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Sabine Traver, Philippe Coulombe, Isabelle Peiffer, James R. A. Hutchins, Magali Kitzmann, et al..
MCM9 Is Required for Mammalian DNA Mismatch Repair. *Molecular Cell*, 2015, 59 (5), pp.831-839.
10.1016/j.molcel.2015.07.010 . hal-02361154

HAL Id: hal-02361154

<https://hal.science/hal-02361154>

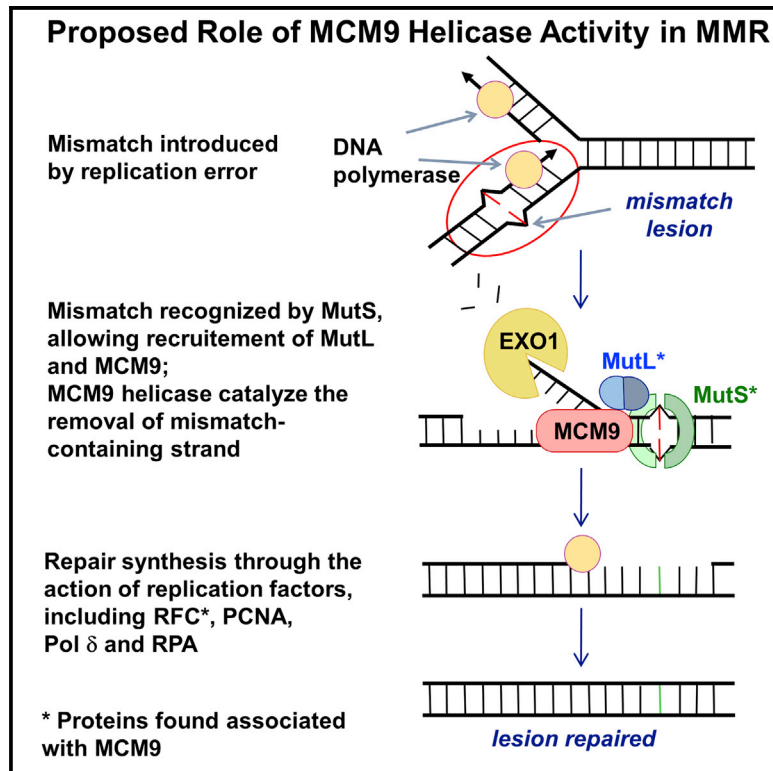
Submitted on 15 Nov 2019

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MCM9 Is Required for Mammalian DNA Mismatch Repair

Graphical Abstract



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In Brief

MCM9 has functions in replication and homologous recombination. Traver et al. find that it also associates with MMR proteins and that MCM9 and its helicase activity are essential for mismatch repair in cells. MCM9 loading is MSH2 dependent and stimulates the recruitment of MLH1 to chromatin.

Highlights

- MCM9 associates with MMR initiation proteins
- MCM9 is required for the MMR reaction
- The MCM9 complex carries a DNA helicase activity required for MMR
- MCM9 loading onto chromatin requires MSH2 and is involved in MLH1 recruitment



MCM9 Is Required for Mammalian DNA Mismatch Repair

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<http://dx.doi.org/10.1016/j.molcel.2015.07.010>

SUMMARY

DNA mismatch repair (MMR) is an evolutionarily conserved process that corrects DNA polymerase errors during replication to maintain genomic integrity. In *E. coli*, the DNA helicase UvrD is implicated in MMR, yet an analogous helicase activity has not been identified in eukaryotes. Here, we show that mammalian MCM9, a protein involved in replication and homologous recombination, forms a complex with MMR initiation proteins (MSH2, MSH3, MLH1, PMS1, and the clamp loader RFC) and is essential for MMR. *Mcm9*^{-/-} cells display microsatellite instability and MMR deficiency. The MCM9 complex has a helicase activity that is required for efficient MMR since wild-type but not helicase-dead MCM9 restores MMR activity in *Mcm9*^{-/-} cells. Moreover, MCM9 loading onto chromatin is MSH2-dependent, and in turn MCM9 stimulates the recruitment of MLH1 to chromatin. Our results reveal a role for MCM9 and its helicase activity in mammalian MMR.

INTRODUCTION

DNA mismatch repair (MMR) is an essential mechanism involved in the accurate transmission of genetic information, being responsible for correcting mistakes made during DNA replication, such as base substitutions and insertion-deletion loops (Kunkel and Erie, 2005; Shah et al., 2010). Defects in this proofreading mechanism lead to microsatellite instability, a phenomenon implicated in most human cancers and used as a marker for defects in the function of proteins of the MMR system (Heinimann, 2013; Rustgi, 2007). MMR impairment causes human nonpolyposis colon cancer (Lynch syndrome) as well as sporadic tumors (Colas et al., 2012; Fishel et al., 1993; Leach et al., 1993). The function of MMR proteins in ensuring chromosomal stability also evolved to participate in meiotic recombination (Baker et al., 1995, 1996; Spies and Fishel, 2015). In addition, the MMR system protects the genome against illegitimate recombina-

tion between divergent sequences (Jiricny, 2013; Rayssiguier et al., 1989).

The MMR process is evolutionarily conserved from bacteria to eukaryotes (see Fukui, 2010; Jiricny, 2013; Radman et al., 1995 for reviews). In the majority of prokaryotes, the mismatched base(s) on the nascent DNA strand are specifically recognized by MutS. The factor MutL is then recruited to the lesion and introduces a nick (incision) to the mismatch-containing strand. A special situation occurs in *E. coli* whereby an incision is catalyzed by MutH, a protein having no known homologs in other organisms (Eisen, 1998). Excision of the mismatched bases is executed through the action of the type II DNA helicase UvrD, exonuclease(s) (RecJ and Exo1), and the single-strand binding protein SSB. In *E. coli*, UvrD binds to a nick introduced by MutH and unwinds the DNA until the mismatch is recognized. The released single-stranded DNA is then degraded by the exonuclease. Finally, DNA synthesis and ligation take place to fill the excised gap, resulting in the effective repair of the lesion.

