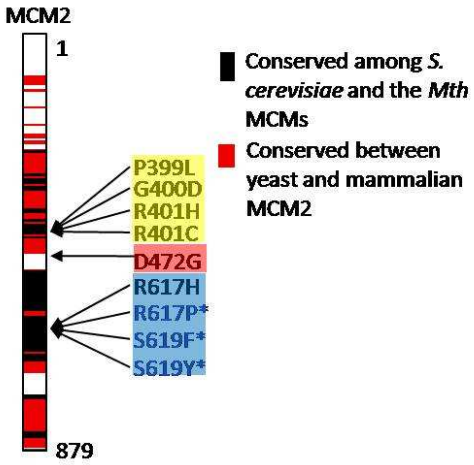


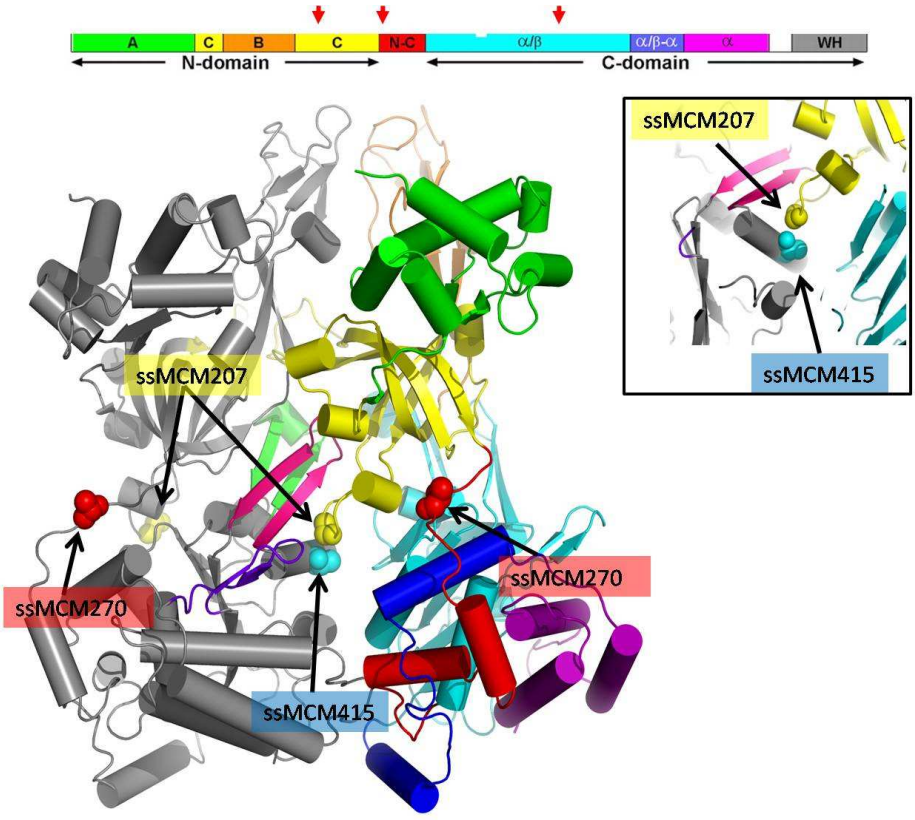
Figure 3.1- Suppression of *mcm10* temperature sensitivity by *mcm2* mutants. Five-fold serial dilutions of wild-type, *mcm10-1*, *mcm10-43*, and the different *mcm2* suppressors in *mcm10-1* or *mcm10-43* background were spotted onto YPD plates and incubated for 1-2 days at either 30°C or 37°C. The *mcm2* mutants are non-allele specific suppressors as they suppress both *mcm10-1* and *mcm10-43*.

Figure 3.2 - (A) The *mcm2* mutations are mostly located at two specific regions of the gene. One set of mutations maps at residues 399-401 and another set maps at residues 617-619. (B) ScG400, ScD472, and ScR617 residues are conserved through archaea and eukaryotes. The corresponding residues in the archaeal *Sulfolobus sulfataricus* MCM structure are G207, D270, and R415. The red arrows indicate the locations of the three residues within the primary structure. G207 and R415 localize close in space at the subunit interface. G207 of one subunit is positioned near R415 of the neighboring subunit, suggesting that the mutations affect interaction between the subunits.

A


MCM2
1
879
Conserved among S. cerevisiae and the Mth MCMs
Conserved between yeast and mammalian MCM2
P399L
G400D
R401H
R401C
D472G
R617H
R617P*
S619F*
S619Y*

B


A
C
B
C
N-C
α/β
α/β-α
α
WH
N-domain
C-domain
ssMCM207
ssMCM415
ssMCM207
ssMCM270
ssMCM270
ssMCM415

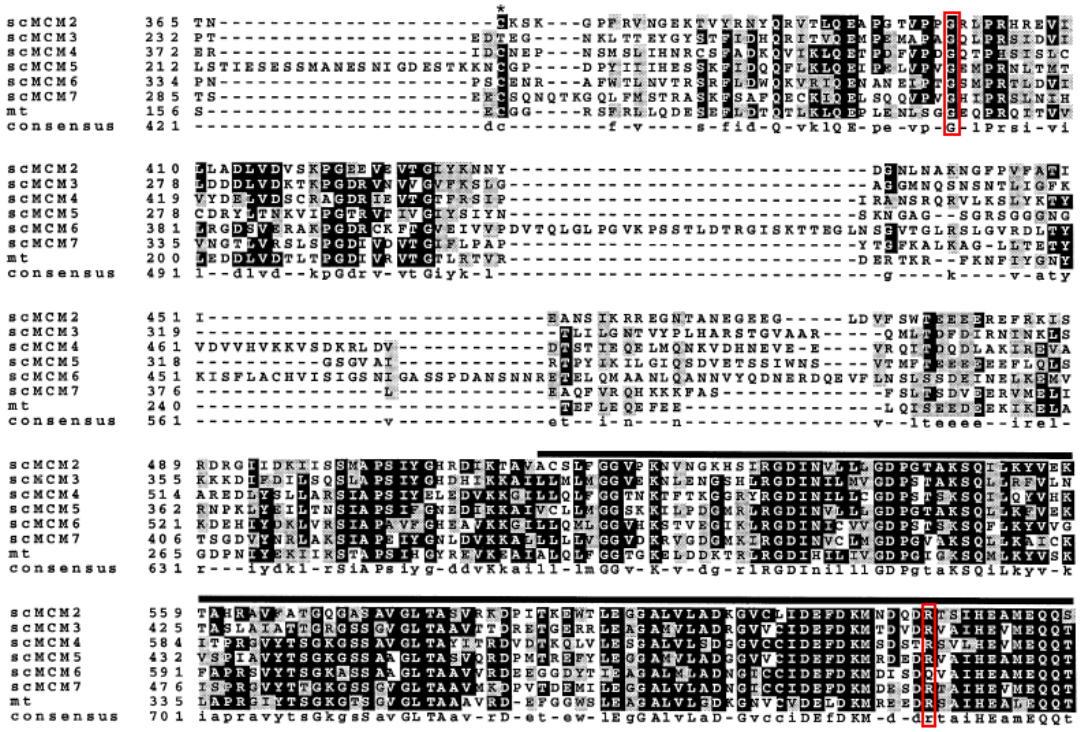


Figure 3.3- Partial sequence alignment of *S. cerevisiae* MCM2-7 subunits and the archaeal *Methanothermobacter thermautotrophicus* (mt) MCM protein. Residues G400 and R617 are conserved in all eukaryotic MCM2-7 subunits and in the archaeal MCM.

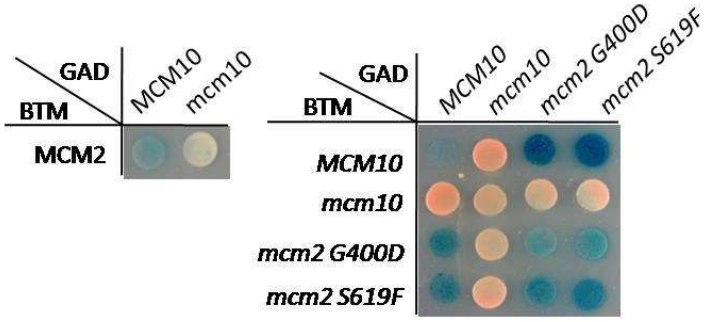
the Mcm2-7 subunits themselves have been shown by yeast two-hybrid experiments (Merchant, Kawasaki et al. 1997; Homesley, Lei et al. 2000). Mcm10 interacts with Mcm2 but this interaction is disrupted in the *mcm10-1* strain. To investigate if the *mcm2* suppressors have restored this interaction, I performed yeast two-hybrid analysis of the Mcm2 suppressors with the Mcm10-1 protein (Fig. 3.4A). The mutants *mcm2-G400D* and *mcm2-S619F* are selected as representatives for this analysis. Plasmid-based constructs with the Gal4 activation domain (GAD) or the Gal4-binding domain (GBD) fused to the wild type and mutant proteins were made. The plasmids were transformed into a wild-type W303 strain with a reporter plasmid. The levels of interaction were assayed by the visualization of β-galactosidase activity. The level of interaction between the mutant *mcm2* construct and wild-type Mcm10 construct was similar to that of wild-type Mcm2 and wild-type Mcm10 interaction. However, I could not detect any β-galactosidase activity between the *mcm10-1* and *mcm2* constructs. This suggests that the protein interaction between Mcm10 and Mcm2 is not restored by the *mcm2* mutations.

Although the *mcm2* suppressors did not restore physical interactions with Mcm10-1, I wanted to know if they restored the stability of the mutant Mcm10 protein at 37°C, a suspected cause of the ts phenotype of *mcm10*. Mcm10 protein levels in the wild-type, *mcm10,* and *mcm10 mcm2* suppressor strains were visualized by Western blots and I found that both Mcm10-1 and Mcm10-43 proteins are labile in the presence or absence of the *mcm2* suppressor mutations (Fig. 3.4B). Therefore, the *mcm2* suppressors do not prevent the degradation of either Mcm10-1 or Mcm10-43 proteins.

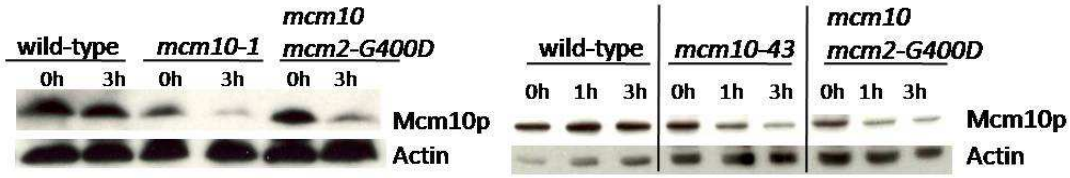
Since the *mcm2* suppressors do not seem to restore their interactions with the mutant Mcm10 protein nor prevent its degradation, I asked if Mcm10 is dispensable in the *mcm2* suppressor strains. I used a *mcm10* knockout strain that was kept viable by a

Figure 3.4 - Mutations in Mcm2 do not restore interaction with Mcm10-1 nor stabilize the mutant Mcm10 protein. (A) The two-hybrid reporter plasmid pSH18-34, pGAD (prey), and pBTM (bait) constructs with *mcm10* and *mcm2* alleles were transformed into a wild-type W303 strain to detect yeast two-hybrid interactions, indicated by blue color. The loss of interaction between *mcm10-1* and *mcm2* is not restored by Mcm2-G400D or Mcm2-S619F proteins. (B) Log phase cells of strains with Myc-tagged Mcm10, Mcm10-1, or Mcm10-43 in wild-type and Myc-tagged Mcm10-1 or Mcm10-43 in *mcm2-G400D* background were exposed to 37°C and collected at various time points for Western blot analysis. Western blots show that mutant Mcm10 protein degrades at the restrictive temperature and the Mcm2 suppressor proteins do not prevent this degradation. (C) Plasmid shuffling was carried out to exchange the wild-type *MCM10* gene in for *mcm2-G400D* in a *mcm10* null strain. *mcm10Δ*/pRS316-*MCM10* (*URA3*) was transformed with an empty pRS315-LEU2, pRS315-Mcm10, and pRS315-*mcm2-G400D* and plated on 5-FOA plates. Control experiment shows that pRS315-*mcm2-G400D* is functional and is able to suppress *mcm10-1* temperature sensitivity.

