

Addressing the Enigma of MCM8 in DNA Replication

Dianne C. Daniel and Edward M. Johnson
Eastern Virginia Medical School
United States of America

1. Introduction

MCM8 is a relatively new protein about which little is currently known regarding its function within the cell. Even so, there is controversy surrounding what its role might be in DNA replication. In this chapter, information regarding the role of MCM8 in DNA replication gleaned from studies carried out in different species will be discussed. In addition, sequence differences in the protein, itself, among different species will be presented. This review will focus on MCM8 in three species, *Homo sapiens*, *Xenopus laevis* and *Drosophila melanogaster*, since the bulk of published experimental data was obtained using these organisms.

2. Structure of MCM8

The discovery of the MCM8 gene was the direct result of sequencing of the human genome. The MCM8 gene was discovered based on direct search for homologies to the genes of other family members (Johnson et al., 2003) and on direct database comparison of sequences in which a hepatitis virus had integrated in DNA from human hepatocellular carcinoma tissue (Gozuacik et al., 2003). MCM8 belongs to the Minichromosome Maintenance (MCM) family of proteins so named based on the role of founding members in the maintenance of centromeric plasmids containing an origin of replication in *Saccharomyces cerevisiae* (Maine et al., 1984). MCM family members other than MCM8 will be discussed in another chapter and will not be further discussed here except for comparative purposes. Two groups simultaneously named the newly discovered protein and gene MCM8, essentially because the names MCM1, MCM2-7 and MCM10 were already taken (Gozuacik et al., 2003; Johnson et al., 2003). MCM 1 and MCM10 are not homologous in sequence to MCM2-7 family members (Tye, 1999). Whereas MCM2-7 family members have a secured position within the mechanistic framework of DNA replication based on studies in both yeast and *Xenopus* (Blow & Laskey, 1988; Chong et al., 1995; Forsburg, 2004; Gomez et al., 2002; Hennessy et al., 1991; Kubota et al., 1995; Labib et al., 2000; Thommes et al., 1997; Tye, 1999; Yan et al., 1993), the role of MCM8 is enigmatic.

In *Homo sapiens*, the MCM8 gene is located at chromosome band 20p12.3-13 and is located contrapodal to a gene encoding a homolog of the yeast GCD10 gene. It is composed of 19 exons (Johnson et al., 2003). MCM8 will be italicized hereafter when specifically referring to nucleic acid. *MCM8* also exists in a variant form (isoform 2) in a choriocarcinoma found

among Expressed Sequence Tags, which could be the result of aberrant splicing (Johnson et al., 2003). Both MCM8 and MCM8 isoform 2 have canonical helicase domains, Walker A and Walker B (Walker et al., 1982). Although other family members have an unusual A-box motif, either AKS or SKS, MCM8 has the canonical A-box motif GKS, indicating the possibility of intrinsic helicase activity (Johnson et al., 2003). Sequences deleted in the MCM8 isoform 2 are near a zinc finger-like motif located N-terminal to the Walker boxes (Fig. 1). The reported *Xenopus laevis* MCM8 homolog contains similar structural motifs, including a Zn finger-type motif, highly homologous to the human one, and Walker A and Walker B motifs. It also contains the canonical Walker A GKS sequence described above for human MCM8 (see Fig. 2). *Xenopus* MCM8 is highly homologous to human MCM8 (74%), with most variability in the N terminus. This variability is in a region of 60 amino acids that are arginine- and glycine-rich in both species (Maiorano et al., 2005).

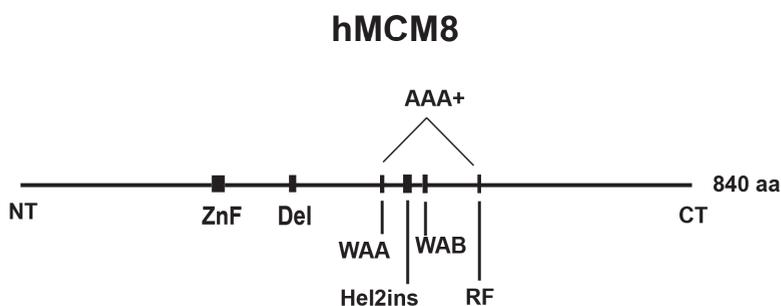


Fig. 1. Diagram showing the conserved structural features for human MCM8. Lengths and positions of the noted motifs are approximately to scale. WAA and WAB are the Walker A and Walker B boxes, respectively (Walker et al., 1982). Hel2ins is the helix-2 insert (Jenkinson & Chong, 2006) and ZnF and RF refer to the zinc finger-like motif and arginine finger motif, respectively (Forsburg, 2004; Tye, 1999). Del refers to the sixteen amino acids deleted in human MCM8 isoform 2 (Johnson et al., 2003).

The *rec* gene in *Drosophila melanogaster* was later reported to be the MCM8 ortholog, and it is located on *Drosophila* Chromosome 3 between *c(3)G* and *spn-E*. The *rec* gene consists of two exons and an intron. Note that *rec* will indicate the gene and REC will indicate the protein. It may have diverged from other MCM8 orthologs through accumulation of more changes as indicated by its longer branch length in the phylogenetic tree compared with other MCMs (Blanton et al., 2005). Based on ancient diversification, MCM8 is concordantly either present or absent along with another family member, MCM9, in various taxa. Exceptions are the *Drosophila* species, which lack MCM9. The sequence divergence of REC is suggested to relate to the lack of MCM9 in this species or to a major change in protein function (Blanton et al., 2005; Liu et al., 2009). MCM9 is not included in all compilations of MCM proteins because MCM9 lacks the signature Walker B box sequence, IDEFDKM, present in all other MCM members. Human MCM9 contains instead the somewhat conserved IDEFNLSL (Shultz et al., 2007). Compared to other MCM family members, MCM9 proteins have a longer and poorly conserved C-terminus (Lutzmann et al., 2005; Shultz et al., 2007). From an evolutionary standpoint, the highly divergent MCM8 ortholog in all species of *Drosophila* may not make it a good model for other eukaryotes (Liu et al., 2009).

MCM8 is not present in yeast where family members MCM2-7 have been extensively studied using genetic approaches (Tye, 1999). The archaeal MCM proteins and MCM2-7 proteins are AAA+ (ATPases associated with a variety of cellular activities) enzymes (Bae et al., 2009; Bochman & Schwacha, 2009, review; Koonin, 1993; Neuwald et al., 1999). The ATPase active site is known as the AAA+ domain (Neuwald et al., 1999). Subdomains of AAA+ proteins contain ATPase active site motifs found in a P loop domain and a lid domain C-terminal to the P loop. The P loop domain consists of Walker A and Walker B boxes and a Sensor I motif. The lid contains the arginine finger motif and a Sensor II motif. In MCM proteins, the AAA+ active sites are formed from the P loop *cis* motif of one subunit and the lid domain *trans* motif of another subunit. Nucleotide binding and hydrolysis can lead to conformational changes that drive mechanical work (Bochman & Schwacha, 2009; Erzberger & Berger, 2006). Conformational changes among subunits of toroidal hexamers, such as the MCM2-7 complex, may be coupled and propagated by the combinational arrangement of active sites (Davey et al., 2003; Bochman & Schwacha, 2009).