In eukaryotes, the MutS (MSH2-MSH6 and MSH2-MSH3 heterodimer) and MutL (MLH1-PMS2, MLH1-PMS1, and MLH1-MLH3 heterodimer) homologs are similarly involved in the recognition and incision of the mismatch-containing strand (Flores-Rozas and Kolodner, 1998; Modrich and Lahue, 1996). In addition, the loading clamp RFC complex and its substrate PCNA are important players in MMR, by first stimulating MutL endonuclease activity (Kadyrov et al., 2006, 2007; Umar et al., 1996) and also by enabling DNA synthesis (with the help of DNA polymerase δ [Beattie and Bell, 2011]). Excision involves the exonuclease Exo1 (Genschel et al., 2002; Wei et al., 2003), as well as the eukaryotic single-strand binding protein RPA (Lin et al., 1998). Interestingly, the helicase activity required for the MMR reaction in eukaryotes has not yet been identified, and its role has been debated (Song et al., 2010). Therefore, one main question remaining regarding the eukaryotic MMR process is whether a helicase activity similar to the prokaryotic UvrD is necessary for the excision step to unwind the damaged nascent DNA before exonuclease-mediated degradation.

MCM9 is the last member of the MCM2-9 family to be discovered. MCM9 belongs to the AAA+ superfamily (Hanson and Whiteheart, 2005) and contains an MCM domain that includes motifs required for ATP hydrolysis, such as the Walker A and B motifs, and also an arginine finger (R-finger) that may confer

DNA helicase activity. MCM9 has a role in DNA replication in *Xenopus laevis* egg extracts (Lutzmann and Méchali, 2008) and also in homologous recombination in the mouse, where it forms a complex with MCM8 (Lutzmann et al., 2012; Nishimura et al., 2012; Park et al., 2013). *Mcm9*^{-/-} mice show gametogenesis defects and impaired homologous recombination (Lutzmann et al., 2012). In chicken and in human cells, MCM9 also forms a complex with MCM8 that is involved in homologous recombination repair (Nishimura et al., 2012). Here, we show that MCM9 forms a tight complex with MMR proteins, and that this complex has a DNA helicase activity that is suppressed by mutations in MCM9 critical for its helicase activity. Moreover, in *Mcm9*^{-/-} cells MMR is impaired, and its activity can be restored by expression of wild-type (WT) MCM9, but not of helicase-dead MCM9. We also identify the step of MMR in which MCM9 is involved and provide a model of the MMR reaction that includes our findings.

RESULTS

MCM9 Interacts with the Components of the MMR Process, and the Complex Has a DNA Helicase Activity

To identify new MCM9 partners in human cells, we generated HeLa S3 cell lines that stably express MCM9 proteins that carry the FLAG-HA epitope at the N or C terminus (Experimental Procedures). The vectors (pOZ-FH-N and pOZ-FH-C, respectively) allow the expression of two proteins from a single transcript, thus ensuring tight coupling between the expression of tagged MCM9 and the selection marker. Moreover, we designed weak plasmid promoters in order to induce a low level of protein expression mimicking the expression of endogenous MCM9 (Figure S1A). Tagged MCM9 was purified from nuclear extracts (Figure S1B; Dignam et al., 1983) by tandem affinity chromatography using anti-FLAG-, then anti-HA-antibody-coupled beads (Figure S1C; Nakatani and Ogryzko, 2003). Mass spectrometry (MS) analysis revealed the presence of 22 specific interacting proteins (Figures 1A and 1B and Table S1), none of which appeared in major public interaction databases (IntAct, BioGRID, and STRING). The MCM9 “interactome” revealed partners implicated in recombination, meiosis, and replication (Table S1), results consistent with previously published functions of MCM9 (Hartford et al., 2011; Lutzmann et al., 2012; Nishimura et al., 2012). As previously reported, MCM9 was associated with MCM8 (Lutzmann et al., 2012; Nishimura et al., 2012). Many of the interactions identified by MS were then confirmed by co-immunoprecipitation (coIP) (Figures 1C and 1D). In addition to the strong interaction between MCM9 and MCM8 (Figures 1B and 1C), the MS results also revealed significant and previously unreported interactions between MCM9 and nearly all the components of the MutS and MutL complexes that form the DNA MMR initiation complex in mammals (Figures 1B and S1D). The interaction between MCM9 and MSH2, MSH3, MLH1, and PMS1 was confirmed by coIP of these partners with endogenous MCM9 from HeLa S3 nuclear extracts (Figure 1D, left panels). Reciprocally, endogenous MSH2, MSH6, and MLH1 also co-immunoprecipitated MCM9 from the same nuclear extracts (Figure 1D, right panels). Moreover, we could detect interaction of endogenous MCM8 with MSH2 and MLH1 (Figure 1E). Similar in-

teractions were also observed using ectopic MCM8 and MSH2 (Figure S1E). These results demonstrate that MCM9 can associate with most proteins from the MMR initiation complex in mammals. In addition, they raise the possibility that it is the dimeric MCM9/8 complex that interacts with MMR components.

We wished to investigate the stoichiometry of the affinity-purified MCM9/8-MMR complex; however, the yield of this complex was insufficient to allow a clear analysis by density gradient sedimentation. We therefore analyzed whether MCM9 as well as MCM8 present in a nuclear extract could be found in complexes of high molecular weight on glycerol gradients. Figure S2 shows that this was indeed the case. First, the majority of MCM9 and MCM8 were found to co-sediment in the gradient. The two proteins fractionated above their monomeric masses (94 kDa for MCM8, 127 kDa for MCM9), being found in fractions corresponding to ~200 kDa to > 669 kDa, suggesting the presence of MCM9/8 dimers through hexamers. The sedimentation patterns of MMR proteins partly overlapped with those of MCM8 and MCM9, consistent with our observed interactions between these factors.