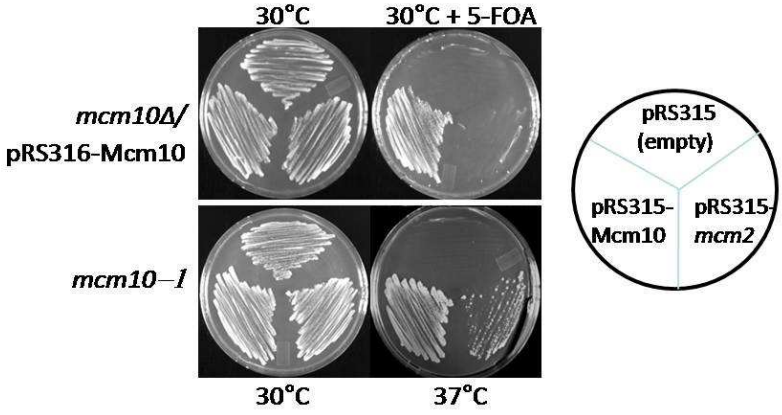
A


GAD
BTM
MCM10
mcm10
MCM2
GAD
BTM
MCM10
mcm10
mcm2 G400D
mcm2 S619F
MCM10
mcm10
mcm2 G400D
mcm2 S619F

B


wild-type
mcm10-1
mcm10
mcm2-G400D
0h 3h 0h 3h 0h 3h
Mcm10p
Actin
wild-type
mcm10-43
mcm10
mcm2-G400D
0h 1h 3h 0h 1h 3h 0h 1h 3h
Mcm10p
Actin

C


30°C
30°C + 5-FOA
mcm10Δ/
pRS316-Mcm10
mcm10–1
30°C
37°C
pRS315
(empty)
pRS315-
Mcm10
pRS315-
mcm2

wild-type copy of *MCM10* on a plasmid (Fig. 3.4C). If *mcm2* suppressors are capable of rendering the cells viable without *MCM10*, I should be able to replace the plasmid carrying *MCM10 URA3* with one carrying the *mcm2-G400D LEU2*. I performed plasmid shuffling by transforming the *mcm10*Δ/pRS316-MCM10 strain with a plasmid that carries *mcm2-G400D* and replica-plating the Leu^+ transformants onto 5-Fluoroorotic Acid (FOA) plates. I found that *mcm10* knockout strains are unable to grow on 5-FOA in the presence or absence of *mcm2-G400D*, suggesting that the *mcm2* suppressor could not bypass all of the essential functions of *MCM10* but only the essential function of *mcm10-1* and *mcm10-43* compromised at the restrictive temperature of 37°C.

mcm2 suppressors suppress origin-specific fork pausing in mcm10

Replication forks in *mcm10-1* pause at unfired origins (Merchant, Kawasaki et al. 1997; Araki, Kawasaki et al. 2003). Accumulation of DNA replication intermediates near the origin sequences of ARS1 or ARS121 have been visualized by two-dimensional (2D) gel electrophoresis. The locations of the pauses suggest that a defect in the elongation machinery may have compromised the fork's ability to move past bound pre-RCs at unfired origins. Since *mcm10* has both initiation and elongation defects, the pause at unfired origins could be due to either problems in initiation at the origin or elongation of the fork through the origin. Also, the pause could be specific to unfired origins or may occur at any replication block sites. To obtain insight into why replication intermediates accumulate in *mcm10*, I used the 2D gel technique to investigate how the *mcm2* mutants affect the replication intermediate pattern in *mcm10* and also whether the replication forks in *mcm10* pause at other replication block sites.

DNA from the strains of interest were purified and processed according to the 2D gel procedure adapted from (Brewer and Fangman 1987). Images of replication

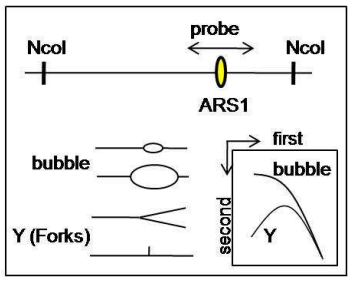
intermediates were obtained and the intensity of the 1n signal, which corresponds to the unreplicated DNA, was used to normalize the amount of replication intermediates when quantification was necessary.

The severity of *mcm10-1* growth defect increases with temperature. Though 30°C is considered as permissive, *mcm10-1* still shows mild growth defect at this temperature compared to when grown at 25°C. As *mcm10-1* displays fork pausing at 30°C, it was of interest whether this phenotype correlates with temperature, that is, *mcm10-1* growth defect. Therefore, DNA was extracted from *mcm10-1* grown at either 25°C or 30°C for comparison of the pause signals. I found that accumulation of the pause structures is more striking at 30°C compared to 25°C (Fig. 3.5A), which suggests that the severity of fork stalling does increase with temperature and therefore, may be the cause of death at the restrictive temperature.

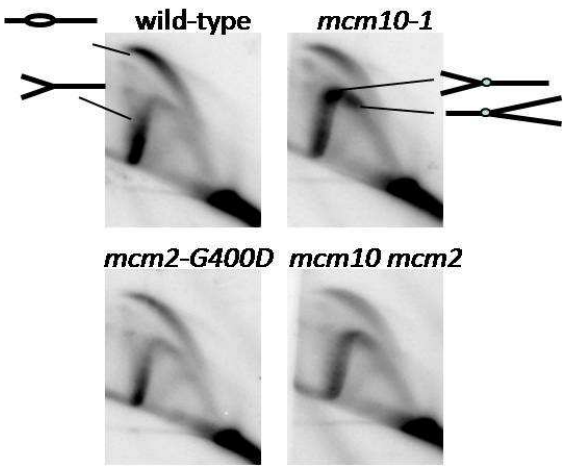
If the fork pausing is indeed associated with *mcm10* temperature sensitivity, it too would be expected to be suppressed by *mcm2*. Therefore, I asked whether the *mcm2* mutants are able to suppress the pause phenotype. Replication intermediates of wild-type, *mcm10-1*, *mcm2-G400D*, and *mcm10-1 mcm2-G400D* strains grown at 30°C were analyzed by 2D gel electrophoresis (Fig. 3.5B). The pause signals observed in *mcm10-1* are no longer observed in the *mcm10-1 mcm2-G400D* strains suggesting that the *mcm2* suppressor has alleviated the fork pausing at unfired pre-RC. Furthermore, the enhancement rather than the suppression of the replication initiation defect in the double mutant suggests that the lethality of *mcm10-1* at the restrictive temperature is not due to replication initiation at origins. Failure to suppress the replication initiation defect, but successful suppression of the pause phenotype is also observed with *mcm2-S619F* (Fig. 3.5C). This suggests that the defect of *mcm10* that leads to replication fork pausing and temperature sensitivity is in replication elongation.

Figure 3.5 - Visualization of replication intermediates using two-dimensional DNA gel analysis. (A) Log phase cultures of *mcm10-1* grown at either 25°C or 30°C were harvested for 2D gel analysis. Replication intermediates accumulate in *mcm10-1* at unfired origins at 30°C. The intensity of replication intermediates increase compared to 25°C. (B) *mcm2-G400D* alleviates the pause phenotype of *mcm10-1*. (C) Quantification of bubble structures normalized to the 1n signal shows that *mcm2-G400D* does not suppress initiation defect. Quantification was done using the ImageQuant software. (D) 2D gel analysis of *mcm2-S619F* mutant grown at 30°C. Initiation defect of *mcm10-1* is not suppressed by *mcm2-S619F*. Accumulation of pause structures is suppressed.

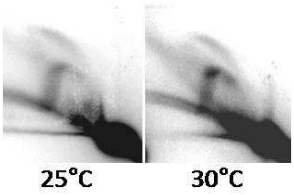
A

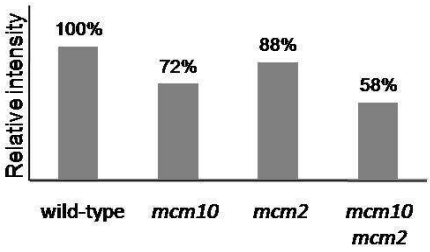

probe
NcoI
NcoI
ARS1
bubble
Y (Forks)
first
bubble
second
Y

B

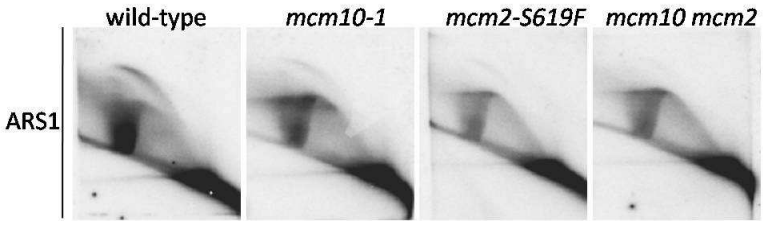

wild-type
mcm10-1
mcm2-G400D
mcm10 mcm2

C


25°C
30°C


Relative intensity
100%
72%
88%
58%
wild-type
mcm10
mcm2
mcm10 mcm2

D


wild-type
mcm10-1
mcm2-S619F
mcm10 mcm2
ARS1

Next, I asked whether replication forks in *mcm10* pauses at other replication fork block sites. Normal replication forks are known to pause at the rDNA locus and regions of active tRNA transcription. In budding yeast, there are about 100-200 tandem repeats of rDNA units on chromosome XII (Petes 1979). Each unit contains the transcribed 35S and 5S rRNA genes, an origin of replication (ARS), and a replication fork barrier (RFB) site (Linskens and Huberman 1988). The RFBs regulate replication as it allows fork progression only in the same direction as rRNA transcription (Brewer, Lockshon et al. 1992; Kobayashi, Hidaka et al. 1992). Pausing at the rDNA locus is mediated by the Fob1 protein that binds tightly to the RFB site (Kobayashi, Heck et al. 1998).

Another well known cause for replication fork pausing is when the replication machinery and transcription machinery collide. This can occur when replication and transcription take place simultaneously at the same DNA region. Replication fork pausing have been observed at sites of active transcription, one of which is the region downstream of *Ty1-17* where the *SUP53* tRNA gene is located. The pause is polar as it is only observed when the replication fork from the nearby origin and the transcription machinery move toward each other (Deshpande and Newlon 1996).

DNA was purified from wild-type and *mcm10-1* strains and probed for either the rDNA locus (Fig. 3.6 A) or the *SUP53* tRNA gene region (Fig. 3.6B) for 2D gel analysis. The intensity of the pause signals in wild-type and *mcm10* were similar, indicating that replication forks in *mcm10* did not display increased pausing phenotype at these regions. Interestingly, a novel pause spot at the rDNA locus appeared in *mcm10*. The position of the spot shows that large Y structures are accumulating at either end of the DNA fragment. The presence of an ARS site in the rDNA locus at one end of the fragment suggests that the pause may be at the origin. The observation that the replication fork in *mcm10-1* does not show increased pausing at other

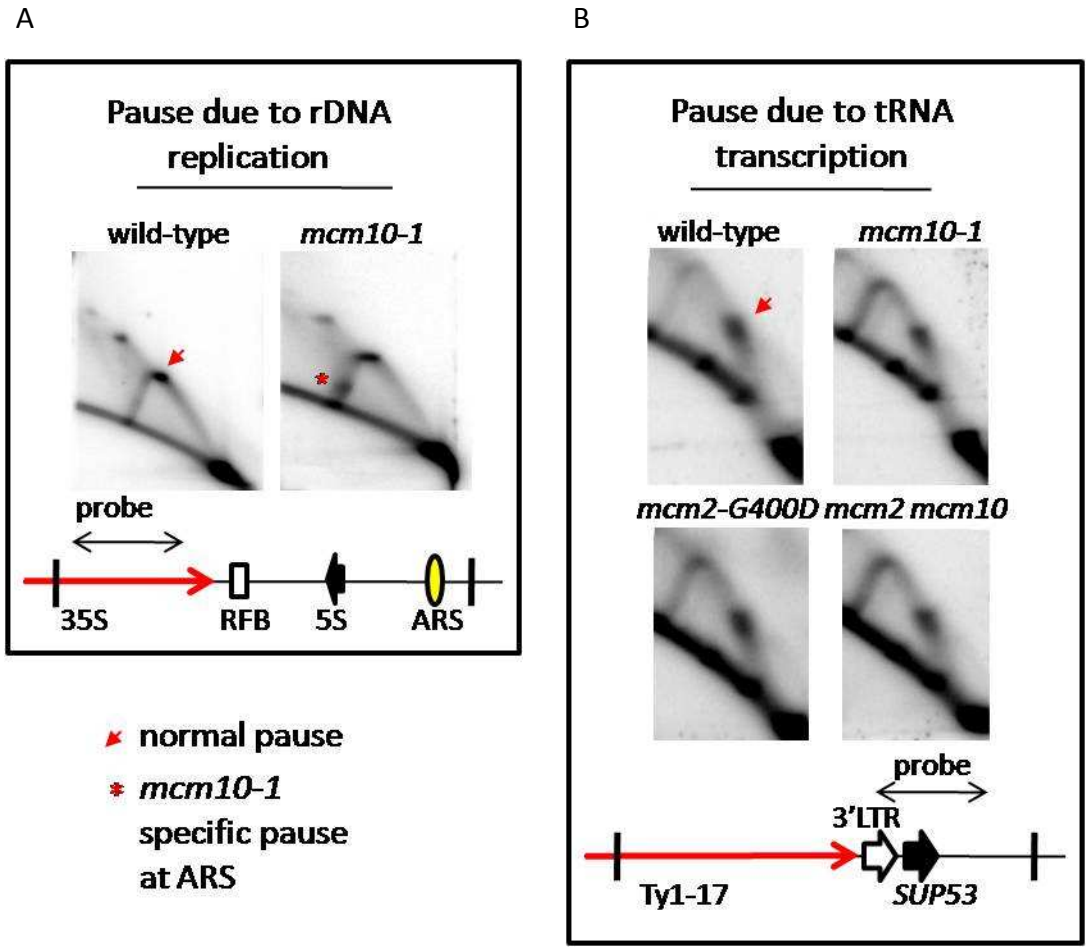


Figure 3.6 - Replication fork pausing phenotype of *mcm10-1* is specific to unfired origins and is not observed at other replication fork block sites. (A) 2D analysis of *SUP53* tRNA region where replication fork pauses due to collision with active transcription. (B) 2D analysis of rDNA locus where replication fork pauses due to the RFB-binding protein Fob1.

replication fork blocks suggests against the notion that fork pausing in *mcm10* is due to a general instability of the fork that causes it to pause easily. Rather, the fork pausing in *mcm10* appears specific for unfired origins, suggesting that elongation through pre-RC complexes require Mcm10.

Replication fork defect of *mcm10* causes DNA damage.

Hydroxyurea (HU) depletes the nucleotide pools and causes replication forks to stall. Methylmethane sulfonate (MMS) is a DNA alkylating reagent that hinders fork progression. Defects in replication fork stabilization and DNA repair have been associated with sensitivity to these chemicals (Desany, Alcasabas et al. 1998; Tercero and Diffley 2001). Sensitivity to HU reflects defects in the replication fork and sensitivity to MMS, which causes DNA damages, can be due to either defects in the fork or in DNA repair. I found that *mcm10-1* is sensitive to both HU and MMS and the *mcm2-G400D* suppressor alleviates this sensitivity to both reagents (Fig. 3.7). The sensitivity to these reagents is more likely to be associated with the defect at the fork rather than with DNA repair because *mcm10-1* did not display increased spontaneous mutation rate by the canavanine assay (data not shown). This further supports that *mcm10* renders the replication fork defective and this is what is being suppressed by the *mcm2* mutant.