A large presensor-I (PS-I) AAA+ superclade is comprised of clade 4 viral superfamily III helicases (e.g., SV40 large T-antigen), the clade 5 HCLR (HslU, ClpX, Lon and Ruv) and the clade 6 H2 insert family in which MCM family members were initially placed (Erzberger & Berger, 2006). The clade 6 H2 insert family has a beta-alpha-beta insertion in helix 2 (Iyer et al., 2004; Jenkinson & Chong, 2006). As in MCM2-7, Fig. 2 sequence alignment shows that the helix-2 insert (Hel2ins) is also present between the Walker A and B boxes in MCM8 in *Homo sapiens*, *Xenopus laevis* and *Drosophila melanogaster*. In addition to the presence of an insertion in helix 2 of clade 6 members, an additional insertion has been reported to define Clade 7 (Erzberger & Berger, 2006). Based on structural studies, this group reported that MCM2-7 members contain a Sensor-II motif within the lid domain that is uniquely repositioned to act in *trans* through a helical insertion positioned N-terminal to it (presensor-II). They used this insert to define a presensor-II (PS-II) insertion clade 7, which includes MCM2-7. The divergent members reported for clade 7 were chosen based on the presence of the helical insertion when the H2 insert family members were examined. Whereas clade 6 H2 insert mutations disrupted interaction of clade 6 member NtrC with its remodeling target, the clade 7 helical insertion is thought to coordinate the stability of adjacent subunits or protomers, perhaps by changing contacts or accessible surface area (Erzberger & Berger, 2006). Our sequence analyses reveal that *Homo sapiens*, *Xenopus laevis* and *Drosophila melanogaster* MCM8 contain a potential helical insertion in PS-II. Further structural information about this MCM8 insert is not available, but it could potentially place MCM8 in AAA+ clade 7. Notably, this region appears much longer in *Drosophila* than in the other two species based on these analyses (not shown). This region in all three species requires further analysis, since it has been described as potentially important in subunit interactions (Erzberger & Berger, 2006) and could help account for active-site differences within MCM2-7 subunits (Bochman & Schwacha, 2009).

3. Sequence variations of MCM8 proteins among and within different species

The following figures show important sequence alignments for the three species to be discussed in this review: *Hs1* is *Homo sapiens* isoform 1, *Hs2* is *Homo sapiens* isoform 2 (Johnson et al., 2003), *Xl* is *Xenopus laevis*, and *Dm* is *Drosophila melanogaster*. All sequence alignments in the figures in Section 3 were performed using the Multalin algorithm (Corpet, 1988).

Fig. 2 is an alignment of the conserved Walker A and Walker B ATPase domains, the AAA+ clade 6 helix-2 insert that is present in MCM2-7 and the canonical MCM arginine finger

domain (SRFD). The conserved motifs are part of the AAA+ domain of many ATP-dependent molecular motors (Koonin, 1993; Neuwald et al., 1999). Note that there is significant homology in three of these four motifs among the three species. Only the helix-2 insert of *Drosophila* MCM8 is completely divergent.

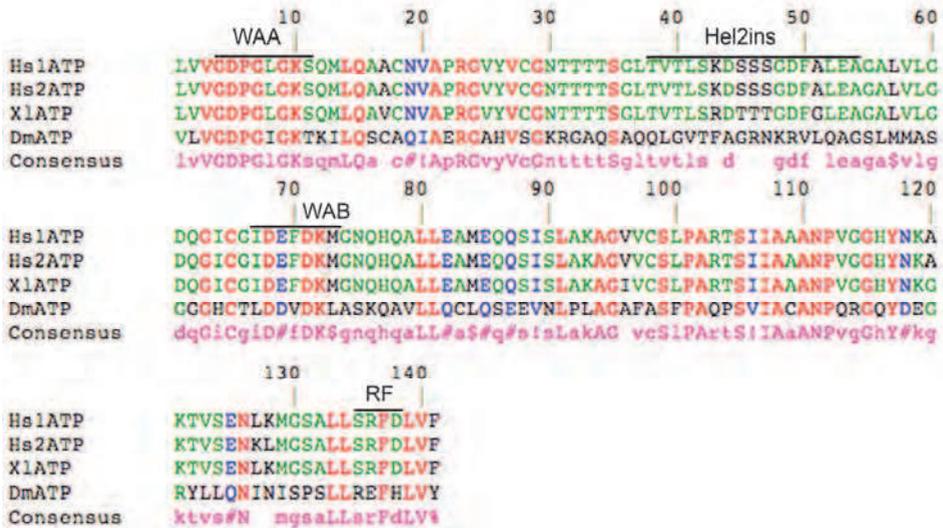


Fig. 2. The Mg-ATP binding domain of MCM8 from three species. Comparative alignment of MCM8 conserved motifs in *Hs* (forms 1 and 2), *Xl* and *Dm*. Abbreviations: WAA (Walker box A), WAB (Walker box B), RF (arginine finger), Hel2ins (helix-2 insert).

The Walker box A is important for P loop conformation and is implicated in ATP binding by interacting with the phosphate moiety (Saraste et al., 1990; Walker et al., 1982). The Walker B motif is thought to contribute to ATP hydrolysis by mediating interaction with ATP through Mg^{2+} . (Koonin, 1993; Walker et al., 1982). The arginine finger is the SRFD amino acid motif found in other MCM2-7 family members (Forsburg, 2004). At the AAA+ active site, the arginine finger is in a different subunit from the one that binds ATP and helps to complete the active site interface between two subunits. It may help coordinate the order of hydrolysis in the MCM2-7 oligomer (Bae et al., 2009; Davey et al., 2003; Davey et al., 2002). The helix-2 insert is implicated in helicase activity (Brewster et al., 2008; Jenkinson & Chong, 2006). The helix-2 insert domain plays a key role in transducing or coupling energy of hydrolysis to unwinding of target. Removal of the helix-2 insert in archaeon *Methanothermobacter thermautotrophicus* (*Mth*MCM) resulted in the loss of DNA unwinding and increased dsDNA-dependent ATP hydrolysis and the affinity for single-stranded and double-stranded DNA. Since this motif is not as conserved as other neighboring AAA+ motifs, its role in MCM activity was predicted to be mechanical (Jenkinson & Chong, 2006). In the Fig. 3, the zinc finger-like motif, a common feature of other MCM family members (Forsburg, 2004; Tye, 1999) is presented for the three species under discussion. Note that this domain in *Drosophila melanogaster* is almost completely divergent from the domain in *Homo sapiens* isoform 1, *Homo sapiens* isoform 2 and *Xenopus laevis*. Only a P residue and the Zn-chelating four C residues are conserved in *Drosophila* MCM8.