We next tested whether MCM9 bound to MMR components (i.e., the MCM9 complex) contained a DNA helicase activity. To this aim, we used the standard M13 helicase assay, which is based on the ability of DNA helicases to unwind radiolabeled fragments annealed to single-stranded circular M13 DNA molecules. Indeed, the MCM9 complex purified from nuclear extracts of HeLa S3 cells that express MCM9-FLAG-HA (MCM9-FH) unwound and displaced the ssDNA fragment annealed to M13 DNA (Figure 2A). However, the observed activity could be driven directly by MCM9 or by associated proteins. To test the involvement specifically of MCM9 in this reaction, we used an MCM9 mutant in which two amino acids (K358A and R482A) that are critical for its helicase activity, and located in the Walker B and R-finger motifs, respectively, were mutated (MCM9 HD, Figure 2B) (Nishimura et al., 2012). MCM9 HD was expressed normally and was still able to interact with MCM8 and MSH2 and bind to chromatin (Figure 2A lower panels, and Figure 2C). However, MCM9 HD did not show detectable helicase activity (Figure 2A). We conclude that MCM9 is responsible for the DNA helicase activity carried out by the MCM9 complex.

Involvement of MCM9 in the MMR Reaction

Cells in which MMR is impaired tend to accumulate errors. Gene sequences are not preserved faithfully through DNA replication, and novel microsatellite fragments are created, leading to microsatellite instability, a hallmark of defective MMR. This can be detected by the appearance of new bands after PCR amplification of DNA regions containing microsatellite repeats. To determine whether MCM9 is involved in the MMR reaction, we analyzed the stability of two known microsatellite markers (D7Mit91 and D14Mit15) (Dietrich et al., 1996) in *Mcm9*^{-/-} and WT immortalized mouse embryonic fibroblasts (MEFs) (Lutzmann et al., 2012). For this purpose, 100 clones were derived from each cell type. Genomic DNA was PCR amplified using D7Mit91 and D14Mit15 primer pairs, and the size of the corresponding products was evaluated by agarose gel electrophoresis. Representative results obtained with 18 clones for each condition are shown in Figure 3A. None of the PCR products generated from WT

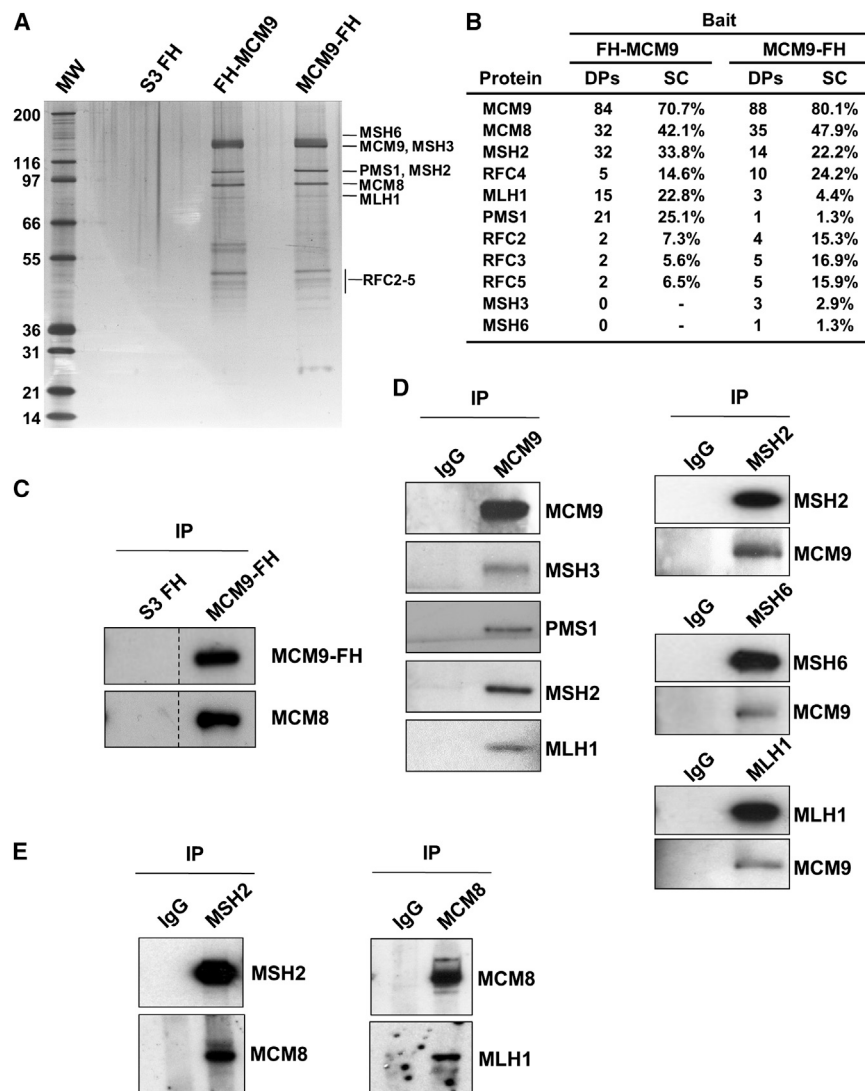


Figure 1. MCM9 Forms a Complex with MCM8 and the Mismatch Repair Initiation Complex

(A) FLAG-HA-MCM9 was tandem affinity purified from nuclear extracts of HeLa S3 cells that express N-terminally (FH-MCM9) or C-terminally (MCM9-FH) FLAG-HA-tagged MCM9, then peptide eluted under native conditions. Eluates were separated by SDS-PAGE and silver stained. The bands corresponding to FLAG-HA-MCM9 and some of its partners identified by MS are highlighted. S3 FH, HeLa S3 cells transfected with FLAG-HA tagged vector alone (control); MW, protein molecular weight markers (kDa).

(B) Proteins identified by MS analysis of complexes tandem affinity purified with MCM9 tagged with FLAG-HA on its N or C terminus. For each protein (gene symbol), the number of distinct peptides (DPs) and percentage sequence coverage (SC) are presented. Proteins classified as background or contaminants have been removed, and only those involved in MMR are shown. The full list of proteins and associated MS data are provided in [Data S1](#).

(C) MCM9-FLAG-HA (MCM9-FH) was immunoprecipitated from nuclear extracts of stably transfected HeLa S3 cells, and immunoreactivity against the HA tag and MCM8 was characterized by immunoblotting.