The defect at the fork could be due to specific damages caused by *mcm10* or a general instability of the fork. While the origin-specific pausing in *mcm10* may discount the general instability of the fork, another assay to assess the general replication proficiency of cells is the minichromosome maintenance (*mcm*) assay. This measures how well the cells are able to replicate and maintain plasmids in the absence of selective pressure. Mutants defective in replication display higher levels of plasmid

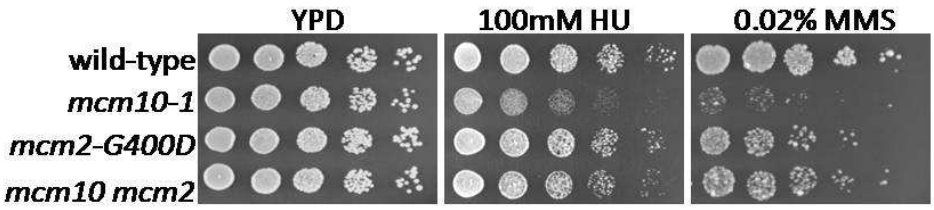


Figure 3.7 – *mcm2* suppressors rescue sensitivity of *mcm10-1* to replication stress. Five-fold serial dilutions of wild-type and mutant strains were spotted onto YPD, YPD with HU, and YPD with MMS and were grown at 30°C for 2 days to assess HU and MMS sensitivity of *mcm10-1*. *mcm10-1* displays sensitivity to HU and MMS. This is rescued by *mcm2*.

loss. Since *mcm10-1* shows a mild *mcm* defect, I was interested in whether the *mcm2* mutants suppress this (Fig. 3.8). If the fork defect, as observed by sensitivity to HU and MMS, is due to the reduced replication proficiency of *mcm10*, that is general instability of the fork, I would expect the *mcm2* mutants to enhance replication proficiency, in other words, suppress the *mcm* defect. However, the *mcm2* mutants displayed varying degrees of *mcm* defect themselves, with *S619F* showing severe *mcm* defect, and none suppressing the *mcm* defect of *mcm10*. Therefore, *mcm2* mutants do not improve overall replication. As helicase mutants with diminished replication proficiency can still suppress *mcm10* ts, the fork defect of *mcm10* does not seem to be due the general instability of the replication fork.

The observation that *mcm10* loses viability as the cells go through S phase at the restrictive temperature (Araki, Kawasaki et al. 2003) suggests that damages accumulate as the defective replication fork progresses. The damages could either be to the DNA as breaks or to the fork structure. If this is the case, proteins that function in DSB repair or resolution of aberrant fork structures should be required.

As shown in Figure 3.9A, the *mre11*, *sgs1*, *exo1*, and *srs2* mutations display synthetic growth defect or lethality with the *mcm10-1* mutation. Even at 30°C, *mre11*, *sgs1* and *exo1* display synthetic growth defects with *mcm10-1* (Fig. 3.9A) and *mcm10 srs2* is synthetically lethal (Fig. 3.9B). The latter is viable only when it carries a plasmid expressing the wild-type *MCM10* gene. These gene products are key factors in repair of double-strand DNA breaks (DSBs). *MRE11* is required for initiation of DSB repair (Paull and Gellert 1998). DNA helicases *SGS1*, *SRS2* and nuclease *EXO1* process ssDNA overhangs during double-strand DNA break repair (Ira, Malkova et al. 2003; Ira, Pellicioli et al. 2004). It has been previously reported that *mcm10-1* is synthetically lethal with yet another DNA helicase/nuclease *dna2-2* (Araki, Kawasaki et al. 2003) that also function in DSB repair.

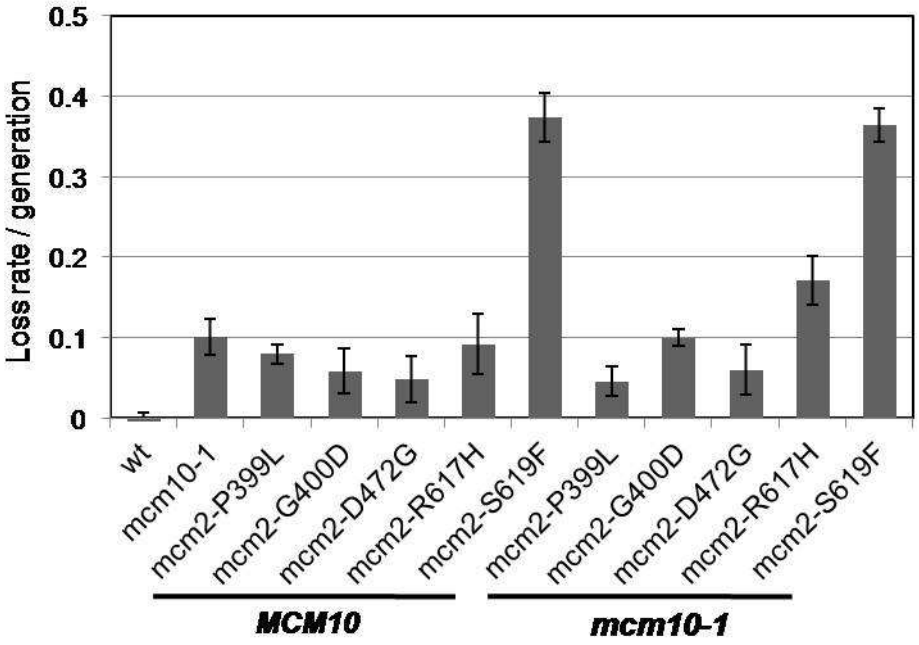


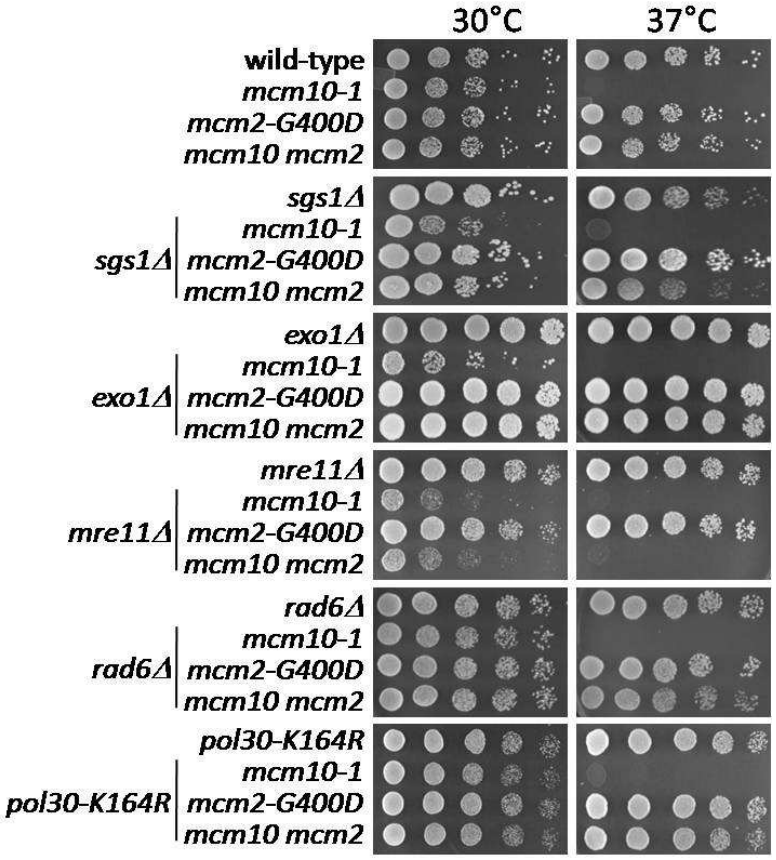
Figure 3.8 - *mcm* assay to measure plasmid loss rate of *mcm2* mutants at 30°C. Each bar shows an average loss rate of three independent cultures. Suppression of *mcm10* MCM defect is not the common mechanism as the *mcm2* single mutants themselves display varying degrees of replication proficiency.

However, Sgs1, Exo1, and Srs2 also function in fork repair as their helicase or nuclease activities are involved in promoting progression and/or resolution of reversed forks and Holliday junction structures. Srs2 is known to disrupt Rad51 binding to ssDNA to prevent aberrant recombination (Krejci, Van Komen et al. 2003; Krejci, Van Komen et al. 2003) and most *srs2* synthetic lethal mutants are rescued by deletion of *rad51* (Gangloff, Soustelle et al. 2000). Indeed, I observed that *rad51Δ* suppresses the *mcm10 srs2* synthetic lethality as well (data not shown). Interestingly, *mcm2-G400D* also rescues this synthetic lethality (Fig. 3.9B right panel). If rescue of *mcm10 srs2* synthetic lethality by *rad51Δ* is due to disruption of Rad51 filament formation and prevention of aberrant recombination events, then *mcm2* may be preventing *mcm10* from producing substrates for Srs2 and/or Rad51. While Srs2 also functions in channeling the repair pathway to PRR, I found no effect of *rad6Δ* or *pol30 K164R*, which are required for PRR, with *mcm10* (Fig. 3.9A). This suggests that the PRR pathway is not essential in *mcm10* and therefore, the absolute requirement for SRS2 seems to be in its other role, in regulating HR.

I observed that *mcm2-G400D* also suppresses *mcm10 sgs1* and *mcm10 exo1* growth defects (Fig. 3.9A), suggesting that the DNA helicases/nucleases that were previously important for viability of *mcm10* are no longer vital in *mcm2-G400D* cells. However, *mcm2-G400D* does not suppress *mcm10 mre11* synthetic defect (Fig. 3.9A), which indicates that DSBs are still occurring in *mcm10 mcm2*. In summary, these results suggest that the role of Sgs1, Exo1, and Srs2 in *mcm10* is different from that of Mre11, which implies that different types of damages are occurring at the replication fork due to *mcm10* defect.

Figure 3.9 - *mcm10-1* displays synthetic growth defects with genes in DSB and fork repair pathway. (A) *sgs1*, *exo1*, and *mre11* display synthetic growth defects with *mcm10-1*. *mcm10 sgs1Δ* and *mcm10 exo1Δ* synthetic growth defects are suppressed by *mcm2-G400D* whereas, that of *mre11 mcm10* is not suppressed. Mutations in the PRR pathway (*RAD6* and *POL30*) do not affect *mcm10*. (B) Deletion of *srs2* is synthetically lethal with *mcm10-1*. *mcm10 srs2* is only viable when it carries a plasmid containing the wild-type *MCM10* gene. The strain is unable to grow on 5-FOA when the plasmid is lost due to the *URA3* marker. *mcm2-G400D* suppresses *mcm10 srs2* lethality and the triple mutant grows well at both 30°C and 37°C.

A


30°C
37°C
wild-type
mcm10-1
mcm2-G400D
mcm10 mcm2
sgs1Δ
sgs1Δ mcm10-1 mcm2-G400D mcm10 mcm2
exo1Δ
exo1Δ mcm10-1 mcm2-G400D mcm10 mcm2
mre11Δ
mre11Δ mcm10-1 mcm2-G400D mcm10 mcm2
rad6Δ
rad6Δ mcm10-1 mcm2-G400D mcm10 mcm2
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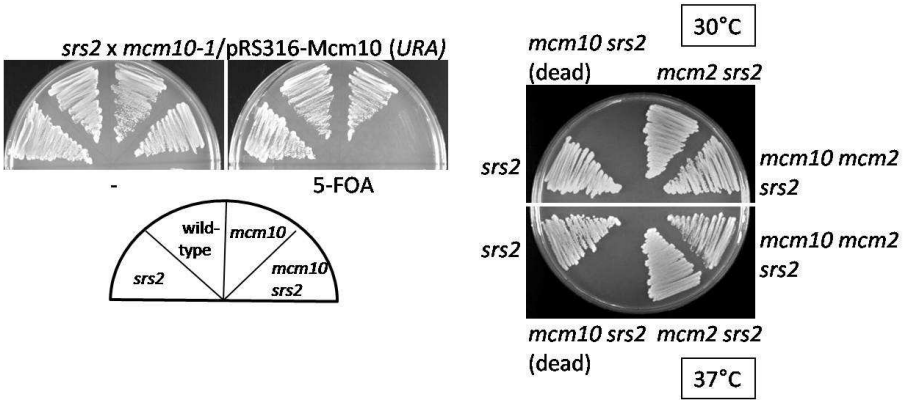
B


srs2 x mcm10-1/pRS316-Mcm10 (URA)
-
5-FOA
wild-type
mcm10
srs2
mcm10 srs2
30°C
(dead)
mcm2 srs2
srs2
mcm10 mcm2
srs2
srs2
mcm10 mcm2
srs2
mcm10 srs2
mcm2 srs2
(dead)
37°C

Discussion

Mcm10 function is essential for viability of cells and temperature sensitive mutant alleles display multiple defects in both initiation and elongation of replication. However, it is not clear what aspects of Mcm10 function, are responsible for lethality at the restrictive temperature. To understand the cause of lethality at the restrictive temperature, suppressors of *mcm10* temperature sensitivity were identified. Multiple *mcm2* point mutations were identified from the screen and most were found to lie in two regions of the gene that are conserved throughout archaea and all six subunits of the MCM2-7 helicase in eukaryotes. Due to the conserved nature of the residues ScG400 and ScR617, the available archaeal MCM helicase structure can be used to deduce how the two residues may be positioned. Structure of the archaeal MCM helicase shows that the corresponding residues SsoG207 and SsoR415 lie closely in space, suggesting that mutations in either residue may have similar effects on altering the helicase. The mutated regions lie at the subunit interface with SsoG207 positioned to interact with the SsoR415 in the neighboring subunit (Brewster, Wang et al. 2008).