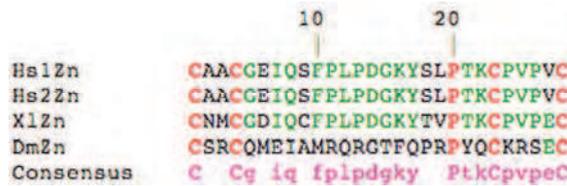


Fig. 3. Homologies among zinc finger domains in three species.

The Zn^{2+} finger motif is not involved directly in DNA binding. It stabilizes folding of the N-terminal domain, and this function is predicted to aid in the formation of the double hexamer in the *Mth*MCM archaeon (Fletcher et al., 2003). For discussion of this motif in eukaryotic MCMs, see the review by Bochman and Schwacha, 2009.

In Fig. 4, the region of *Homo sapiens* isoform 1 MCM8 found to be deleted in choriocarcinoma is aligned to compare the two *Homo sapiens* isoforms with *Xenopus laevis* and *Drosophila melanogaster*. Note that *Xl* and *Hs1* are highly homologous in the region of the deletion. The deletion occurs in the *Hs2* variant, which has thus far only been reported in a case of choriocarcinoma (Johnson et al., 2003). A similar region is missing in *Dm* MCM8. This deletion most likely does not eliminate DNA binding because *Dm* MCM8 is reportedly involved in DNA repair synthesis in meiosis (Matsubayashi & Yamamoto, 2003; Blanton et al., 2005).

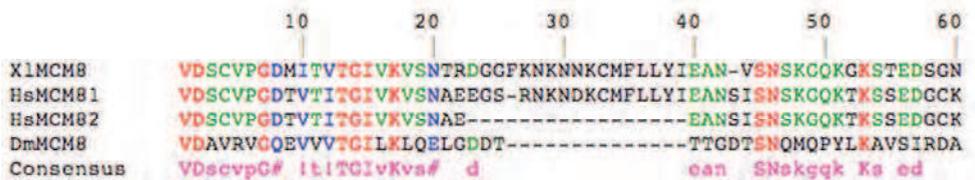


Fig. 4. A deletion in *Hs* MCM8 isoform 2 is also present in *Dm* MCM8.

Based on archaeon *Mth*MCM mutational analysis, N- and C-terminal sequences were found to play a regulatory role in ATP hydrolysis with effects on substrate binding and on processivity (Jenkinson & Chong, 2006). Whether this particular region of deletion in *Hs* isoform 2 has similar regulatory effects remains to be determined.

4. Research regarding MCM8 protein function in DNA replication in *Homo sapiens* and *Xenopus laevis*

A report on MCM8 from human studies resulted from the integration of hepatitis B virus at a site that was subsequently identified as being the sequence coding for MCM8 (Gozaucik et al., 2003). The group developed a rabbit polyclonal antibody against the N-terminus of the protein and cloned the MCM8 gene using RT-PCR. Further investigation was carried out using cultured cells for *in vitro* studies. Following the release of density arrested Hs27 newborn foreskin fibroblasts, MCM8 mRNA accumulated from G1 through S phase. The MCM8 protein was detectable in these same cells throughout the cell cycle. Although no nuclear localization sequence could be found, MCM8 was detected in the nuclei of Hs27 and HeLa cells. Both nucleosolic and structure-bound MCM8 were observed. Detergent-permeabilized nuclei released nucleosolic MCM8 into the lysate, whereas the remaining structure-bound MCM8 portion in the pellet could be released by increasing salt

concentrations. Unlike the MCM3 control, MCM8 was structure bound in S, but not G1, based on HeLa cell synchronization at G2/M, late G1 and early S phases using nocodazole, mimosine and aphidicolin, respectively. Using these same cells, this group did not pull down MCM8 during immunoprecipitation procedure using antibodies against MCM3 or MCM4. MCM8 was expressed in Hs27, HeLa, HEK-293 and HuH7 cell lines, and was more highly expressed in the hepatocellular carcinoma-derived HuH7 cells than in normal (nonproliferating) liver tissue. The authors proposed a specific role for MCM8 in DNA replication based on its being structure bound in S phase and their lack of detection of MCM8 with MCM3 and MCM4, members of the G1 pre-replication complex (pre-RC).

Another group reported on a new human *MCM8* gene following a comparison of *MCM* family sequences against sequence tags expressed mRNAs (Johnson et al., 2003). By arranging these expressed cDNA segments contiguously, they identified an open reading frame (ORF) that was not identical to any known *MCM*. DNA sequencing of IMAGE cDNA clones led to confirmation of the ORF. They also searched the HTGS database of human genomic sequences to locate a BAC clone containing a unique gene encoding the new ORF with chromosomal location on 20p12.3-13. *MCM8*, comprised of 19 exons, was found to be located contrapodal to another gene comprised of 11 exons that encodes a homolog of yeast gene product GCD10. The sequences between the two transcription units were found to be TATA-less and highly GC-rich with multiple CpG units and to contain E2F, Sp1 and Pur binding elements. Notably, MCM8 was reported to have a canonical Walker A helicase domain as distinct from MCM2-7. This group also identified an MCM8 variant in a choriocarcinoma that is devoid of sixteen amino acids that are located N-terminal to the conserved helicase domain. They prepared a monoclonal antibody that specifically detects MCM8. Using this antibody, a fraction of MCM8 was found by this group to coisolate through several steps with MCM6 and MCM7 from HeLa cells. There was also an MCM8 gradient fraction not coincident with other MCMs that could be free MCM8. In addition, antibodies against MCM4, MCM6, and MCM7 coimmunoprecipitated MCM8 from HeLa cells as detected with anti-MCM8 antibody using immunoblotting. Using RT-PCR with commercially prepared cDNA, this group reported expression of *MCM8* mRNA in placenta, lung, liver, pancreas and heart. The same procedure using matched normal and tumor cDNAs involving cases of colon adenocarcinoma showed that *MCM8* mRNA expression was reduced relative to noncancerous tissue from the same patient. Results of their studies led these authors to propose a role for MCM8 in DNA replication or repair processes. They also proposed that MCM8 might substitute for another MCM at certain times in development or during the cell cycle. Due to a unique helicase motif among MCM proteins, they proposed that such a substitution might add a regulatory dimension to the function of the MCM complex.