(D) Left panels: endogenous MCM9 was immunoprecipitated from HeLa S3 nuclear extracts, and immunoreactivity against MCM9, MSH3, PMS1, MSH2, and MLH1 was characterized by immunoblotting. Right panels: endogenous MSH2, MSH6, or MLH1 was immunoprecipitated from HeLa S3 nuclear extracts, and immunoreactivity against MCM9 and these three proteins was characterized by immunoblotting using an antibody against human MCM9 ([Supplemental Information](#)), and MSH2, MSH6, and MLH1 antibodies. (E) Endogenous MSH2 (left panels) or MCM8 (right panels) was immunoprecipitated from HeLa S3 nuclear extracts, and immunoreactivity against MCM8 and MLH1 was characterized by immunoblotting as indicated.

clones showed changes in the length of the two microsatellites. Conversely, novel allele lengths, characteristic of MMR defects, were observed in 15% of PCR products corresponding to D7Mit91, and in 9% of PCR products corresponding to D14Mit15 in MCM9^{-/-} cells ([Figure 3B](#)). This phenotype is strikingly similar to that observed in *Msh2*^{-/-} cells ([de Wind et al., 1995](#)) and strongly suggests that MCM9 is involved in the processing of slipped replication intermediates.

To monitor the MMR reaction in a different way, we performed an assay based on the reversion of a mutated codon in the open reading frame of the enhanced green fluorescent protein (EGFP) sequence to quantify MMR activity in living cells by flow cytometry ([Lei et al., 2004](#)). We constructed a heteroduplex plasmid where the (+) strand corresponds to the WT sequence of EGFP, while the (-) strand has a mutation or an insertion (CACA) resulting in the premature termination of the reading frame ([Figures 3C and S3A–S3D](#); see [Supplemental Information](#)). In MMR-proficient cells (like HCT116+chr3 cells [[Koi et al., 1994](#)]), the WT

open reading frame was recovered from the (+) strand, and EGFP was produced ([Figure S3E](#)). In contrast, MMR-defective cells (HCT116 cells) do not efficiently repair the template and yield consequently low fluorescence ([Figure S3E](#)). Importantly, a similar fluorescence signal was observed when using the WT EGFP homoduplex, showing that the transfection efficiencies of the control cell lines were comparable ([Figure S3E](#)).

Using this assay, we detected MMR activity in immortalized WT MEFs in which the MMR pathway is intact ([Figure 3D](#)). Conversely, in MCM9^{-/-} cells, EGFP was poorly expressed compared to WT cells, indicating MMR impairment. Similar results were obtained with two other heteroduplex constructs carrying a single C/T mismatch or a small CA insertion ([Figures S3E and S3F](#)). MMR deficiency in MCM9^{-/-} cells was significantly restored upon transfection of a construct encoding WT MCM9 ([Figure 3D: MCM9^{KO} + WT](#)). Taken together, these observations provide strong genetic evidence for the involvement of MCM9 in MMR.

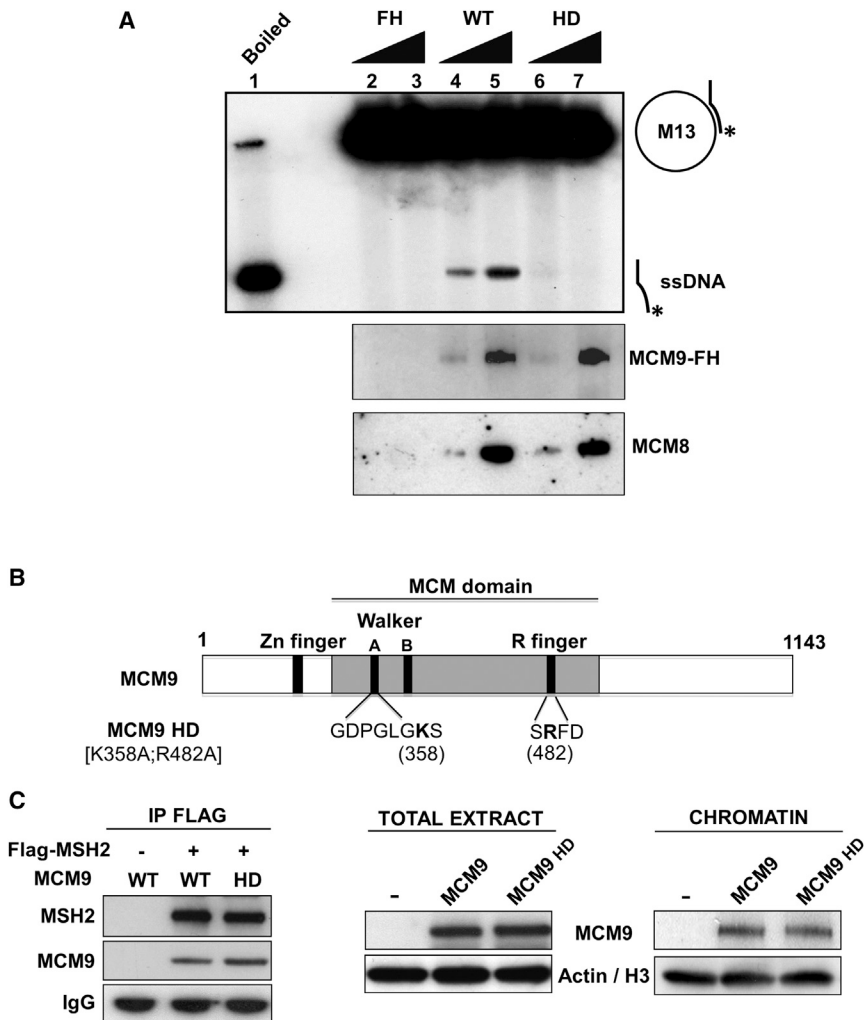


Figure 2. MCM9 Complex Displays Specific DNA Helicase Activity

(A) Upper panel: the DNA helicase activity of the purified MCM9 WT and HD complexes was assayed by evaluating the displacement of a ^{32}P -labeled 40-mer oligonucleotide annealed to ssM13 DNA, followed by acrylamide gel electrophoresis and autoradiography. The annealed M13 DNA substrate was incubated at 32°C with increasing amounts of tandem affinity-purified FLAG-HA tag only (FH, lanes 2 and 3), WT MCM9-FLAG-HA (WT, lanes 4 and 5), or helicase-dead MCM9-FLAG-HA (HD, lanes 6 and 7) for 1 hr. The displacement of the annealed substrate by heat denaturation is also shown (Boiled, lane 1). Lower panels: purified MCM9-FLAG-HA WT and HD used in this assay were analyzed by immunoblotting using FLAG or MCM8 antibodies.