A recent biochemical study examined the function of the regions where the suppressor mutations are located (Barry, Lovett et al. 2009). It is suggested that these regions play a role in mediating communication between the N-terminus domains that are important for processivity and the C-terminus domains that contain the ATPase/helicase activity. Therefore, the suppressor mutations may be affecting the processivity of the helicase. In fact, the corresponding *ScG400D* and *ScR617H* mutations have been introduced into the archaeal helicase by Roxane Bouten in the Kelman lab at UMBI for *in vitro* helicase assays and the mutant helicases were confirmed to display weaker helicase activity (Fig 3.10) (personal communication with Dr. Zvi Kelman). Though the study is of the archaeal MCM helicase, the

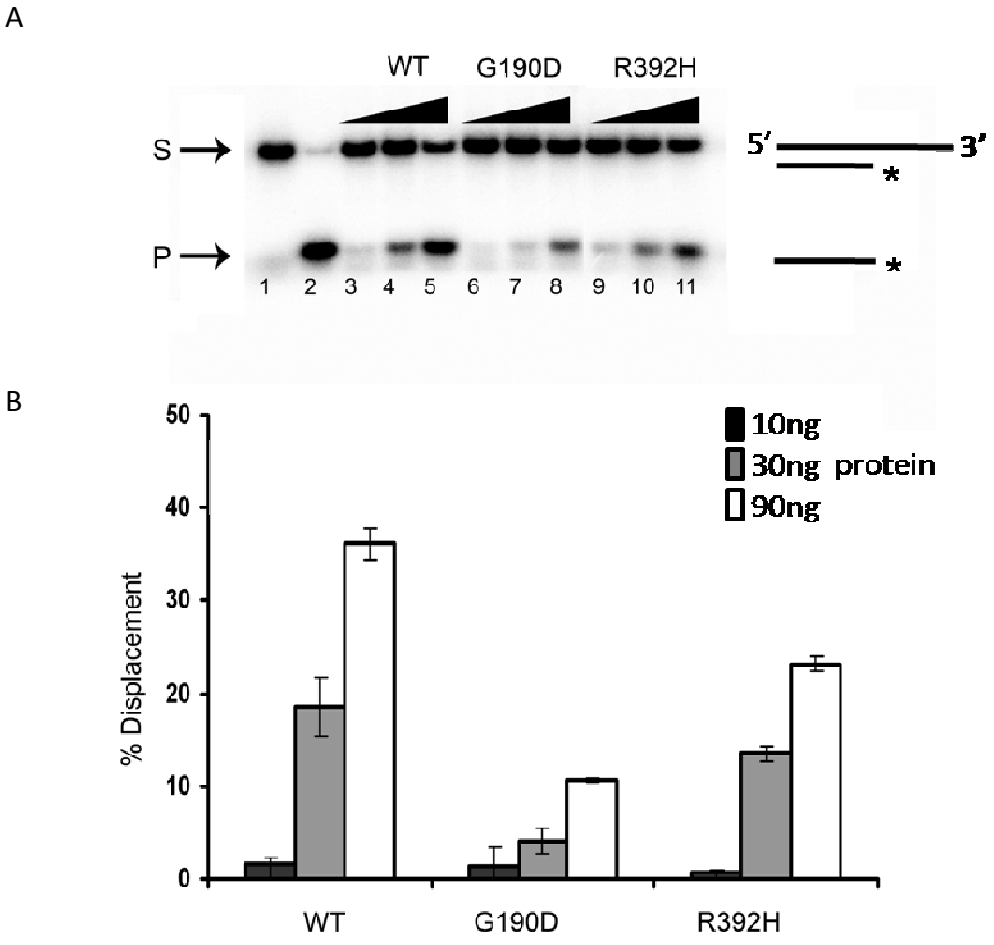


Figure 3.10 - The corresponding suppressor mutations in the *M. thermautotrophicus* MCM proteins affect the helicase activity. Purification of mutant proteins and helicase assays were performed by Roxane Bouten at UMBI using wild-type and mutant MCM proteins. A linear dsDNA made by hybridization of a 50-mer and 25-mer ssDNA was used as the substrate. The extent of helicase activity was determined by measuring the displacement of the radio-labeled 25-mer from the 50-mer. (A) Representative gel. Lanes 3-5 Wild-type MCM protein; lanes 6-8, *G190D* (*ScG400D*) mutant mtMCM protein; lanes 9-11, *R392H* (*ScR617H*) mutant mtMCM protein. Lane 1, substrate only; lane 2, boiled substrate. Lanes 3, 6, and 9, 10ng (8.7 nM as monomers) MCM protein; lanes 4, 7, and 10, 30 ng (26 nM as monomers) MCM protein; lanes 5, 8, and 11, 90 ng (78 nM as monomers) MCM protein. S: substrate, P: product (B) Average of three independent experiments.

observation that the budding yeast *mcm2* mutants all display varying degrees of *mcm* defect strongly suggest that the mutations may have similar effects on the eukaryotic helicase. Therefore, this provides the basis for speculating how the different *mcm2* mutations commonly suppress *mcm10* temperature sensitivity as we can hypothesize that the decreased helicase activity is important for suppression of *mcm10* ts.

mcm2 suppressors do not restore the interaction with the mutant Mcm10 protein, nor do they stabilize the mutant Mcm10 protein itself. However, the *mcm2* suppressor is unable to sustain a *mcm10* knockout strain. Therefore, the expression of Mcm10, albeit unstable, is required for viability. The unstable protein is likely to be essential for replication initiation since, the *mcm2* suppressors do not restore the replication initiation activity of *mcm10-1* to wild-type level visualized by the 2D gel analysis while various defects in replication elongation of *mcm10-1* are suppressed by the *mcm2* mutants. The *mcm2* mutant suppresses the replication fork pausing phenotype and HU and MMS sensitivity of *mcm10*. Double mutants of *mcm10* with *mrc1* or *tof1* display additive HU sensitivity which further suggests Mcm10 is required for replication fork stability.

However, the defect at the fork does not seem to be due to a general instability of the fork because we do not detect increased fork pausing at normal pause sites in *mcm10* and some *mcm2* suppressors demonstrate obvious defects in replication that result in high plasmid loss. These observations suggest that lethality is the consequence of specific damages caused by *mcm10*. Synthetic growth defect and lethality of *mcm10-1* with DNA helicase/nucleases Sgs1, Exo1, and Srs2 that commonly function in dsDNA break repair or resolution of aberrant fork structures are all suppressed by *mcm2* (Ira, Malkova et al. 2003; Mimitou and Symington 2008; Zhu, Chung et al. 2008). However, while *mcm2-G400D* can suppress the synthetic effect of *mcm10* with *sgs1*, *exo1*, and *srs2*, it fails to suppress *mcm10 mre11* synthetic defect.

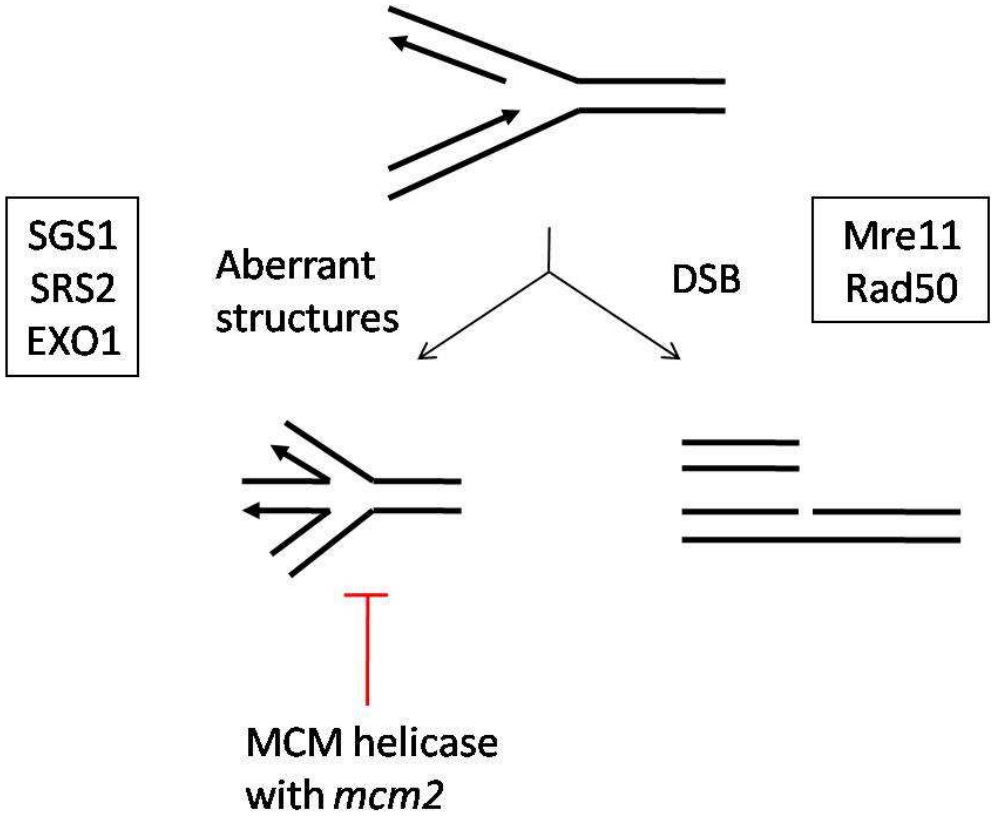


Figure 3.11 - Types of DNA lesion or aberrant fork structure in *mcm10* that may arise from uncoordinated MCM helicase and polymerase activity. The differential suppression by *mcm2* suggests that Sgs1, Srs2, and Exo1 may function in resolving aberrant fork structures that are produced when coordination between the wild-type MCM helicase and polymerases is lost. The mutation in the helicase may prevent such structures from occurring. Failure to suppress synthetic effects of *mcm10* with either *mre11* or *rad50* suggests DSBs still arise from fork progression, possibly due to Mcm10 protein instability.

The former are proteins that function as helicases or nucleases and are important for repair of aberrant fork structures in addition to their role in DSB repair while Mre11 is a major protein in DSB repair. One possible explanation for the difference in suppression could be that all the proteins function in DSB repair, but *mcm2* only substitutes for the role of Sgs1, Exo1, and Srs2, which function downstream of Mre11. However, this is unlikely because the roles of Sgs1 and Exo1 are particularly well defined in the DSB repair pathway and it is doubtful that a mutant helicase can carry out the functions of a nuclease. Therefore, this suggests that the roles of Sgs1, Exo1, and Srs2 in *mcm10* are different from that of Mre11 and their critical requirement may be in fork repair rather than DSB repair (Fig. 3.11). Therefore, it seems that while *mcm2* does not prevent DSBs, it does prevent formation of aberrant fork structures which abrogates the need for other helicase and nuclease that function in fork repair. The latter is especially interesting as it suggests interplay of the different helicases at the fork. The observation that a mutation in the main replicative helicase renders the different helicases known to assist in fork progression to be unnecessary suggests that the helicases may function in balancing or counteracting each other. Together, these results provide us with scenarios of what damages are caused by the defective *mcm10* to cause instability of the replication fork and how the mutant helicase may suppress this.

CHAPTER 4

CHECKPOINT PATHWAY FUNCTIONS IN SUPPRESSION OF *MCM10*

Introduction

Checkpoint pathways function as a surveillance mechanism to ensure the integrity of the genome by coordinating vital processes such as replication, repair, and cell cycle progression. In particular, the S phase checkpoint pathway responds to stalled replication forks or DNA damages to maintain the stability of the replication fork (Tercero, Longhese et al. 2003). Proteins involved in the S phase checkpoint pathway can either be trans factors that become activated due to replication stress or are part of the replication machinery. Mec1 and Rad53 are key players of the checkpoint response that are recruited to the fork in the event of replication stress (Allen, Zhou et al. 1994; Weinert, Kiser et al. 1994; Sanchez, Desany et al. 1996; Sun, Fay et al. 1996). Mrc1 and Tof1 associate and travel with the fork functioning as fork stabilizers because they are required for stable association of other fork components with the fork. In addition to its function in stabilizing the fork by direct interaction, Mrc1 functions also in mediating the checkpoint response (Alcasabas, Osborn et al. 2001; Katou, Kanoh et al. 2003). Loss of these checkpoint proteins result in severe instability of the replication fork as 2D gel experiments have shown stalled forks to collapse in *mec1* or *rad53* (Lopes, Cotta-Ramusino et al. 2001; Cobb, Bjergbaek et al. 2003; Katou, Kanoh et al. 2003) and uncoupling of the fork components and DNA synthesis activities are observed in *mrc1* or *tof1* (Katou, Kanoh et al. 2003).

The main signal for checkpoint activation is believed to be the exposure of

RPA-coated ssDNA. This can arise when DNA damage or nucleotide depletion hinders the progression of the polymerase in such way that the polymerase activity is uncoupled from the helicase activity. The accumulation of ssDNA then recruits and activates Mec1 (Zou and Elledge 2003). Phosphorylation of Mec1 results in Rad53 activation by means of adapter proteins Mrc1 (Alcasabas, Osborn et al. 2001; Tanaka and Russell 2001) and Rad9 (Weinert and Hartwell 1988). However, other proteins such as Sgs1 are also known to function in Rad53 activation (Bjergbaek, Cobb et al. 2005).