Investigators performing studies in human HeLa and 293T cells reported that MCM8 has an important function during G1 in pre-RC assembly (Volkening & Hoffmann, 2005). This group prepared N- and C-terminal rabbit polyclonal antibodies against MCM8. Based on biochemical fractionation of HeLa cells following cell synchronization with nocodazole, MCM8 was found to accumulate on chromatin in G1 prior to binding of the MCM2-7 complex. MCM8 was found to be chromatin-bound throughout the cell cycle paralleling the binding of Cdc6 and Orc2. Following transfection of 293T cells with expression constructs for Ha-MCM8, HA-Cdc6 and HA-ORC2 (hemagglutinin-derived tag), MCM8 was found to interact with proteins Cdc6 and Orc2, both of which are components of the pre-RC. HA-MCM8 was not, however, found to interact with MCM2 or MCM6, also components of the pre-RC. Interactions were verified for these endogenous proteins in HeLa extracts

using anti-MCM8 antibody for immunoprecipitation followed by Western blots with antibody against Cdc6 or Orc2. Small hairpin RNAs were used to down-regulate endogenous MCM8 in HeLa cells, and this down regulation led to a delay in the entry of these cells into S phase as verified by flow cytometry. The authors proposed that this delay has to do with a role for MCM8 in G1 progression. The down-regulation of MCM8 was found to lead to a reduction of Cdc6 and MCM2-7 complex loaded onto chromatin. All these findings led these authors to report that interaction of MCM8 and Cdc6 is required for assembly of the pre-RC.

Using the *Xenopus* model, a group reported that MCM8 is required for efficient replication of chromosomal DNA in the *Xenopus* cell free replication assay (Maiorano et al., 2005). This group identified the *Xenopus* MCM8 homolog and studied its function using *Xenopus* egg extracts and demembranated sperm nuclei. They prepared a rabbit polyclonal antibody against a *Xenopus* MCM8 N-terminal peptide. Immunoprecipitation procedure using an anti-MCM3 antibody was performed and followed by Western detection using an anti-MCM8 antibody. MCM8 was not found to associate with soluble MCM3 or to complex with other components of the MCM2-7 complex present in S phase egg cytosol. The investigators isolated detergent-resistant chromatin fractions over a time course from S phase egg extracts to which demembranated sperm nuclei had been added. MCM8 was found to bind this chromatin at initiation of DNA replication after chromatin binding by Cdt1 and MCM2. In addition, they also isolated chromatin from membrane-depleted egg extracts, competent only for formation of the pre-RC, but not for initiation of DNA replication. MCM8 did not bind this chromatin. This group used immunofluorescence microscopy and Western blotting of chromatin fractions to determine the effect on MCM8 of adding aphidicolin at the time of initiation of DNA replication (which is not inhibited by aphidicolin) or during elongation (which is inhibited due to inhibition of DNA polymerase alpha). Although MCM3 was present in the labeled nuclei after both aphidicolin treatments, MCM8 was only present during elongation. Although MCM3 was present when S-CDK inhibitor, p21, was added at initiation (where p21 proteins blocks initiation of DNA synthesis but not formation of the pre-RC) and at the elongation time points, MCM8 was only present during elongation. Based on these experiments, investigators proposed that MCM8 binds chromatin after the pre-RC licensing step and at initiation of DNA synthesis. Immunodepleting MCM8 did not affect the chromatin loading of MCM3, a member of the pre-RC. The authors interpreted these data to mean that MCM8 is not required for replication licensing. This group also showed that recombinant MCM8 prepared from a baculovirus expression system possessed *in vitro* DNA helicase and ATPase activities. The helicase activity was lost by creating a mutation in the ATP binding site. Knockdown of MCM8 resulted in a 40% reduction in DNA synthesis compared to controls and in a slow replication phenotype. Nuclear assembly, however, was not affected. After depletion of MCM8, replication products were found to be short DNA chains similar to those produced in the presence of low concentrations of aphidicolin, which slows down replication. They found that depletion of MCM8 reduced chromatin bound Replication Protein A (RPA) 34 subunit and DNA polymerase alpha. In extracts depleted of MCM8, DNA synthesis could be rescued by reconstitution with ATP bound MCM8. Using immunofluorescence microscopy, MCM8 was found to colocalize with the RPA34 subunit and with replication foci. The authors proposed that MCM8 is a helicase that facilitates RPA recruitment as well as the processivity of DNA polymerases. The investigators proposed a function for MCM8 in the elongation step of DNA replication in regulating

fork movement that is distinct from the exclusive role of MCM8 in pre-initiation studies in HeLa and 293T cells noted by Volkening & Hoffmann, 2005.

These apparently contradictory roles for MCM8 in initiation versus elongation may be at least partially resolved by more recent findings. In human studies using HeLa cells, MCM8 was reported to colocalize with certain proteins involved in different aspects of DNA replication (Kinoshita et al., 2008). Using HeLa cells synchronized by a double-thymidine block, this group performed immunoprecipitation procedure with an anti-MCM7 antibody followed by Western detection using an anti-MCM8 monoclonal antibody, which they had previously prepared and characterized to be MCM8-specific. The resulting data revealed that the association between MCM8 and MCM7 peaked in mid G1, at the time of assembly of the pre-replication complex. Double chromatin immunoprecipitation (ChIP), developed by this group to determine the presence of two proteins on a specific segment of DNA at an origin of replication, was then used to show association of MCM8 with proteins involved in DNA replication. Using HeLa cells synchronized by double thymidine block, cell cycle studies were combined with double ChIP procedure. Genomic sequences upstream of the *c-MYC* gene were targeted through double ChIP using an antibody against Cdc6 for the first ChIP and followed by an anti-MCM8 antibody for the second ChIP. This procedure localized MCM8 with Cdc6, a protein reported to be involved in the subsequent loading of MCM proteins into the pre-RC (Kinoshita & Johnson, 2004; Kneissl et al., 2003; Lei & Tye, 2001). This localization was on specific DNA segments flanking the approximate center of the *c-MYC* replication initiation zone (Vassilev & Johnson, 1990), and occurred during both G1 and S phases, but not continuously. The investigators found a role for MCM8 in elongation was likely to be discontinuous from any role in initiation due to an MCM8 off signal in regard to its interaction with Cdc6 at the G1/S border and an MCM8 on again signal at the beginning of S phase. They also used double ChIP to show that MCM8 is present simultaneously at the *c-MYC* initiation zone with chromatin-bound Cdk2, a G1-S phase kinase essential for G1 to S phase transition. Immunogold electron microscopy was performed using mid-S phase HeLa cells, and MCM8 was strongly localized to heterochromatin, which replicates during this time. In these same samples, MCM8 was also shown to be in close localization with RPA70, a protein involved in elongation, not initiation. Based on these findings, the authors proposed distinct roles for MCM8 in DNA replication during G1 and S phases of the cell cycle. In addition, they suggest that MCM8 may also participate in processes distinct from replication initiation and elongation.