(B) Schematic illustration of functional domains of MCM9. The human MCM9 helicase-dead (HD) mutant was generated by mutating two residues (highlighted in bold), in the Walker A and R-finger motifs, to alanine.

(C) HeLa cells were co-transfected with FLAG-MSH2 and human WT MCM9 (MCM9) or the helicase-dead mutant (MCM9 HD). MSH2 was immunoprecipitated using an anti-FLAG antibody. MSH2, MCM9, and MCM9 HD were detected by immunoblotting using MSH2 and MCM9 antibodies, respectively. IgG, actin, and histone H3 were used as loading controls.

The MCM9 Helicase Activity Is Required for MMR

MCM9 belongs to the MCM family that includes MCM2–7. These proteins form a complex that acts as a DNA helicase at replication forks. In bacteria, MMR requires a DNA helicase activity that is delivered by UvrD (Kunkel and Erie, 2005). In eukaryotes, the nature of such helicase has remained elusive. We thus asked whether the MMR reaction itself requires MCM9 and whether this reaction is dependent on its helicase activity. In contrast to WT MCM9, the helicase-dead MCM9 HD mutant did not restore MMR in MCM9 $^{-/-}$ cells in the EGFP assay (Figures 3D, MCM9 $^{\text{KO}}$ + MCM9 HD). We concluded that a functional MCM9 helicase domain in the MCM9 complex is essential for the MMR reaction.

MSH2-Dependent MCM9 Recruitment to Chromatin Stimulates MLH1 Chromatin Binding

In *E. coli*, MutS and MutL are responsible for recruiting the helicase UvrD to the mismatched region (Kunkel and Erie, 2005; Jiricny, 2013). We thus asked whether the essential MutS and MutL factors (MSH2 and MLH1, respectively) were required for MCM9 recruitment. siRNA-mediated depletion of MSH2 (Figure 4A, left

panel) but not of MLH1 (Figure 4A, right panel) abolished MCM9 recruitment to chromatin. Consistent with this observation, overexpression of MSH2 induced a proportional increase of MCM9 association with chromatin (Figure 4B). These results suggest that MSH2 acts upstream of

MCM9 and is important for regulating its recruitment to chromatin (see Figure S4 for a model).

MSH2 binding to chromatin was not affected by MCM9 depletion (Figure 4C). However, siRNA-mediated depletion of MCM9 significantly decreased the recruitment of MLH1 to chromatin (Figure 4C), to a level of 40%–50% of control cells. Moreover, MCM9 overexpression induced a proportional increase of MLH1 association with chromatin (Figure 4D). Taken together, these results show that MCM9 recruitment to chromatin is regulated by MSH2 and that MCM9 stimulates the binding of MLH1 to chromatin (see Figure S4).

DISCUSSION

Our results demonstrate that MCM9, a protein involved in DNA replication and homologous recombination, also has a role in the DNA MMR reaction. Specifically, MCM9 can form complexes with subunits of the MutS and MutL complexes, helps the recruitment of the MutL complex to chromatin, and is involved in microsatellite stability and MMR in a helicase-dependent manner.

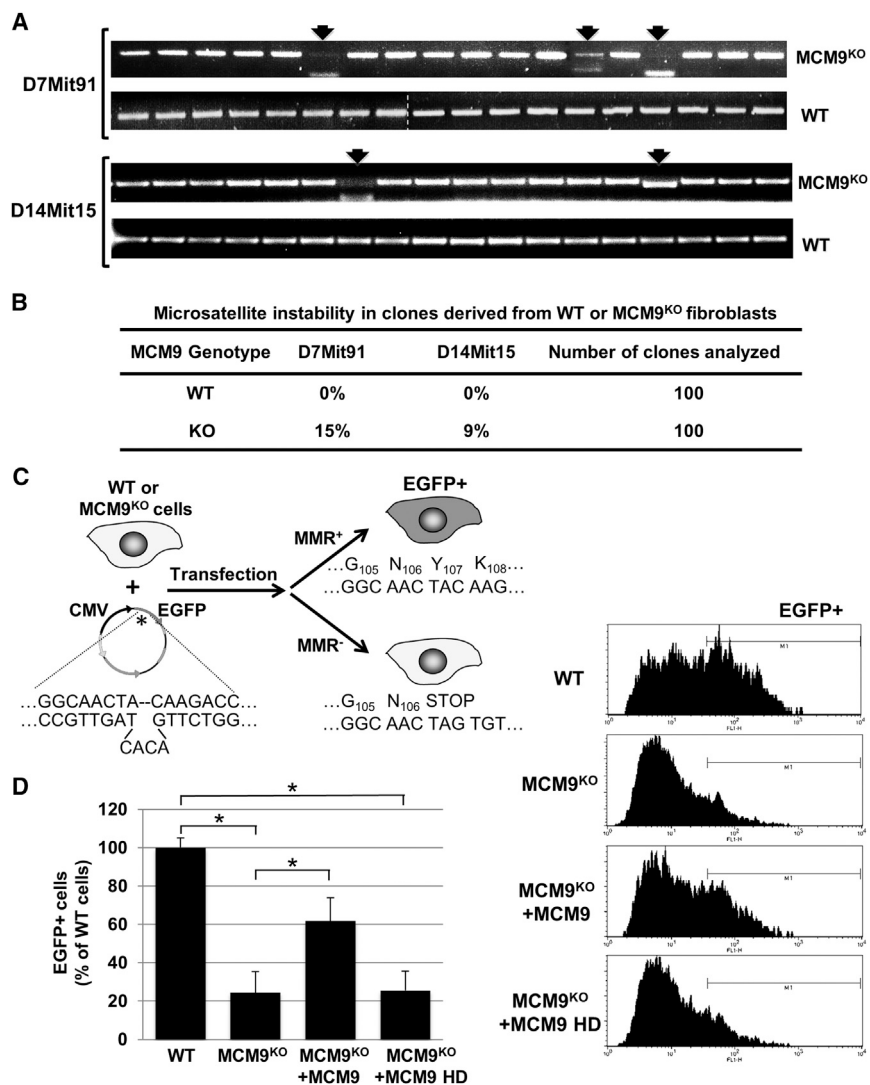


Figure 3. MCM9 Is Required for MMR in a Helicase-Dependent Fashion

(A) *Mcm9*^{-/-} cells show microsatellite instability. The microsatellites D7Mit91 and D14Mit15 were PCR-amplified from genomic DNA isolated from subclones derived from immortalized *Mcm9*^{-/-} (MCM9^{-/-}) or WT (WT) MEFs. The PCR products from genomic DNA of 18 subclones were run on 3% agarose gels. Arrows indicate microsatellite alleles with detectable length changes. Dotted line indicates two agarose gels joined together.