I have found that activation of the checkpoint pathway is required for viability of *mcm10 mcm2* during replication stress. While the checkpoint pathway is activated in both *mcm10* and *mcm10 mcm2*, only in the latter does checkpoint activation function successfully in stabilizing the fork. Therefore, the mutation in the helicase seems critical in mediating stabilization of the fork by the checkpoint pathway. This tells us that the physical stabilization of the fork by Mcm10 can be substituted by a mechanistic stabilization that involves the helicase and the checkpoint proteins.

Result

Checkpoint proteins are required for the suppression of *mcm10-1*

The nature of the various *mcm10* phenotypes that are suppressed by *mcm2* strongly suggests that the defect in the replication fork is the cause of cell death at the restrictive temperature. However, the suppressors do not suppress the temperature sensitivity by restoring physical interaction between the mutant Mcm10 and Mcm2 proteins, or by preventing degradation of the mutant Mcm10 protein (Fig. 3.4). Therefore, the mechanism by which mutations in *mcm2* restore viability of *mcm10*

cells at the restrictive temperature must involve mechanisms that compensate for the function of Mcm10 at the fork. One possible scenario is that Mcm10 is an important fork stabilizer. Mutations in factors that can stabilize the fork independently of Mcm10 would appear as suppressors of *mcm10-1.* Another is that Mcm10 may be essential for fork repair and the suppressor has gained the function to facilitate fork repair by alternative mechanisms. These hypotheses may be tested by candidate mutations from the different DNA repair and checkpoint pathways that negate or weaken the suppression of *mcm10* temperature sensitivity by the *mcm2* mutants. I introduced deletions of *mec1*, *rad53*, *rad52*, *rad51*, *mrc1*, *tof1*, *rad6*, *dnl4*, *rad9*, ex*o1*, *mre11*, *sgs1*, *srs2*, and *ddc1* into the wild type, *mcm10-1*, *mcm2-G400D*, and *mcm10 mcm2* strains (Fig. 4.1). The gene deletions that have a negative effect on suppression were *sgs1*, *rad52*, *mre11*, *rad53*, and *mec1*. Since both *rad53* and *mec1* also require *sml1* deletion for viability, I confirmed that *sml1* is not responsible for the negative effect on suppression (Fig. 4.1A).

As Mre11 is an important player in DSB repair, the complete failure of *mcm2* to suppress *mcm10* ts in the absence of Mre11 suggests that *mcm10 mcm2* display DSBs at levels similar to those seen in *mcm10*. The negative effect of *sgs1* and *rad52* on suppression further adds to the notion that *mcm2* does not completely prevent DNA damages. Sgs1 functions in regulating HR and Rad52 is a key protein in HR (Onoda, Seki et al. 2001; Symington 2002; Liberi, Maffioletti et al. 2005) . Since Sgs1 can be either a positive or negative regulator, it was of interest whether the requirement for Sgs1 and Rad52 for suppression of *mcm10* ts stems from their cooperation in mediating HR. If Sgs1 function in anti-recombination, deletion of *RAD52* in *sgs1Δ* will cancel out the effects of the single mutants. If Sgs1 functions in promoting HR, *sgs1 rad52* double mutant should display synthetic effects. It has been shown in previous studies that *rad52Δ* rescues the defects of mutations in combination with

Figure 4.1 - Serial dilutions of strains are spotted onto YPD and incubated at 30°C or 37°C. (A) Deletion of *SGS1*, *MEC1*, *RAD53*, and *Rad52* has negative effect on suppression. *mcm10 mre11* has synthetic growth defect and *mcm2* fails to suppress this. (B) Gene deletions that have no significant effects on suppression of *mcm10* temperature sensitivity.

A

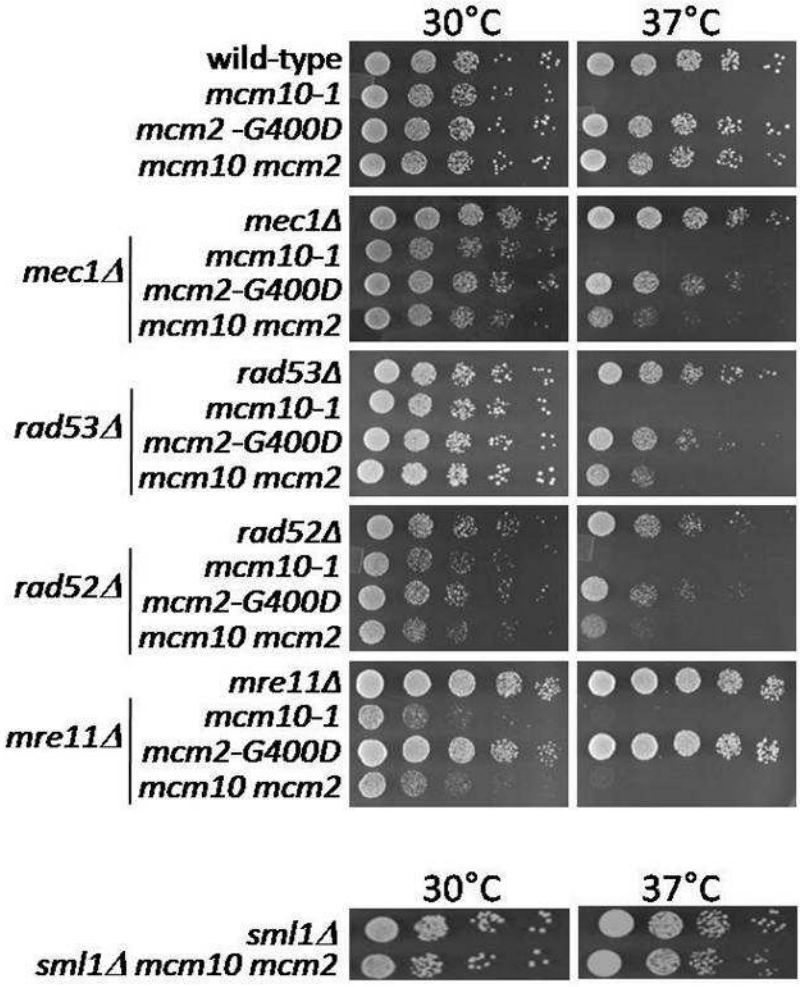
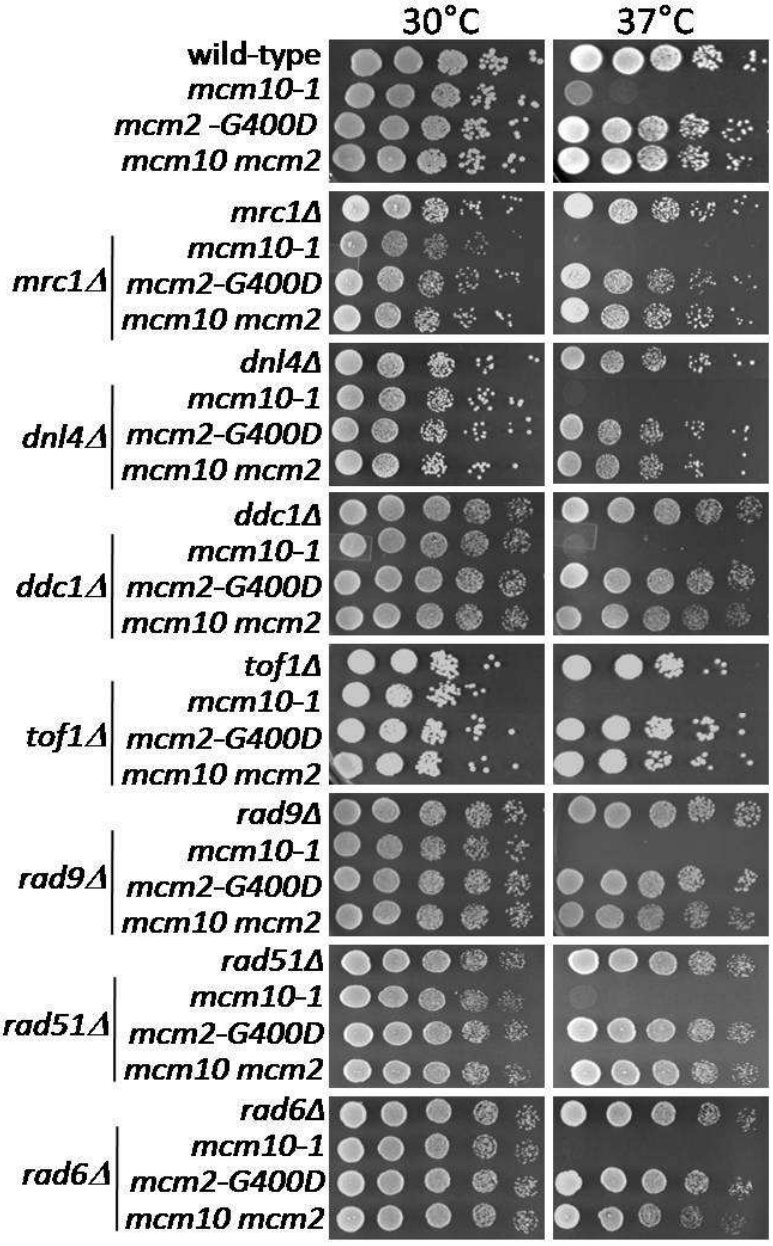

30°C
37°C
wild-type
mcm10-1
mcm2 -G400D
mcm10 mcm2
mec1Δ
mec1Δ
mcm10-1
mcm2-G400D
mcm10 mcm2
rad53Δ
rad53Δ
mcm10-1
mcm2-G400D
mcm10 mcm2
rad52Δ
rad52Δ
mcm10-1
mcm2-G400D
mcm10 mcm2
mre11Δ
mre11Δ
mcm10-1
mcm2-G400D
mcm10 mcm2
30°C
37°C
sml1Δ
sml1Δ mcm10 mcm2

Figure 4.1 – (Continued)

B

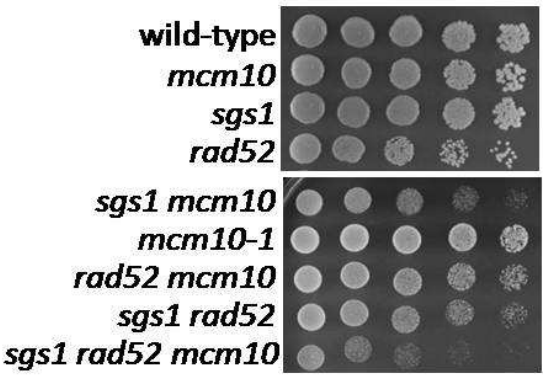


sgs1, such as *srs2 sgs1*, that result in hyperrecombination. However, with *mcm10 sgs1*, deletion of *RAD52* does not rescue, but rather displays a synthetic effect (Fig. 4.2A). This suggests that these proteins function together to promote HR in *mcm10* and is likely to also do so in *mcm10 mcm2*.

Rad53 and Mec1 are both key players in the checkpoint signaling pathway that have a role in stabilizing stalled forks as well as transducing signals to downstream effectors. For review see (Branzei and Foiani 2008). Deletion of *rad53* and *mec1* greatly diminished the ability of the *mcm2* mutants to suppress *mcm10* temperature sensitivity. This suggested that the checkpoint pathway is activated in *mcm10 mcm2*. Recruitment of Rad53 to replication forks requires activation by phosphorylation.

To investigate if Rad53 is indeed activated, I examined the phosphorylation state of Rad53 in *mcm10-1*, *mcm2-G400D*, and *mcm10 mcm2* mutants. Log phase cells were grown at 37°C for 2 hours and collected for protein extraction and western blot (Fig. 4.3). I found that Rad53 is hyperphosphorylated in *mcm10* at 37°C. Previous work showed that a shift to 37°C causes an irreversible loss of viability upon return to permissive temperature in *mcm10* cells (Araki, Kawasaki et al. 2003). This suggests that Rad53 activation is due to degradation of Mcm10p, which leads to irreparable damage of DNA. In *mcm10 mcm2*, Rad53 is also phosphorylated, though the shift due to Rad53 phosphorylation is much weaker. While Rad53 is activated in both *mcm10* and *mcm10 mcm2*, the consequences of its activation are drastically different as *mcm10* loses viability while *mcm10 mcm2* is phenotypically similar to that of wild-type. It is likely that the former accumulates irreversible DNA damages whereas damages are reversible in the latter. Rad53 phosphorylation is not observed in mcm2, ruling out the possibility that the mutation in the helicase activates the checkpoint pathway. Therefore, it seems that the *mcm2* mutant helicase in combination with *mcm10* causes activation of Rad53 that prevents or fixes the damage by *mcm10*. Our

A



B

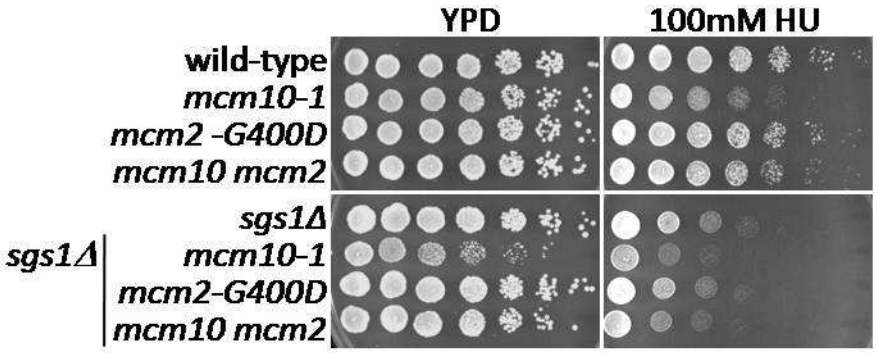


Figure 4.2 - (A) Five-fold dilution of strains spotted onto YPD plate and grown at 30°C. *SGS1* and *RAD52* have synthetic effect with *mcm10-1* suggesting that Sgs1 functions in promoting recombination in *mcm10*. (B) Five-fold dilution of strains spotted onto YPD plate with and without 100 mM HU and grown at 30°C. Deletion of *SGS1* does not affect viability of *mcm10*, *mcm2*, or *mcm10 mcm2* in HU other than the additive sensitivities of the single mutants.