5. MCM8 protein function in DNA synthesis in *Drosophila*

Following the first two reports identifying human MCM8, the *Drosophila rec* gene was identified as a new member of the MCM family and was reported to be required for meiotic recombination in this species (Matsubayashi & Yamamoto, 2003). This group recognized the MCM domain and the putative Zn-finger motif through comparison with MCM2-7. They reported that *rec* mutations result in a very low level of meiotic recombination with primary non-disjunction at high frequency. These defects could be reversed in transformants carrying a wild type transgene. They identified molecular lesions consistent with induced mutant phenotypes. The investigators used DNA damage via methylmethane sulfonate or X-rays to determine that in somatic cells, the *rec* gene plays a limited or no role in DNA recombination and repair. They report that a role in pre-meiotic DNA replication is unlikely because electron microscopy revealed normal

synaptonemal complexes in *rec* mutants. In addition, there was normal oogenesis and oocyte development except for the lack of recombination. The authors suggested a role for *Dm* MCM8 in meiotic recombination.

Studies conducted in the *Drosophila* model by a second group revealed that the MCM8 orthologue REC is required for sufficient DNA synthesis to permit formation of a necessary meiotic crossover intermediate to drive this process (Blanton et al., 2005). This group found that in the absence of MCM8, recombination occurred through synthesis-dependent strand annealing to generate noncrossover products only. They reported that in meiotic recombination, REC functions at an intermediate step, but is not essential for pre-meiotic S phase. They found evidence for this in genomic DNA, where formation of normal synaptonemal complexes between homologous chromosomes in female *rec* mutants provided the supporting data. *Rec* mutant females displayed twice the rate of noncrossover gene conversions, but there was little crossover repair of double strand breaks. There was an increase in noncrossover events, but crossovers did not follow the normal distribution. There was a significant reduction in tract length in these mutants, which the authors suggest is due to diminished DNA repair synthesis. A role for *rec* in nonmeiotic cells was not reported. These authors proposed a model in which REC functions in meiotic crossover at the repair synthesis step.

Another group using *Drosophila* S2 cells reported a role for REC in DNA replication (Crevel et al., 2007). This group used dsRNA interference to deplete MCM2-8 family members. The depletion of MCM8 resulted in a reduction in fork number by 30%. They did not observe a significant effect on cell cycle or viability when analyzing flow cytometry profiles. They co-depleted MCM8 and MCM5, but did not observe a synergistic effect on cell cycle distribution. Based on an immunoblot, depletion of MCM8 did not have an effect on Orc5, MCM2, MCM5 or Cdc45 loading in chromatin. In a similar experiment, the investigators found a decrease in the amount of chromatin-bound PCNA detected, which they reported as evidence for a role for MCM8 in DNA replication in *Drosophila* S2 cells.

6. Role of MCM8 during the cell cycle

Reports differ as to where in the cell cycle MCM8 exerts its activity. Two different groups find that human MCM8 associates with Cdc6 in cultured cells, using two different approaches and three unique antibodies (Kinoshita et al., 2008; Volkening & Hoffmann, 2005). The two procedures included immunoprecipitation with anti-HA for exogenously expressed proteins or with anti-MCM8 polyclonal antibody for pull down of endogenous MCM8 (Volkening & Hoffmann, 2005) and by double ChIP of cell cycle fractions using monoclonal antibodies against Cdc6 and against MCM8 (Kinoshita et al., 2008). Cdc6, along with Cdt1, is involved in the subsequent loading of MCM2-7 onto the ORC complex loading deck during formation of the pre-RC (Forsburg, 2004, review). MCM8 was found to bind to chromatin throughout the cell cycle paralleling the binding of Cdc6 (Volkening & Hoffmann, 2005). Although MCM8 was found to interact with Orc2 based on the same immunoprecipitation experiments, Orc2 binding to chromatin was not affected by MCM8 silencing. Down regulation of MCM8 led to a concurrent decrease in Cdc6 on chromatin and delay of entry into S phase based on flow cytometry profiles leading the authors to suggest that MCM8 is required to load Cdc6 to chromatin (Volkening & Hoffmann, 2005). Cell cycle analysis of chromatin binding of Cdc6 during MCM8 down regulation is, however, not available. These additional data are needed to determine whether Cdc6 chromatin binding

was inhibited in G1 or S or both. In addition, MCM8 was found to be concurrently present with Cdc6 at sequences in the *c-MYC* replication initiation zone in HeLa cells when double chromatin immunoprecipitation (ChIP) with anti-Cdc6 antibody was followed by ChIP using anti-MCM8 antibody. Cell cycle studies using double ChIP revealed that these proteins are both present at the *c-MYC* initiation zone sequence in G1 as well as in S phase, but not continuously due to an *on to off* signal at the G1/S border followed by an *off to on* signal (Kinoshita et al., 2008). Using the same double ChIP procedure, MCM8 was found to be present with Cdk2 on specific sequences at the initiation zone of the *c-MYC* gene. Cdk2 is required for transition into S phase, and this association of MCM8 with Cdk2 supports a role for MCM8 in the transition. During this same time range (G1/S), there was little to no association of MCM8 with Cdc6 on these same sequences. This transition period requires further study.

MCM8 was also found to colocalize with RPA subunits during S phase by two groups (Kinoshita et al., 2008; Maiorano et al., 2005). RPA binds to single-stranded DNA and therefore serves as a marker for unwound strands of replicating DNA. MCM8 was found to colocalize with the RPA34 subunit during S phase in the *Xenopus* replication model (Maiorano et al., 2005). In HeLa mid-S phase cells, dual immunogold electron microscopy studies showed that MCM8 colocalized with RPA70, RPA large subunit (Kinoshita et al., 2008). When MCM7 and MCM8 were compared in this study, there was a difference in the amount of MCM7 and MCM8 that colocalized with RPA70 where colocalization is defined as two beads within 10 nm distance of each other. MCM7 was most often present within 10 nm distance of RPA70. Whereas MCM8 was sometimes within 10 nm of RPA70, it was most often present within 100 nm distance of RPA70. Thus, both in the *Xenopus* replication assay and in HeLa cells, MCM8 colocalizes with or near a protein involved in DNA replication during S phase. Although MCM2-7 proteins have not generally been shown to do so, MCM8 was shown to colocalize with replication foci in *Xenopus* nuclei labeled with bromodeoxyuridine (Maiorano et al., 2005).