(B) Percentage of WT and MCM9^{-/-} cell clones showing D7Mit91 and D14Mit15 microsatellite instability with 100 clones analyzed for each cell type.

(C) Schematic illustration of the method used to assay MMR in vivo. The heteroduplex containing a CACA insertion within the coding sequence of EGFP is shown. Without MMR activity, this plasmid yields a truncated EGFP protein due to the presence of a premature STOP codon. In contrast, MMR activity leads to removal of the CACA insertion, restoration of the full EGFP coding sequence, and production of fluorescent protein.

(D) Assessment of MMR activity of WT or MCM9-deficient MEFs using our in vivo assay. WT or *Mcm9*^{-/-} knockout MEFs (MCM9^{KO}) were co-transfected with EGFP heteroduplexes (with the CACA insertion) and the pcDNA3-mCherry plasmid, then analyzed by flow cytometry. For rescue experiments, human WT MCM9 (MCM9) or the helicase-dead mutant (MCM9 HD) were transiently transfected 2 days before heteroduplex transfection; 24 hr after the last transfection, EGFP-positive cells were quantified by flow cytometry. Left panel: quantification of the MMR competence of MEFs (i.e., the proportion that restored active EGFP protein by repairing the mismatch on the EGFP heteroduplex). Error bars represent standard deviations; * indicates $p < 0.005$ (Student's *t* test). Right panels: flow cytometry representation of the results: transfected cells are gated for red fluorescence, and EGFP-positive cells (green fluorescence; black bar) were counted.

The MCM9 interactome obtained by tandem affinity purification and mass spectrometry analysis (Figures 1A, 1B, and S1) revealed some new MCM9 partners implicated in meiosis, replication, and repair, such as the meiosis checkpoint protein HORMAD1, which modulates DNA double-strand break repair during female meiosis (Shin et al., 2013), and the Replication Factor C (RFC) family, which is implicated in DNA replication and repair, including MMR. Nevertheless, our results show that the more significant MCM9 interactors are MCM8 (Lutzmann et al., 2012; Nishimura et al., 2012; Park et al., 2013) and several proteins with established roles in the MMR process. In mouse (Lutzmann et al., 2012), chicken (Kanemaki, 2013; Nishimura et al., 2012), and human cells (Lutzmann et al., 2012; Park et al., 2013), MCM8 and MCM9 form a complex and stabilize each other. Thus, the knockout of one of the two proteins in mice strongly decreases the amount of the other protein, whereas they stabilize each other when overexpressed (Lutzmann et al., 2012). This interdependence between the levels of

these two proteins complicates the analysis of the specific role of each individual subunit. However, our results clearly show that the DNA helicase activity of the complex is dependent on MCM9 activity.

In *E. coli*, UvrD is a DNA helicase essential for removing replicated DNA containing a mismatch. The presence of a DNA helicase with similar function in eukaryotes has been debated (Song et al., 2010). Indeed, MMR proteins can interact with many proteins that have helicase activity, such as the Werner helicase (WRN) (Saydam et al., 2007), the Bloom helicase (BLM) (Pedrazzi et al., 2003), REQL1 (Doherty et al., 2005), and FANCD1 (Peng et al., 2007), but none of these is essential for MMR. For instance, the WRN helicase interacts with MSH2-MSH6 (MutS α), MSH2-MSH3 (MutS β), and MLH1-PMS2 (MutL α). Both MutS α and MutS β stimulate the helicase activity of WRN, and a G/T mismatch enhances the stimulatory effect of MutS α on WRN-mediated DNA unwinding. However, cell-free extracts from lymphoblastoid cell lines derived from patients with Werner