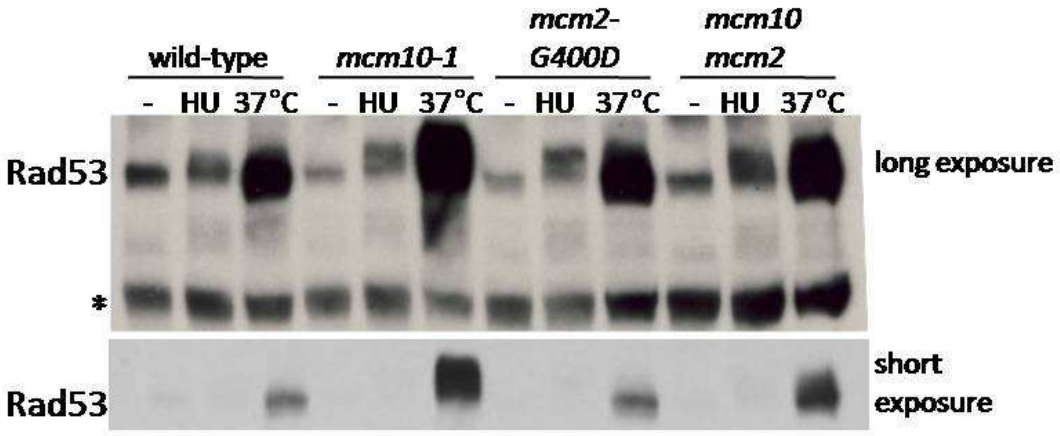


Figure 4.3 –Rad53 phosphorylation in *mcm10-1* and *mcm10 mcm2*. 3xHA-tagged Rad53 strains with *mcm10-1* and *mcm2-G400D* mutations were grown to log-phase, arrested by α-factor for 1.5 hour, and released into fresh media with or without HU at 30°C for 1 hour or without HU at 37°C for 1 hour. Samples were collected for western blot using anti-HA antibodies to assay the phosphorylation state of Rad53. Exposure of *mcm10-1* to 37°C leads to hyperphosphorylation of Rad53. Suppression of ts by *mcm2* is accompanied by decrease in Rad53 phosphorylation to a more moderate state. Asterisk indicates non-specific band.

results suggest that the unstable replication fork in *mcm10* is stabilized by *mcm2* by a mechanism that involves activation of the checkpoint pathway.

While activated Rad53 can stabilize the replication fork by direct interaction, it can also slow down S phase to provide more time for repair of any damages (Paulovich and Hartwell 1995). In order to determine whether Rad53 activation in *mcm10 mcm2* is accompanied by a slower S phase, FACS analysis was carried out. For overall ratio of cells in G1, S, or G2, log phase cells of wild-type, *mcm10*-1, *mcm2-G400D*, and *mcm10 mcm2* strains were collected without alpha-factor arrest. For cell cycle progression, log phase cultures were arrested in G1 phase by alpha-factor for 2 hours. Cells were spun down and resuspended in fresh YPD media containing 100 µg/ml of pronase (Sigma) for rapid alpha-factor degradation and release into S phase. The G1-arrested cells were released at either 30°C or 37°C. For the latter, the cells were pre-incubated at 37°C for 1h before release to allow Mcm10 degradation to occur before onset of S phase. This was done to enhance the effect of Mcm10 degradation on replication progression during a single replication cycle. Samples at different time points were collected for FACS analysis. At 30°C, the overall ratio of G1/S/G2 is similar for all strains with two peaks, one at G1 and the other at G2, with the G2 peak being slightly stronger (Fig. 4.4). However, at 37°C, in *mcm10* the G1 peak is much greater than the G2 peak suggesting that the cells have difficulty entering S phase. *mcm2-G400D* seems to fix this problem, though not completely, as the G1 peak is still stronger than G2. Closer inquiry of how the cell cycle progresses was carried out by arresting the cells at G1 and releasing into S phase (Fig. 4.5). At 37°C, with Mcm10 depletion, significant delay in S phase entry and progression was observed as published (Merchant, Kawasaki et al. 1997). While *mcm2* does not show any difference in cell cycle progression from that of wild-type, a slight delay of S phase progression in *mcm10 mcm2* was observed. Whether entry into S

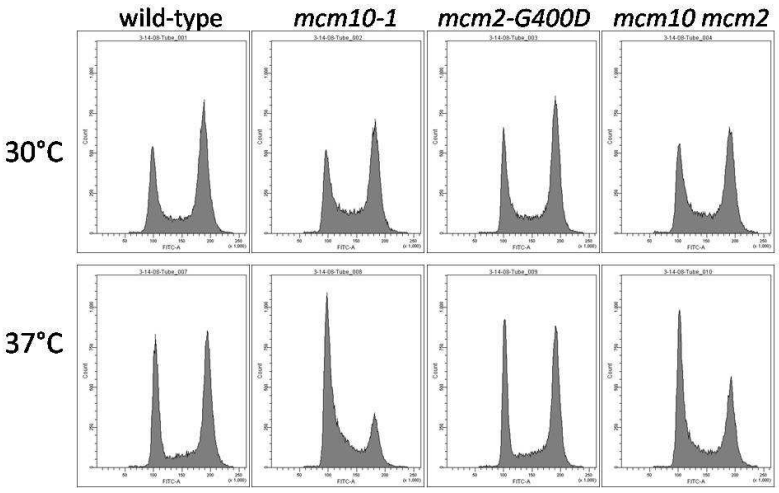


Figure 4.4 - FACS analysis of log phase cells at 30°C and 37°C. For 37°C samples, log phase cultures were exposed to the restrictive temperature (37°C) for 1h.

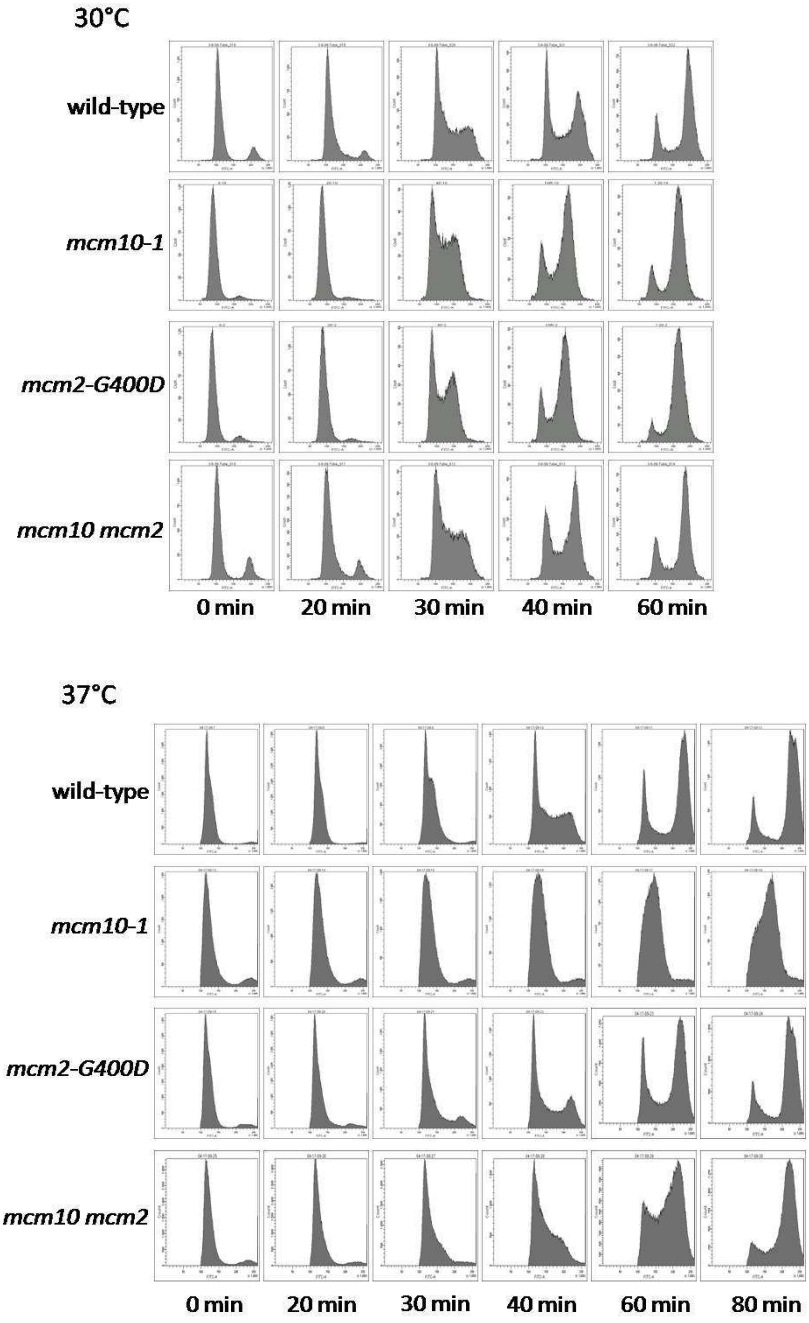


Figure 4.5 - Visualization of cell cycle progression by FACS analysis. Cells were synchronized by arresting in G1 phase with alpha-factor and releasing into S phase.

phase is delayed is not clear as wild-type, *mcm2*, and *mcm10 mcm2* all seem to enter into S phase at the 30 minute time point. However, the delay in progression was evident as *mcm10 mcm2* cells were still in S phase while wild-type or *mcm2* cells were already into G2 at the 60 minute time point. Though the delay in S phase progression in *mcm10 mcm2* could be due to the regulatory function of the checkpoint pathway, it is also possible that it could simply be due to the failure of *mcm2* to completely suppress the replication defects of *mcm10*.

Mutations in *MCM2* stabilize Cdc17p in *mcm10* cells in a checkpoint-dependent manner

Cdc17 is the catalytic subunit of polα-primase which is the only DNA polymerase that has the capability of de novo DNA synthesis (Burgers 1998). The primase is required for priming the leading strand synthesis during initiation and also the Okasaki fragments on the lagging strand throughout elongation. In budding yeast, it is suggested that Mcm10 functions as a linker between polα-primase and the helicase because Mcm10 is required for Cdc17 stabilization and its association with the chromatin (Ricke and Bielinsky 2004). In both *mcm10-1* and *mcm10-temperature degron (td)* mutant, Mcm10 protein degradation at 37C was accompanied by Cdc17 degradation with similar kinetics (Ricke and Bielinsky 2004). I found that the *mcm2* suppressors do not suppress degradation of the mutant Mcm10 protein (Fig. 3.4B). However, since the primase activity is indispensable for DNA replication, I reasoned that suppression of *mcm10* temperature sensitivity by *mcm2* may be accompanied by restoration of Cdc17 function directly or indirectly. Since Cdc17 degrades in both *mcm10-1* and *mcm10-td*, it is the loss of Mcm10 stability that leads to Cdc17 instability rather than the specific mutation in *mcm10-1*. Therefore, *mcm10-43,* which also displays Mcm10 instability, should show the same phenotype. Therefore, I

performed western blot experiments in Cdc17-3xHA-tagged wild-type, *mcm10-1*, *mcm10-43*, *mcm10-1 mcm2-G400D*, and *mcm10-43 mcm2-G400D* strains to determine the stability of Cdc17p. Indeed, I found that Cdc17 is also unstable in *mcm10-43* and degrades at 37°C. However, while the *mcm2* suppressor fails to stabilize Mcm10 in *mcm10-1* and *mcm10-43* cells, it is able to stabilize Cdc17 (Fig. 4.6A and B).

Since Mcm10 is suggested to be a chaperone for Cdc17 stability, it was of interest how Cdc17 is stabilized despite Mcm10 instability. I had noticed that suppression of *mcm10-1* ts by *mcm2-G400D* was greatly diminished in a *mec1* or *rad53* null background. The same is observed with *mcm10-43* (Fig 4.6D). This suggested that the checkpoint function may be required for Cdc17 stability. I tested this idea by looking at Cdc17 stability in *mec1*, a strain defective in checkpoint activation. I carried out Western blot of Cdc17-3xHA to determine protein stability in *mec1 mcm10 mcm2* (Fig. 4.6C). I found that Cdc17 is no longer stable when Mec1 function is lost. This suggests that stability of Cdc17 in *mcm10 mcm2* depends on the checkpoint pathway that functions in stabilizing the replication fork.

Checkpoint functions are required during other replication stress

Though the HU sensitivity of *mcm10-1* is not very strong, double mutants of *mcm10-1* with *mrc1Δ* or *tof1Δ* have increased sensitivity to HU. However, the role Mcm10 plays seems to be distinct from Mrc1 or Tof1. HU sensitivity of *mcm10 mcm2 mrc1* is similar to that of *mrc1Δ* alone, which indicates *mcm2* suppresses only *mcm10* phenotype and not *mrc1*. I noticed that *mcm10 mrc1* shows hypersensitivity to HU, but since Mrc1 is known to function in both directly stabilizing the replication fork and as an adapter for Rad53 activation, I wanted to determine loss of which of these functions were responsible for hypersensitivity. I used the separation of

Figure 4.6 - *mcm2* mutant stabilizes Cdc17p in a Mec1-dependent manner (A) Log-phase cells were incubated at either room temperature or 37°C for 90 minutes and collected for western blot analysis of Cdc17 tagged with 3x HA at the C-terminus. Cdc17 is unstable in both *mcm10-1* and *mcm10-43* at 37°C and this is stabilized by *mcm2-G400D*. Asterisk indicates non-specific band. (B) Log-phase cells of *mcm10-43 mcm2-G400D* were incubated at 37°C for various time periods and subjected to western blot for detection of 3x HA-tagged Cdc17 and 13x Myc-tagged Mcm10-43. Cdc17 displays stability in *mcm10-43 mcm2-G400D* despite Mcm10-43 instability. Asterisk indicates non-specific band. (C) Log-phase cells of *mec1* and *mec1 mcm10-43 mcm2-G400D* were incubated at 37°C for various time periods and collected for western blot of Cdc17. Loss of Mec1 function in *mcm10-43 mcm2-G400D* leads to degradation of Cdc17, suggesting that stabilization of Cdc17 by *mcm2* is dependent on the checkpoint pathway. Asterisk indicates non-specific band. (D) Spot dilution of *mcm10-43* and *mec1* mutants show that Mec1 deletion has negative effect on suppression of *mcm10-43* by *mcm2-G400D*, similar to that of *mcm10-1*.