The major disagreement among studies regards whether MCM8 interacts with the other MCM2-7 family members. This has been addressed in the *Xenopus* model by Maiorano et al., 2005, who investigated whether MCM8 is present along with other members of the MCM complex when the pre-RC forms in demembranated sperm nuclei in egg extracts or when either initiation or elongation stages of replication are specifically inhibited in these nuclei. No association of MCM8 with MCM2 or MCM3 could be found in these studies. The data were in agreement with that of Gozuacik et al., 2003, where chromatin-bound MCM8 could not be detected in pull down lysates from the pellet 1 fraction containing the MCM2-7 complex using anti-MCM3 and anti-MCM4 antibodies. This same group specifically synchronized HeLa cells at G2/M, late G1 and early S phases using nocodazole, mimosine and aphidicolin, respectively. Following this specific synchronization, they did not detect MCM8 in the late G1 pellet fraction remaining after removal of the Triton X-100-extractable supernatant when using anti-MCM8 polyclonal antibody. Volkening et al., 2005, using HA-tagged MCM8, Cdc6 and Orc2, carried out transfection studies to analyze MCM8 association with Cdc6 and Orc2. Although association of MCM8 with Cdc6 and Orc2 was found, association of MCM8 with MCM2 and MCM6 was not found even though these proteins are also members of the pre-RC. They found that MCM8 loaded onto chromatin in G1 prior to the other MCMs in a profile similar to Cdc6 and Orc2. Using double thymidine block of HeLa cells and immunoprecipitation with anti-MCM7 antibody followed by Western detection using anti-MCM8 monoclonal antibody,

Kinoshita et al., 2008, found an association of MCM8 with MCM7 during both G1 and S phases of the cell cycle.

Perhaps differences in experimental protocols or synchronization procedures may have led to different end results when investigating the association of MCM8 with members of the MCM2-7 complex. Three of the studies were completed in human cells (Gozuacik et al., 2003, Kinoshita, 2008 #4; Volkening & Hoffmann, 2005). The third study was done using the *Xenopus* replication model (Maiorano et al., 2005). There is the possibility of species-specific differences or of questions regarding the ability of one model to recapitulate the same process in the other. There may be weak binding that is lost under certain conditions. More experiments where proteins are cross-linked to DNA, such as in the CHIP procedure, may be informative. It is possible that antibodies raised against different epitopes may not yield the same result due to differential accessibility of the epitopes when the MCM8 protein is folded or part of an oligomer or when it is part of a multi-protein complex. More work is needed to resolve the differences, which may be due in part to the multi-dimensional and dynamic aspects of this complex machinery.

7. Summary of MCM8 and interacting partners involved in DNA replication

Evidence described in Section 6 indicates a role for MCM8 in DNA replication during G1 and S phases of the cell cycle. This evidence is based on the apparent interaction of MCM8 with proteins forming the pre-RC during G1 or with proteins involved in elongation during S phase. Whereas all six MCM proteins of the MCM2-7 complex are essential for DNA replication fork progression (Labib et al., 2000), the exact role of these proteins during the cell cycle is not known. The role of MCM8 is also unknown as is its active assembly and configuration. In contrast to members of the MCM2-7 complex, MCM8 has its own intrinsic helicase activity based on studies using the *Xenopus* replication assay (Maiorano et al., 2005). Whether MCM8 could replace one or more members of the MCM2-7 complex at some time during development or during the cell cycle is unknown. The canonical Walker A helicase motif of MCM8, unique among MCMs, could indicate a regulatory role if replacing another family member (Johnson et al., 2003).

There is strong evidence for an interaction between MCM8 and Cdc6. Two independent groups confirmed the interaction of MCM8 with Cdc6 in human cells (Johnson et al., 2003; Volkening & Hoffmann, 2005). There are questions regarding whether the interaction occurs only during G1 phase prior to loading of other MCMs onto chromatin (Volkening & Hoffmann, 2005) or, if indeed, MCM8 is assembled onto chromatin during G1 (Maiorano et al., 2005). An interruption in MCM8 interaction with Cdc6 at the G1/S border could indicate that MCM8 may be involved in a switch mechanism or perform a different function during G1 than in S phase (Kinoshita et al., 2008). There is also strong evidence for the interaction of MCM8 with RPA during S phase based on immunofluorescence microscopy (Maiorano et al., 2005) and immunogold electron microscopy (Kinoshita et al., 2008). There is evidence that much of MCM8 acts with MCM6 and MCM7, while a significant fraction of MCM8 is independent (Johnson et al., 2003). Which MCM partners MCM8 might act with functionally is presently not known.

Studies in *Drosophila* point to a role in meiotic recombination (Matsubayashi & Yamamoto, 2003) and at the repair synthesis step in meiosis crossover (Blanton et al., 2005). Future studies should address whether there is a similar role for MCM8 in other species or whether this is a specific function of *Drosophila* MCM8 brought about by sequence divergence. In

Drosophila S2 cells, MCM8 was found to have a role in replication (Crevel et al., 2007), but direct interactions with proteins involved in DNA replication were not studied.

Based on transfection studies carried out in human HCT116 cells along with semiquantitative RT-PCR, MCM8 mRNA was shown to be upregulated by exogenous E2F1 (Hayashi et al., 2006). This group also used chromatin immunoprecipitation to demonstrate that E2F1 and NF-Y each directly associate with the human MCM8 promoter in HCT116 cells. The investigators carried out transfection of HeLa cells using MCM8 promoter-luciferase constructs and an expression vector for E2F1 in a dual luciferase reporter assay to demonstrate that transcriptional activation required an E2F-binding motif near the site for transcription initiation. Activation of the human MCM8 promoter was achieved by E2F1-4 transcription factors in these assays.

Fig. 5 presents MCM8 interacting partners detected by various experimental procedures based predominately on cell cycle studies as described in this review. MCM8 interaction with Cdk2 is also included since Cdk2 is a G1- S phase kinase that is essential for transition into S phase.

MCM8 Interacting Partners		
G1	G1/S	S
Orc2	MCM8 switch?*	Cdc6
Cdc6	G1 on to off	RPA34
	S off to on	RPA70
		Cdk2

*E2F interaction with MCM8 promoter

Fig. 5. Proteins reported to interact with MCM8 during the cell cycle. References and experimental approaches used: *Orc2*, Volkening and Hoffmann, 2005, immunoprecipitation (IP); *Cdc6*, Volkening and Hoffmann, 2005, IP; *Cdc6*, Kinoshita et al., 2008, double chromatin immunoprecipitation, (ChIP); *RPA34*, Maiorano et al., 2005, immunofluorescence microscopy; *RPA70*, Kinoshita et al., 2008, immunogold electron microscopy; *Cdk2*, Kinoshita et al., 2008, double ChIP; *Cdc6/MCM8 switch G1 on to off, S off to on*, Kinoshita et al., 2008, double ChIP; *MCM8 promoter (E2F, NF-Y)*, Hayashi et al., 2006, ChIP.

Determination of whether MCM8 acts as an AAA+ molecular motor in the three model species discussed will provide some insight into the enigma of MCM8 function during the cell cycle and in DNA replication. The evolutionary advantage for adding a new member to the MCM family and what sets it apart from the MCM2-7 complex are important questions to answer as researchers investigate the MCM8 active three-dimensional configuration and interactive targets in *Hs*, *Xl* and *Dm*.