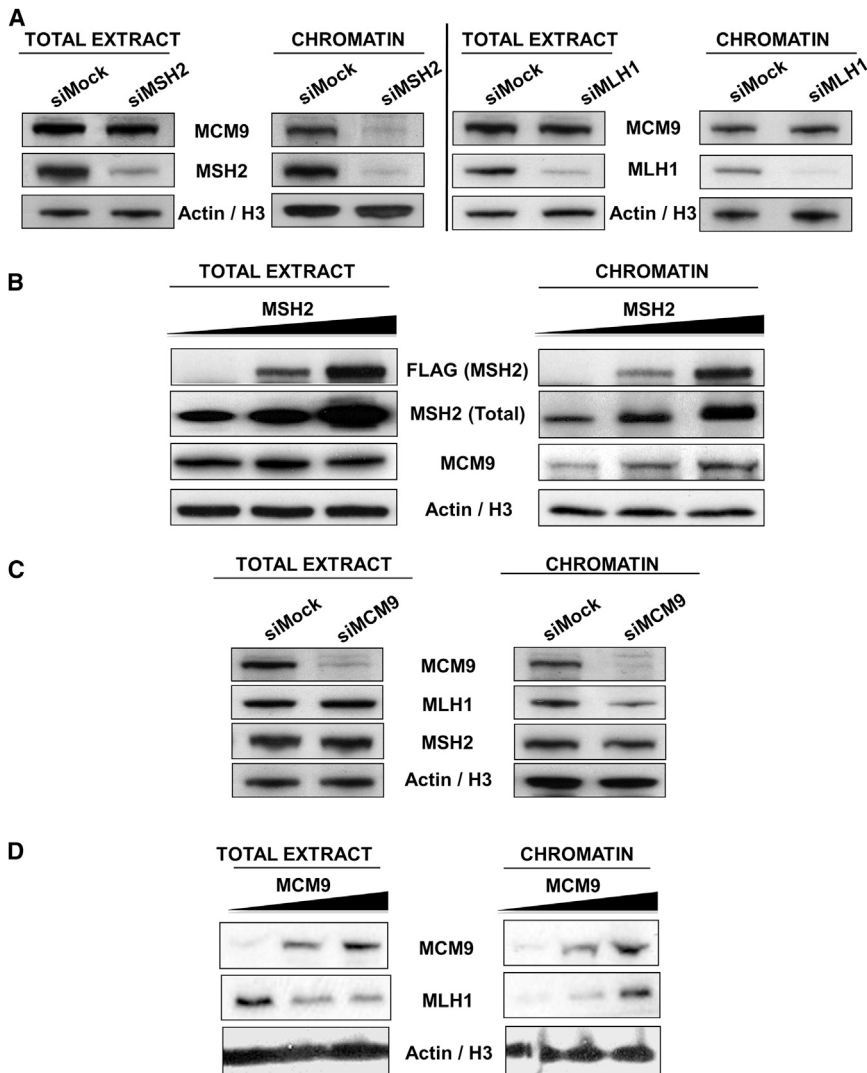


Figure 4. MCM9 Is Recruited to Chromatin by MSH2 and Participates in MLH1 Recruitment

(A) Left panels: immunoblot analysis of MCM9 binding to chromatin and in total extracts after transfection with control siRNA or siRNA against *MSH2*. *MSH2* depletion was confirmed by immunoblotting using an *MSH2* antibody. Right panels: immunoblot analysis of MCM9 binding to chromatin and in total extracts after transfection with control siRNA or siRNA against *MLH1*. Depletion of *MLH1* was assessed by immunoblotting using an *MLH1* antibody.

(B) HeLa S3 cells were transiently co-transfected with GFP-MCM9 and increasing amounts of FLAG-*MSH2*. Immunoblot analysis using an anti-MCM9 antibody was performed to determine the amount of GFP-MCM9 bound to chromatin. The overall expression of *MSH2* was revealed using an *MSH2* antibody.

(C) Immunoblot analysis of *MLH1* and *MSH2* binding to chromatin and in total extracts, after transfection with control siRNA, or siRNA against *MCM9*. Depletion of *MCM9* was confirmed by immunoblotting using an antibody against *MCM9*.

(D) Immunoblot analysis using an anti-*MLH1* antibody was performed to determine the amount of *MLH1* bound to chromatin in three different HeLa S3 cell lines that stably express increasing amounts of *MCM9* (determined using an anti-*MCM9* antibody). Histone H3 and actin were used as loading controls for chromatin and total extracts, respectively.

syndrome and lacking the WRN helicase were all proficient in MMR, indicating that WRN is not necessary in these cells for the MMR reaction (Saydam et al., 2007).

MCM9 belongs to the MCM2-7 DNA helicase family and appeared early in eukaryotic evolution (Aves et al., 2012; Liu et al., 2009). Intriguingly, in some phyla (for instance fungi) MCM9 is lost together with MCM8, suggesting a functional link between the two MCM proteins. Indeed, our proteomic approach has revealed a strong association between MCM9 and MCM8. Moreover, our data suggest that MCM9 could act as the functional eukaryotic homolog of UvrD, thus providing a helicase activity required for an efficient MMR reaction.

Based on our results, we propose a revised model for MMR in mammals that includes MCM9 (Figure S4). The mismatch lesion is recognized by MSH2 complexes, which then recruit MCM9 and MLH1 to chromatin. Here, MCM9 stimulates the loading of MLH1 onto chromatin. The enzymatic activity of MutL was shown to be stimulated by the clamp loader RFC (Kadyrov et al., 2006, 2007), found to be associated with MCM9. Thus,

by bringing RFC to mismatch sites, MCM9 might have a role in the incision step by participating in MutL activation. Moreover, we envisage that MCM9, through its helicase activity, might be involved in the excision of the mismatch-containing strand. In the situation where the mismatch-containing strand possesses nicks on both sides, MCM9 helicase activity could in principle be sufficient to excise the mismatch strand (Song et al., 2010). However, the degradation of the mismatch-containing strand through the hydrolytic activity of Exo1 is known to be important for excision and MMR (Bregenhorn and Jiricny, 2014; Schaetzlein et al., 2013; Shao et al., 2014). The enzymatic action of MCM9 is expected to yield a 5' flapped single-stranded DNA that would be an optimal substrate on which Exo1 could act (Song et al., 2010). After excision, the resulting DNA gap would be filled in a Pol δ -dependent manner (Longley et al., 1997). Because the PCNA loading clamp RFC is important for Pol δ activity (Bambara et al., 1997; Gibbs et al., 1997), MCM9 association with RFC could facilitate DNA repair synthesis.

It is firmly established that MMR components, such as MSH2, MSH3, or MSH6, play a role during HR to ensure the fidelity of the recombination reaction (Abuin et al., 2000; de Wind et al., 1995; Elliott and Jasin, 2001). In the absence of these factors, HR does take place, but can occur even between sequences showing

differences, a process called homeologous recombination (reviewed in [Spies and Fishel, 2015](#)). In addition to its role in MMR, MCM9 is also required for efficient HR ([Lutzmann et al., 2012](#); [Nishimura et al., 2012](#); [Park et al., 2013](#)). It is therefore difficult to test its implication in homeologous recombination, as HR itself is impaired. Are the functions of MCM9 in HR and MMR related? Our model proposes that MCM9 helps the resection of the mismatch-containing strand ([Figure S4](#)). Interestingly, MCM9 or MCM8 KO cells, in response to a replication fork block, show defects in chromatin recruitment of Rad51, Mre11, and RPA ([Lutzmann et al., 2012](#); [Park et al., 2013](#)), again suggesting that resection in HR repair is dependent on MCM9/8.