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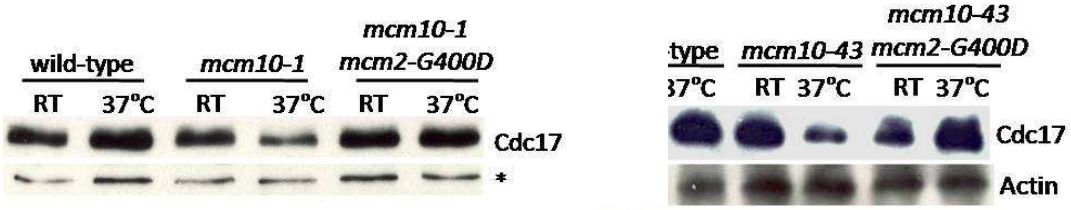
wild-type
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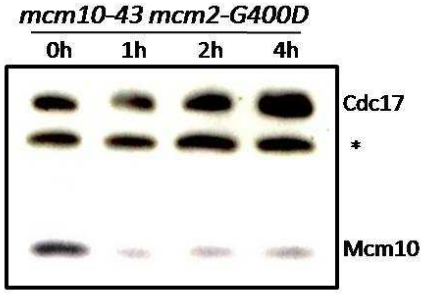
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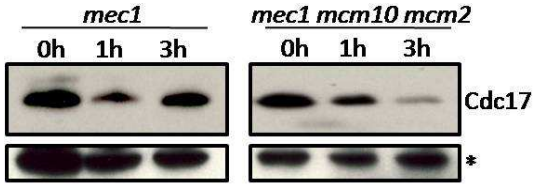
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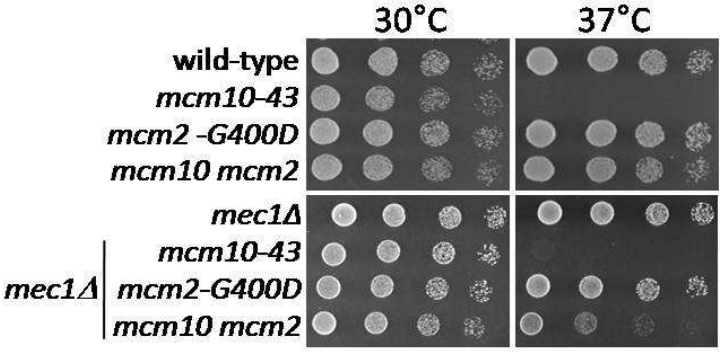

30°C
37°C
wild-type
mcm10-43
mcm2 -G400D
mcm10 mcm2
mec1Δ
mec1Δ
mcm10-43
mcm2-G400D
mcm10 mcm2

function mutant *mrc1AQ* which is competent for replication but not for checkpoint activation to test this (Fig. 4.7) (Osborn and Elledge 2003). By comparing *mcm10*, *mcm2*, and *mcm10 mcm2* in the *mrc1* null background to *mrc1AQ* background, I found that hypersensitivity of *mcm10 mrc1*to HU is due to Mrc1 function in directly stabilizing the replication fork as *mcm10 mrc1AQ* was no longer hypersensitive and was restored to that of *mcm10* alone. Sensitivity of either *mcm2* or *mcm10 mcm2* to HU in the *mrc1AQ* background was similar to that of *mrc1* null, indicating that Mrc1 function in mediating the checkpoint response is important in these mutants. In other words, the loss of Mrc1 checkpoint function renders the *mcm2* and *mcm10 mcm2* more sensitive to HU. Therefore, it seems that the mutant helicase is more dependent on the checkpoint functions than the wild-type and that the checkpoint pathway is activated more readily by the mutant helicase. This shows that the mechanism by which the replication fork is stabilized in *mcm10 mcm2* during replication stress depends heavily on checkpoint activation.

The role of Tof1 seems to be more vital, because in *tof1* null mutants, *mcm2* fails to suppress HU sensitivity of *mcm10,* suggesting that *mcm2* cannot compensate for the loss of Tof1 function when forks stall in HU. This observation is interesting since Tof1 has been shown to be important for the stability of paused complexes, while Mrc1 is more important for the stability of progressing replication forks. In summary, the findings suggest that activation of checkpoint is the key mechanism by which *mcm2* suppresses *mcm10* defect during replication stress.

Interactions between MCM10 and MCM2-7 subunits are altered in *mcm2-G400D*

Interaction between Mcm10 and the subunits of the MCM helicase has been demonstrated by yeast two-hybrid assays in previous studies (Merchant, Kawasaki et al. 1997; Douglas 2003). While *mcm2-G400D* maintains its interaction with Mcm10

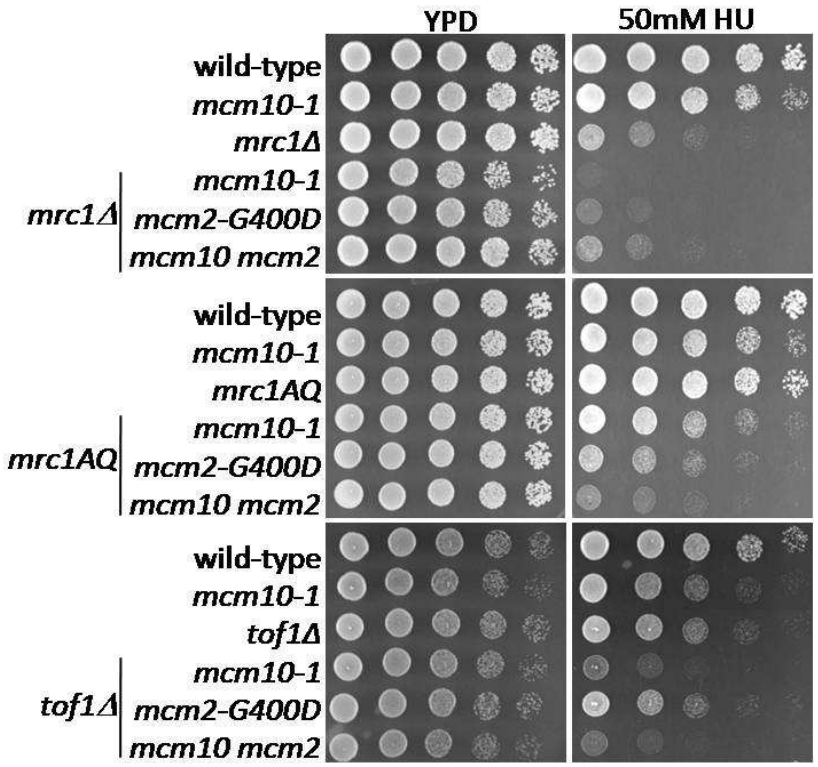


Figure 4.7 - Combinations of *mcm10*, *mcm2*, *mrc1Δ*, and *tof1Δ* mutations were spotted onto YPD or YPD with 50mM HU. Both *mcm10 mrc1Δ* and *mcm10 tof1Δ* are more sensitive to HU than the single mutants. *mcm2-G400D* suppresses only *mcm10* phenotype and not *mrc1Δ* as *mcm10 mcm2 mrc1Δ* HU sensitivity is similar to *mrc1Δ* alone. *mcm10 mrc1Δ* hypersensitivity to HU is due to the loss of replication fork stabilizing function of *mrc1Δ* as *mcm10 mrc1AQ* is no longer hypersensitive. However, the loss of checkpoint function of *mrc1AQ* severely affects viability of *mcm10 mcm2* in HU.

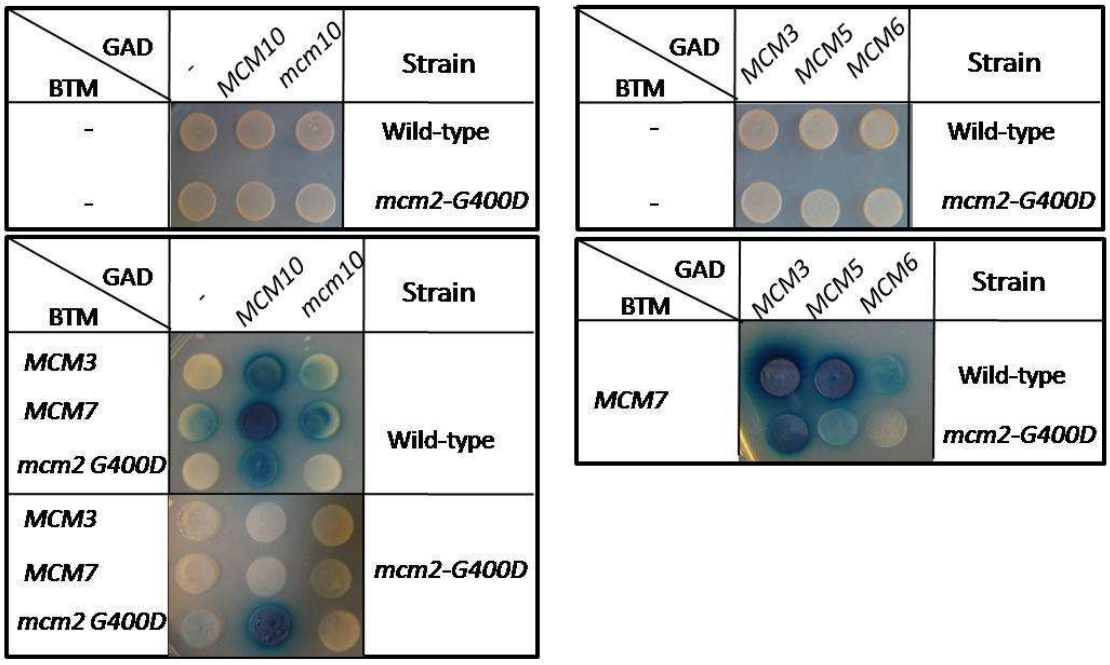
(Fig. 3.4A), it was of interest whether the interaction between Mcm10 and the other helicase subunits were changed by the *mcm2* mutation.

The two-hybrid constructs of Mcm10 and MCM helicase subunits were transformed in either wild-type or *mcm2-G400D* strains carrying the reporter plasmid. Interactions between the constructs were observed by the appearance of blue colonies when grown on X-gal plates (Fig. 4.8). Interestingly, interactions between the constructs Mcm10 and Mcm3 or Mcm10 and Mcm7 that are observed in wild-type background were no longer observed when the same plasmids were transformed into *mcm2-G400D*. Interaction between the constructs Mcm10 and *mcm2-G400D* were observed in both backgrounds, serving as a control. The results suggest that Mcm2 may be mediating the interaction between Mcm10 and the other MCM helicase subunits and this is disrupted by the mutation in Mcm2. We do not know whether the interaction between Mcm10 and MCM helicase is required for stimulation or inhibition of helicase activity. However, the functional implication of this is that in either *mcm2* or *mcm10 mcm2*, MCM helicase would be independent of Mcm10.

Discussion

Polymerase α-primase is essential for priming DNA synthesis and is required throughout elongation on the lagging strand. Mcm10 is required for chromatin association of Polα and also functions as a chaperone for Cdc17 stability (Ricke and Bielinsky 2004). Therefore, it was of interest to determine how the *mcm2* suppressor stabilizes Cdc17 despite Mcm10 instability. The requirement for Mec1 function in Cdc17 stabilization shed light on this. Stabilization of Cdc17 seems to be one of the effects that lead to fork stabilization by checkpoint activation. It is possible that Cdc17 is directly targeted by the checkpoint proteins because it has been shown that

A



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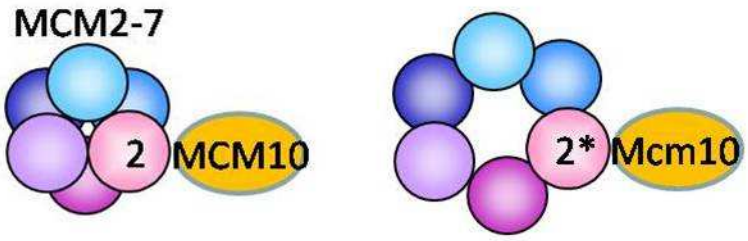

Figure 4.8 - Interactions between Mcm10 and MCM helicase subunits are altered by *mcm2-G400D*. (A) MCM3 or MCM7 interaction with MCM10 is no longer observed in *mcm2-G400D* background. Interaction between the MCM helicase subunits are maintained, albeit weaker. (B) Model to explain the loss of interaction. MCM10 interaction with the helicase may be mediated by MCM2. Tight interaction between the MCM helices subunits may allow interaction between MCM10 and other MCM helicase subunits. Loose interaction between the MCM helicase subunits may result in loss of interaction between MCM10 and MCM3 or MCM7.

the Polα acts downstream of Rad53 activation as another subunit of the polymerase has been shown to be phosphorylated due to Rad53 activation (Marini, Pellicioli et al. 1997). Therefore, while normally Mcm10 functions in stabilizing Polα, in the events that Mcm10 cannot carry out this function, another pathway may be evoked to substitute for this critical activity. Though direct interaction between Cdc17 and the mutant Mcm2 protein is another possible way of stabilizing Cdc17, two-hybrid interactions between Cdc17 and either wild-type or mutant Mcm2 have given only negative results (data not shown).