8. Acknowledgment

This project was supported by grant funding from Virginia's Commonwealth Health Research Board to DCD and by NIH (NINDS and NCI) funding to EMJ.

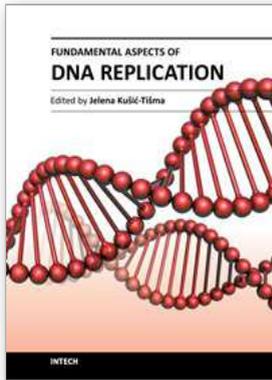
9. References

- Bae, B., Chen, Y.H., Costa, A., Onesti, S., Brunzelle, J.S., Lin, Y., Cann, I.K., & Nair, S.K. (2009). Insights into the architecture of the replicative helicase from the structure of an archaeal MCM homolog. *Structure* 17, 211-222, 0969-2126 (Print) 0969-2126 (Linking).
- Blanton, H.L., Radford, S.J., McMahan, S., Kearney, H.M., Ibrahim, J.G., & Sekelsky, J. (2005). REC, Drosophila MCM8, drives formation of meiotic crossovers. *PLoS Genet* 1, e40, 1553-7404 (Electronic).
- Blow, J.J. & Laskey, R.A. (1988). A role for the nuclear envelope in controlling DNA replication within the cell cycle. *Nature* 332, 546-548, 0028-0836 (Print) 0028-0836 (Linking).
- Bochman, M.L. & Schwacha, A. (2009). The Mcm complex: unwinding the mechanism of a replicative helicase. *Microbiol Mol Biol Rev* 73, 652-683, 1098-5557 (Electronic) 1092-2172 (Linking).
- Brewster, A.S., Wang, G., Yu, X., Greenleaf, W.B., Carazo, J.M., Tjajadia, M., Klein, M.G., & Chen, X.S. (2008). Crystal structure of a near-full-length archaeal MCM: functional insights for an AAA+ hexameric helicase. *Proc Natl Acad Sci U S A* 105, 20191-20196, 1091-6490 (Electronic) 0027-8424 (Linking).
- Chong, J.P., Mahbubani, H.M., Khoo, C.Y., & Blow, J.J. (1995). Purification of an MCM-containing complex as a component of the DNA replication licensing system. *Nature* 375, 418-421, 0028-0836 (Print) 0028-0836 (Linking).
- Corpet, F. (1988). Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res* 16, 10881-10890, 0305-1048 (Print) 0305-1048 (Linking).
- Crevel, G., Hashimoto, R., Vass, S., Sherkow, J., Yamaguchi, M., Heck, M.M., & Cotterill, S. (2007). Differential requirements for MCM proteins in DNA replication in Drosophila S2 cells. *PLoS One* 2, e833, 1932-6203 (Electronic) 1932-6203 (Linking).
- Davey, M.J., Indiani, C., & O'Donnell, M. (2003). Reconstitution of the Mcm2-7p heterohexameric subunit arrangement, and ATP site architecture. *J Biol Chem* 278, 4491-4499, 0021-9258 (Print) 0021-9258 (Linking).
- Davey, M.J., Jeruzalmi, D., Kuriyan, J., & O'Donnell, M. (2002). Motors and switches: AAA+ machines within the replisome. *Nat Rev Mol Cell Biol* 3, 826-835, 1471-0072 (Print) 1471-0072 (Linking).
- Erzberger, J.P. & Berger, J.M. (2006). Evolutionary relationships and structural mechanisms of AAA+ proteins. *Annu Rev Biophys Biomol Struct* 35, 93-114, 1056-8700 (Print) 1056-8700 (Linking).
- Fletcher, R.J., Bishop, B.E., Leon, R.P., Sclafani, R.A., Ogata, C.M., & Chen, X.S. (2003). The structure and function of MCM from archaeal M. Thermoautotrophicum. *Nat Struct Biol* 10, 160-167, 1072-8368 (Print) 1072-8368 (Linking).
- Forsburg, S.L. (2004). Eukaryotic MCM proteins: beyond replication initiation. *Microbiol Mol Biol Rev* 68, 109-131, 1092-2172 (Print) 1092-2172 (Linking).
- Gomez, E.B., Catlett, M.G., & Forsburg, S.L. (2002). Different phenotypes in vivo are associated with ATPase motif mutations in Schizosaccharomyces pombe minichromosome maintenance proteins. *Genetics* 160, 1305-1318, 0016-6731 (Print) 0016-6731 (Linking).

- Gozuacik, D., Chami, M., Lagorce, D., Faivre, J., Murakami, Y., Poch, O., Biermann, E., Knippers, R., Brechot, C., & Paterlini-Brechot, P. (2003). Identification and functional characterization of a new member of the human Mcm protein family: hMcm8. *Nucleic Acids Res* 31, 570-579, 1362-4962 (Electronic) 0305-1048 (Linking).
- Hayashi, R., Goto, Y., Haga, A., Kobayashi, D., Ikeda, R., & Yoshida, K. (2006). Comparative genomics on MCM8 orthologous genes reveals the transcriptional regulation by transcription factor E2F. *Gene* 367, 126-134, 0378-1119 (Print) 0378-1119 (Linking).
- Hennessy, K.M., Lee, A., Chen, E., & Botstein, D. (1991). A group of interacting yeast DNA replication genes. *Genes Dev* 5, 958-969, 0890-9369 (Print) 0890-9369 (Linking).
- Iyer, L.M., Leipe, D.D., Koonin, E.V., & Aravind, L. (2004). Evolutionary history and higher order classification of AAA+ ATPases. *J Struct Biol* 146, 11-31, 1047-8477 (Print) 1047-8477 (Linking).
- Jenkinson, E.R. & Chong, J.P. (2006). Minichromosome maintenance helicase activity is controlled by N- and C-terminal motifs and requires the ATPase domain helix-2 insert. *Proc Natl Acad Sci U S A* 103, 7613-7618, 0027-8424 (Print) 0027-8424 (Linking).
- Johnson, E.M., Kinoshita, Y., & Daniel, D.C. (2003). A new member of the MCM protein family encoded by the human MCM8 gene, located contrapodal to GCD10 at chromosome band 20p12.3-13. *Nucleic Acids Res* 31, 2915-2925, 1362-4962 (Electronic) 0305-1048 (Linking).
- Kinoshita, Y. & Johnson, E.M. (2004). Site-specific loading of an MCM protein complex in a DNA replication initiation zone upstream of the c-MYC gene in the HeLa cell cycle. *J Biol Chem* 279, 35879-35889, 0021-9258 (Print) 0021-9258 (Linking).
- Kinoshita, Y., Johnson, E.M., Gordon, R.E., Negri-Bell, H., Evans, M.T., Coolbaugh, J., Rosario-Peralta, Y., Samet, J., Slusser, E., Birkenbach, M.P., & Daniel, D.C. (2008). Colocalization of MCM8 and MCM7 with proteins involved in distinct aspects of DNA replication. *Microsc Res Tech* 71, 288-297, 1059-910X (Print) 1059-910X (Linking).
- Kneissl, M., Putter, V., Szalay, A.A., & Grummt, F. (2003). Interaction and assembly of murine pre-replicative complex proteins in yeast and mouse cells. *J Mol Biol* 327, 111-128, 0022-2836 (Print) 0022-2836 (Linking).
- Koonin, E.V. (1993). A common set of conserved motifs in a vast variety of putative nucleic acid-dependent ATPases including MCM proteins involved in the initiation of eukaryotic DNA replication. *Nucleic Acids Res* 21, 2541-2547, 0305-1048 (Print) 0305-1048 (Linking).
- Kubota, Y., Mimura, S., Nishimoto, S., Takisawa, H., & Nojima, H. (1995). Identification of the yeast MCM3-related protein as a component of *Xenopus* DNA replication licensing factor. *Cell* 81, 601-609, 0092-8674 (Print) 0092-8674 (Linking).
- Labib, K., Tercero, J.A., & Diffley, J.F. (2000). Uninterrupted MCM2-7 function required for DNA replication fork progression. *Science* 288, 1643-1647.