Whereas the mechanistic details of MCM9's involvement await future studies, our results have unraveled a new function for MCM9, as a helicase required in mammalian MMR, and have strengthened its essential role in the maintenance of genome stability ([Hartford et al., 2011](#); [Lutzmann et al., 2012](#); [Nishimura et al., 2012](#)). This function may be emphasized in light of recent data showing the involvement of MCM9 and MCM8 deficiency in short stature, ovarian failure, and compromised DNA repair ([AlAsiri et al., 2015](#); [Wood-Trageser et al., 2014](#)).

EXPERIMENTAL PROCEDURES

Affinity Purification of MCM9-Containing Protein Complexes

To generate stable cell lines that express MCM9 tagged with the double FLAG-HA epitope at the N or C terminus, we used the retroviral vectors pOZ-FH-N and pOZ-FH-C, respectively. FLAG-HA-tagged MCM9 and associated proteins were isolated from nuclear extracts ([Dignam et al., 1983](#)) by tandem affinity purification based on the FLAG and HA tags, according to the method of Nakatani and Ogryzko ([Nakatani and Ogryzko, 2003](#)). Detailed experimental procedures are available in [Supplemental Experimental Procedures](#).

Immunoprecipitations and Immunoblotting

Immunoprecipitations were carried out using cell extracts and analyzed by immunoblotting with specific antibodies, as detailed in [Supplemental Experimental Procedures](#).

Chromatin Isolation

For chromatin isolation, cells were lysed in CSK buffer (10 mM PIPES [pH 6.8], 100 mM NaCl, 300 mM sucrose, 1 mM MgCl₂, 1 mM EDTA, 0.5 mM DTT, 0.3% Triton X-100, 1 mM ATP, protease inhibitors) on ice for 10 min. Lysates were centrifuged at 3,500 *g* at 4°C for 5 min. Pellets were then homogenized in CSK buffer, extracted on ice for 10 min, centrifuged, and solubilized in Laemmli buffer.

Microsatellite Analysis

Subclones of immortalized WT or *Mcm9*^{-/-} cells were generated by seeding statistically one cell per well. These clones grew for approximately 30 divisions. Genomic DNA was then isolated using a QIAGEN kit and PCR-amplified using two end-labeled primer pairs (*D7Mit91* and *D14Mit15*) ([Dietrich et al., 1996](#)). Amplified products were electrophoresed on 3% agarose gels, observed under UV illumination, and photographed.

MMR Assay

Immortalized WT or *Mcm9*^{-/-} cells were transfected with 400 ng of homo- or heteroduplex together with the mCherry plasmid in large excess in 10-cm culture plates using Lipofectamine (Life Technologies). For rescue experiments, constructs encoding human (WT or HD mutant) MCM9 were transfected with Lipofectamine 2 days before heteroduplex transfection. Forty-eight hours after the last transfection, flow cytometry analyses were performed using a

FACSCalibur with green (FL1) and red (FL3) fluorescence plots. The method used for the preparation of heteroduplexes for MMR assays in live cells is described in [Supplemental Experimental Procedures](#).

siRNA Transfection

A total of 3 μM of siGENOME Non-Targeting siRNA for the mock condition or 3 μM ON-TARGETplus SMARTpool for human *MCM9*, *MSH2*, or *MLH1* (Dharmacon, GE Healthcare) was transfected in cells with Oligofectamine (Life Technologies) for 48 hr and analyzed by immunoblotting.

DNA Helicase Activity Assay

DNA helicase activity was assayed using single-stranded M13 DNA as a substrate (Biolabs), annealed to a 40-mer branched oligonucleotide, as previously described ([Lee and Hurwitz, 2001](#)). Five femtomoles of ³²P-labeled annealed substrate were incubated with the purified MCM9 complex in a reaction mixture (20 μl) containing 25 mM HEPES-NaOH (pH 7.5), 25 mM sodium acetate, 10 mM magnesium acetate, 4 mM ATP, 1 mM DTT, and 0.1 mg/ml BSA at 32°C for 1 hr. The reaction was stopped by addition of 5 × loading buffer (100 mM EDTA, 0.5% SDS, 0.1% xylene cyanol, 0.1% bromophenol blue, 25% glycerol) and separated on a 12% polyacrylamide gel in 1 × TBE at 150 V for 90 min. The gel was then dried and visualized by autoradiography.

ACCESSION NUMBERS

Mass spectrometry data have been deposited in the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository ([Vizcaino et al., 2009](#); [Vizcaino et al., 2013](#)) with the dataset identifier ProteomeXchange: PXD000212.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, one table, and a list of proteins and peptides identified and can be found with this article online at <http://dx.doi.org/10.1016/j.molcel.2015.07.010>.

AUTHOR CONTRIBUTIONS

S.T. contributed to research design; performed TAP-tag, biochemical, and functional MMR experiments; prepared draft figures; and co-wrote the manuscript. P.C. contributed to research design, set up the microsatellite instability and the EGFP-based MMR assays, performed biochemical analysis and helicase assay, prepared figures, and co-wrote the manuscript. I.P. assisted with cell culture and extract preparation. J.R.A.H. performed bioinformatical analysis on the MCM9 interactome and participated in the manuscript preparation. M.K. performed glycerol gradient experiments and set up the helicase assay. D.L. contributed to the setting up of the TAP-tag protocol. M.M. proposed and supervised the project, contributed to research design, analyzed the results, and co-wrote the manuscript.

ACKNOWLEDGMENTS

We wish to thank Josef Jiricny for sending some reagents used in this study and for helpful exchanges. We wish to thank Ross Tomaino (Taplin Mass Spectrometry Facility, Harvard University) for help and advice on MS analysis and the PRIDE repository team for their assistance. We thank the Vectorology Platform of Montpellier, especially Céline Lemmers for her help with cell infections. We also thank Bijan Sobhian for technical advice with TAP-tag experiments and Angelos Constantinou for helpful discussions. The research leading to these results received funding from the European Research Council (FP7/2007-2013 Grant Agreement no. 233339). This work was also supported by the Fondation ARC, and the FRM. P.C. and J.R.A.H. were supported by post-doctoral fellowships from FRM. P.C. was also supported by a post-doctoral fellowship from ARC. D.L. was supported by a studentship from FRM and the ANRS.

Received: March 11, 2015
 Revised: May 23, 2015
 Accepted: July 15, 2015
 Published: August 20, 2015

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