The importance of the checkpoint pathway in *mcm10 mcm2* was observed during two different replication stress conditions. One is at 37°C with the loss of Mcm10 that functions in stabilizing the fork. The other is in HU, when polymerases stall due to lack of nucleotide. The loss of checkpoint function in *mrc1AQ* severely affected the viability of *mcm10 mcm2 mrc1AQ* in HU. In both cases, activation of checkpoint seemed critical for viability of *mcm10 mcm2*. Interestingly, the mediators of checkpoint activation seem to be different. The functions of Mrc1 and Tof1 are important in HU while they do not affect viability of *mcm10 mcm2* at 37°C. On the other hand, Sgs1, which is important for viability of *mcm10 mcm2* at 37°C, does not affect viability in HU (Fig. 4.2B). Sgs1 also function in activating the checkpoint and therefore, may be the checkpoint mediator at 37°C (Bjergbaek, Cobb et al. 2005). Mrc1, Tof1, and Sgs1 all travel with the fork (Cobb, Bjergbaek et al. 2003; Katou, Kanoh et al. 2003) and may differently activate the checkpoint in response to the fork structures that result from the different stress conditions. However, due to the wide range of roles that Sgs1 plays, it is uncertain how this protein functions in *mcm10* suppression. It could be involved in fork repair along with Mre11 and Rad52. In fact, activation of the checkpoint pathway could be assisting in efficient repair of the forks in *mcm10 mcm2*.

Stabilization of the replication fork in *mcm10 mcm2* appears to be dependent on checkpoint activation. In order to determine whether the role of *mcm2* mutations is to simply activate the checkpoint pathway, I tried to see whether *mcm10-1* ts can be suppressed when grown in YPD containing HU, which would activate the checkpoint pathway. I found that growing *mcm10* in different amounts of HU does not suppress the temperature sensitivity (Fig. 4.9). However, there is a caveat to this experiment as *mcm10-1* itself is sensitive to HU and the reagent would create problems for the replication fork. Regardless, a previous observation that loss of checkpoint activation does not completely abrogate suppression, that is *mcm10 mcm2 mec1* or *mcm10 mcm2 rad53* strains still maintain viability at 37°C, suggest that suppression of *mcm10-1* ts by *mcm2* involves more than checkpoint activation. Therefore, the mutations in *mcm2* must render the helicase capable of mediating fork stabilization by both substituting for the role of Mcm10 as well as activating the checkpoint pathway. How does the mutant helicase achieve these ends? A simple explanation can be that the mutations affect the helicase activity to make it less processive. Indeed, *G400D* and *R617H* mutations introduced into the archaeal helicase renders the helicase to display weaker helicase activity in *in vitro* helicase assays (Fig. 3.10).

I show a model of how *mcm2* may suppress *mcm10* in figure 4.10. In a normal replication fork, Mcm10, by interaction with both Polα and the MCM helicase, may function in coupling the helicase activity to the polymerase activity (Fig. 4.10A). When Mcm10 is defective, association of Polα with the chromatin would be unstable. In addition, when exposed to either 37°C or HU, the helicase would be uncoupled from polymerase activity causing wide-spread ssDNA exposure and fork collapse. Checkpoint activation may fail to stabilize the fork in such conditions (Fig. 4.10B). The suppressor mutations may alter the processivity of the helicase. The handicapped

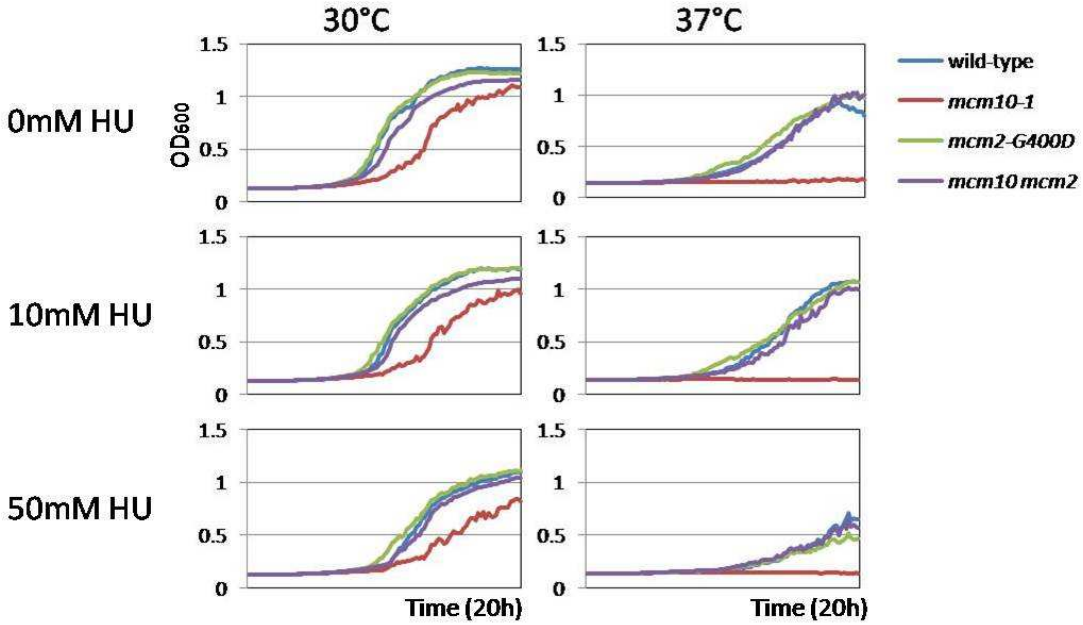

Figure 4.9 - Activation of checkpoint pathway by growth in HU does not suppress temperature sensitivity of *mcm10-1*. Growth curves at 30°C or 37°C were obtained by using the Tecan microplate reader. 150 microliter cultures were added to the 96-well plates and incubated for 20h with intermittant shaking. Each growth curves are an average of 3 independent readings.

Figure 4.10 - Model of mechanism by which *mcm2* suppresses *mcm10* temperature sensitivity. (A) Mcm10 functions in coupling helicase to polα-primase in a normal replication condition. (B) Defect in Mcm10 causes unstable polα-primase association with the chromatin and failure to coordinate the helicase activity with the polymerase activity. This leads to long stretches of ssDNA that causes fork collapse and checkpoint activation. (C) Mutation in the helicase affects the processivity and allows the polymerase to keep up without a coupling factor. The defect in coupling would result in chronic exposure of ssDNA that makes the complex prone to checkpoint activation. However, the replication fork would not collapse. The checkpoint activation, in turn, stabilizes the components of the fork and helps fork progression despite the absence of a major fork stabilizing factor, Mcm10.

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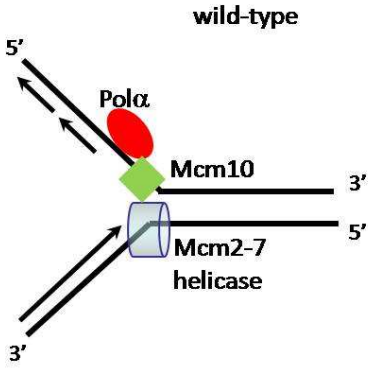

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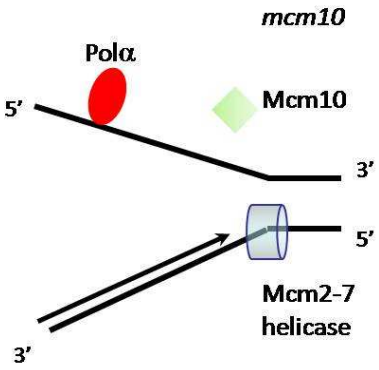
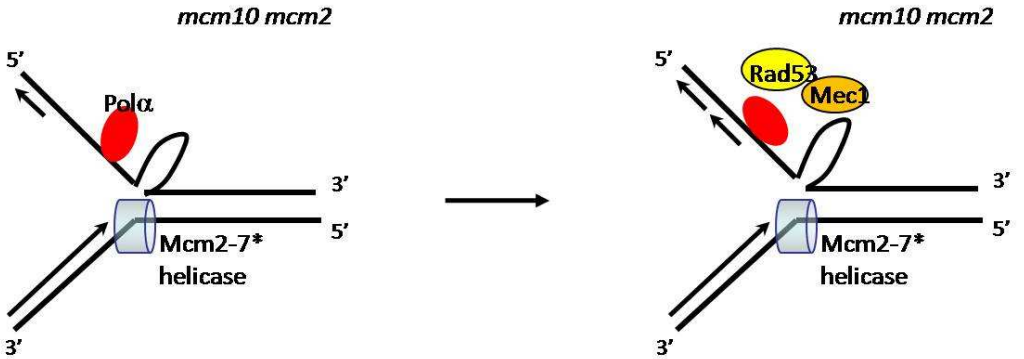


Unstable association of polα and uncoupling of helicase from polα leads to accumulation of long stretches of ssDNA.

Fork collapse due to DSBs or other damages.

C



Decreased helicase activity shortens ssDNA exposure.
Fork is "prone to checkpoint activation", but held together.

Checkpoint activation further stabilizes fork.

helicase may decrease the extent of ssDNA accumulation. As a result, the replication complex may be more prone to checkpoint activation due to chronic ssDNA exposure, but does not fall apart (Fig. 4.10C). Replication stress in the form of either HU or Mcm10 degradation may both increase the chance of helicase-polymerase uncoupling that requires the checkpoint pathway for fork stability.

With the results presented here, I propose that *mcm2* mutants suppress most of the defects of *mcm10* associated with elongation by bypassing Mcm10 requirement at the fork and at the same time, activating the checkpoint pathway. In other words, the loss of physical stabilization at the fork rendered by the Mcm10 defect can be compensated by a mechanistic stabilization that arises from a mutant helicase in coordination with the surveillance system. This hypothesis points to the dynamics of fork components in adapting to the defects of one another and the integration of different cellular pathways such as replication, repair, and checkpoints to maintain the integrity of the genome.

CHAPTER 5

PERSPECTIVES

In this thesis, I have investigated the cause of *mcm10* defect that leads to temperature sensitivity and the mechanism by which this is suppressed by mutations in the MCM helicase. Various evidence suggests that suppression by *mcm2* pertains to fixing the defects at the fork due to *mcm10* by both bypassing the requirement for Mcm10 during elongation and activating the checkpoint pathway to reinforce the stability of the fork. The defect at the fork seems to be the cause of specific DNA damage for several reasons. First, *mcm10* displays synthetic effects with genes involved in DSB and fork repair even at the permissive temperature. Also, in mammalian cells, mutation in *mcm10* causes increased phosphorylation of H2AX, which is indicative of DSB occurrence (Chattopadhyay and Bielinsky 2007). Lastly, the complete loss of suppression rendered by *mre11Δ* at 37°C suggests that repair of DSB is important in *mcm10 mcm2*.

Our model proposes that the mutation affects the helicase activity to allow stabilization and repair of the replication fork. In the absence of Mcm10 that stabilizes Polα association at the fork and links the lagging strand polymerase with the helicase, the decreased rate of the helicase may help prevent fork collapse. In addition, the loss of the coupling factor may cause persistent exposure of ssDNA that renders the fork prone to checkpoint activation, which will function to stabilize the fork and mediate repair.

Though we have evidence that the suppressor mutations do indeed decrease the helicase activity of the archaeal MCM helicase, whether they have the same effect in the eukaryotic MCM helicase is yet to be tested. So far we only have indirect evidence

in the form of the *mcm* defect, which suggests that the mutant helicases do not function as well as the wild-type. In addition to this uncertainty, the relationship between overall helicase activity and the rate of the enzyme needs to be further clarified as decrease in helicase activity is not simply an indication of a slower rate. There are other factors that can result in decrease of the helicase activity besides the rate of the helicase movement. DNA binding properties such as association or dissociation rates or processivity, that is how long it goes without dissociation, are other factors that can contribute to the overall helicase activity.

Another experiment that can be carried out to illuminate the defect at the fork is electron microscopy to directly visualize the fork structure and ssDNA accumulation. In *mcm10*, it is expected that large stretches of ssDNA is exposed at 37°C due to the instability of Polα and the failure to couple the lagging strand synthesis with the helicase activity. Whether ssDNA can be detected in *mcm10 mcm2* is uncertain as it will depend on the degree of ssDNA accumulation and the resolution of the technique.

Lastly, it will be of interest to understand the nature of fork repair that seems to occur in *mcm10 mcm2*. The requirement for Mre11 and Rad52 suggests that *mcm2* does not completely prevent DNA damages from occurring in *mcm10* deficient cells. It seems that HR is utilized for repair of DSB. As DSB at the fork are likely to create structures that resemble broken chromosomes, these may be repaired by BIR, a subpathway of HR. It is known that Mcm10 is required for BIR. Therefore, it will be of interest to know whether *mcm2* suppresses *mcm10* defect in BIR. It is suggested that the MCM helicase does not function in BIR when the length of DNA to be repaired by BIR is short (Aparicio, Weinstein et al. 1997; Labib, Tercero et al. 2000). The low processivity and higher dissociation activity had suggested that other non-replicative helicases are employed during this process (Paques, Leung et al. 1998).

However, when the gap to be repaired is large (>5kb) or when only one end of the DSB has homologous sequences such as in re-establishment of the replication fork, the MCM helicase is required (personal communication with Dr. Jim Haber). Therefore, further investigation into whether BIR plays an important role in *mcm10* suppression will be interesting. As many models are continuously emerging to explain how replication forks are stabilized and repaired, these findings will hopefully help us in understanding this vital process.

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