- Lei, M. & Tye, B.K. (2001). Initiating DNA synthesis: from recruiting to activating the MCM complex. *J Cell Sci* 114, 1447-1454, 0021-9533 (Print) 0021-9533 (Linking).
- Liu, Y., Richards, T.A., & Aves, S.J. (2009). Ancient diversification of eukaryotic MCM DNA replication proteins. *BMC Evol Biol* 9, 60, 1471-2148 (Electronic) 1471-2148 (Linking).
- Lutzmann, M., Maiorano, D., & Mechali, M. (2005). Identification of full genes and proteins of MCM9, a novel, vertebrate-specific member of the MCM2-8 protein family. *Gene* 362, 51-56, 0378-1119 (Print) 0378-1119 (Linking).
- Maine, G.T., Sinha, P., & Tye, B.K. (1984). Mutants of *S. cerevisiae* defective in the maintenance of minichromosomes. *Genetics* 106, 365-385, 0016-6731 (Print) 0016-6731 (Linking).
- Maiorano, D., Cuvier, O., Danis, E., & Mechali, M. (2005). MCM8 is an MCM2-7-related protein that functions as a DNA helicase during replication elongation and not initiation. *Cell* 120, 315-328, 0092-8674 (Print).
- Matsubayashi, H. & Yamamoto, M.T. (2003). REC, a new member of the MCM-related protein family, is required for meiotic recombination in *Drosophila*. *Genes Genet Syst* 78, 363-371, 1341-7568 (Print) 1341-7568 (Linking).
- Neuwald, A.F., Aravind, L., Spouge, J.L., & Koonin, E.V. (1999). AAA+: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Res* 9, 27-43, 1088-9051 (Print) 1088-9051 (Linking).
- Saraste, M., Sibbald, P.R., & Wittinghofer, A. (1990). The P-loop--a common motif in ATP- and GTP-binding proteins. *Trends Biochem Sci* 15, 430-434, 0968-0004 (Print) 0968-0004 (Linking).
- Shultz, R.W., Tatineni, V.M., Hanley-Bowdoin, L., & Thompson, W.F. (2007). Genome-wide analysis of the core DNA replication machinery in the higher plants *Arabidopsis* and rice. *Plant Physiol* 144, 1697-1714, 0032-0889 (Print) 0032-0889 (Linking).
- Thommes, P., Kubota, Y., Takisawa, H., & Blow, J.J. (1997). The RLF-M component of the replication licensing system forms complexes containing all six MCM/P1 polypeptides. *Embo J* 16, 3312-3319, 0261-4189 (Print) 0261-4189 (Linking).
- Tye, B.K. (1999). MCM proteins in DNA replication. *Annu Rev Biochem* 68, 649-686, 0066-4154 (Print) 0066-4154 (Linking).
- Vassilev, L. & Johnson, E.M. (1990). An initiation zone of chromosomal DNA replication located upstream of the c-myc gene in proliferating HeLa cells. *Mol Cell Biol* 10, 4899-4904, 0270-7306 (Print) 0270-7306 (Linking).
- Volkening, M. & Hoffmann, I. (2005). Involvement of human MCM8 in prereplication complex assembly by recruiting hcdc6 to chromatin. *Mol Cell Biol* 25, 1560-1568, 0270-7306 (Print) 0270-7306 (Linking).
- Walker, J.E., Saraste, M., Runswick, M.J., & Gay, N.J. (1982). Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *Embo J* 1, 945-951, 0261-4189 (Print) 0261-4189 (Linking).

Yan, H., Merchant, A.M., & Tye, B.K. (1993). Cell cycle-regulated nuclear localization of MCM2 and MCM3, which are required for the initiation of DNA synthesis at chromosomal replication origins in yeast. *Genes Dev* 7, 2149-2160, 0890-9369 (Print) 0890-9369 (Linking).



Fundamental Aspects of DNA Replication

Edited by Dr. Jelena Kusic-Tisma

ISBN 978-953-307-259-3

Hard cover, 306 pages

Publisher InTech

Published online 26, September, 2011

Published in print edition September, 2011

DNA replication, the process of copying one double stranded DNA molecule to produce two identical copies, is at the heart of cell proliferation. This book highlights new insights into the replication process in eukaryotes, from the assembly of pre-replication complex and features of DNA replication origins, through polymerization mechanisms, to propagation of epigenetic states. It also covers cell cycle control of replication initiation and includes the latest on mechanisms of replication in prokaryotes. The association between genome replication and transcription is also addressed. We hope that readers will find this book interesting, helpful and inspiring.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Dianne C. Daniel and Edward M. Johnson (2011). Addressing the Enigma of MCM8 in DNA Replication, *Fundamental Aspects of DNA Replication*, Dr. Jelena Kusic-Tisma (Ed.), ISBN: 978-953-307-259-3, InTech, Available from: <http://www.intechopen.com/books/fundamental-aspects-of-dna-replication/addressing-the-enigma-of-mcm8-in-dna-replication>

INTECH

open science | open minds

InTech Europe

University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

© 2011 The Author(s). Licensee IntechOpen. This chapter is distributed under the terms of the [Creative Commons Attribution-NonCommercial-ShareAlike-3.0 License](#), which permits use, distribution and reproduction for non-commercial purposes, provided the original is properly cited and derivative works building on this content are distributed under the same license.